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Novel targets and clinically relevant models for ovarian cancer

Alkema, Nicolette

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Chapter 2

Studying platinum sensitivity and resistance in high-grade serous ovarian cancer: different models for different questions

N.G. Alkema¹, G.B.A. Wisman¹, A.G.J. van der Zee¹, M.A.T.M. van Vugt²,
S. de Jong²

Departments of Gynaecologic Oncology¹ and Medical Oncology², Cancer Research Centre Groningen, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands.

Resubmitted to Drug Resistance Updates

Abstract

Ovarian cancer, with high-grade serous ovarian cancer (HGSOC) as the most common histological subtype, has the highest mortality among all gynaecological cancers. HGSOC is generally diagnosed in an advanced stage with the majority of cases showing platinum resistant relapses. Therefore, 5-year survival rates for advanced stage disease remained low over the last decades. Genomic interrogation of large numbers of patient samples has improved our understanding about the complexity of HGSOCs in terms of genetic aberrations and intra-tumoural heterogeneity and underscored their lack of targetable mutations. Clearly, experimental models are required in which resistance to platinum therapy and the use of novel therapeutics can be studied. Several novel genetically engineered mouse models (GEMMs) have recently emerged, in which pathogenic mutations are introduced that mimic human pathogenesis. With patient-tailored therapy as a new treatment modality in multiple cancer types, also more personalised models for HGSOC are arising. DNA repair status of the tumour can be obtained using *ex vivo* tumour tissue slices and novel therapeutic strategies can be tested using patient-derived xenografts (PDXs). Previously, tracking changes in tumours during treatment and after relapse was hardly possible because of the invasiveness of serial sampling without contributing to personal care of patients. This problem might be solved in the near future by profiling tumours and analysing circulating cell-free tumour DNA or circulating tumour cells. This review will focus on recently developed models and platforms suitable for investigating responses to platinum-based chemotherapy, with *BRCA1/2*-mutation carriers and BRCAness ovarian cancer patients as an example. We will discuss how various models can be combined to study resistance mechanisms, to predict responses by measuring DNA repair capacity of tumours and to use synthetic lethality as a way to improve therapy outcomes in specific subgroups among ovarian cancer patients.

Introduction

Due to late symptoms, ovarian cancer is diagnosed at an advanced stage in the majority of cases and therefore has the highest mortality rate among all gynaecological cancers (1). In the 1980s, first-line treatment consisted of cisplatin in combination with cyclophosphamide. Soon after, carboplatin was introduced, which was found to have a more favourable toxicity profile than cisplatin, while resulting in comparable outcome in survival (2-3). By forming inter- and intrastrand DNA adducts, platinum-based chemotherapy induces lethal damage to dividing cells, such as cancer cells, by interfering with DNA replication. Paclitaxel replaced cyclophosphamide as part of first-line regimen in the 1990s when proven to give significant survival benefit (4). Current standard of care for patients with advanced stage ovarian cancer consists of surgical debulking of tumour mass combined with neo-adjuvant or adjuvant treatment with 3-weekly paclitaxel and carboplatin for 6 cycles (5). To date, the extent of cytoreduction after debulking surgery is considered the most important prognostic factor for survival when combined with a platinum-based regimen (6). Despite high initial response rates, the majority of advanced stage patients will relapse, with the progression free interval being a direct predictor of sensitivity to second-line platinum therapy (7-8). The majority of relapses is platinum resistant (i.e. with a short progression free interval less than 6 months) and shows little to no long-lasting responses to other agents. As a result, survival rates have not improved over the last decades, with a 5-year survival rate of 19-28% in advanced stage disease (1).

A big leap in achieving upfront selection of ovarian cancer patients is the radical change in the definition of ovarian cancer, which is no longer conceived as one entity. It is now widely accepted that histological subtypes of ovarian cancer, i.e. high-grade serous, low-grade serous, endometrioid, mucinous and clear cell carcinoma, are derived via different routes of tumourigenesis, divided in low-grade and high-grade pathways (9). Low-

grade, slow growing tumours encompass histological subtypes such as low-grade serous, endometrioid, mucinous and some clear cell carcinomas, and are now recognised to harbour mutations in *KRAS*, *BRAF*, *PTEN*, *CTNNB1* and *TGFBR2* (10-11). The tumour suppressor gene *ARID1A* is mutated in 46%-57% and 30% of clear cell and endometrioid ovarian cancers, respectively, without showing mutations in high-grade serous ovarian cancer (HGSOC), indicating a difference in pathogenesis (12-13). The presence of *KRAS* and *BRAF* mutations solely in low-grade serous carcinomas and borderline tumours, but not in HGSOC, again underscores that these tumours develop through independent pathways (14). HGSOC is characterised by an almost ubiquitous presence of *TP53* mutations (10). In 20% of the HGSOC cases a mutation in *BRCA1* or *BRCA2* is found, predisposing these women to hereditary breast and ovarian cancer. *BRCA1* and *BRCA2* are both important players in homologous recombination (HR), which together with the nucleotide excision repair (NER) pathway, is one of the two most common pathways for repairing platinum-induced DNA damage. In addition to *BRCA1/2* mutations, aberration of the HR pathway can also happen via *BRCA1* promoter hypermethylation (15-18). Remarkably, HGSOC were shown to have relatively few other gene mutations (10). In striking contrast, a remarkable degree of copy number aberrations is present, suggesting disruption of DNA repair pathways in an early stage of tumour development (19). In line with the ensuing genomic instability, a high degree of intra-tumoural heterogeneity with remarkable genomic rearrangements was observed using spatially taken samples of primary tumour and metastatic sites (20-21).

The different histological subtypes are known for their variation in platinum sensitivity. The majority of HGSOC patients respond well to first-line platinum-based chemotherapy whereas clear cell, mucinous and low-grade serous tumours are known to be more resistant (22-23). Especially HGSOC patients that harbour a *BRCA1* or *BRCA2* mutation are known to be sensitive

towards platinum-based chemotherapy resulting in a significant better response rate and longer survival (24). A further sub-classification was made for HGSOC based on gene expression (10, 25). These expression subtypes (termed 'differentiated', 'immunoreactive', 'mesenchymal' and 'proliferative') were shown to have prognostic value, with those patients harbouring the mesenchymal signature having the poorest outcome. However, this classification is not exclusive and multiple signatures can be found within one tumour (26).

Resistance to platinum-based chemotherapy is hypothesised to be a Darwinian evolutionary process, driven by selective pressure of chemotherapy (27). It has been postulated that at time of presentation already minor subpopulations of intrinsically resistant cancer cells are present (28). Because of the high level of genomic instability in HGSOC, the development of intrinsically resistant subclones during the numerous cell divisions before clinical presentation seems a plausible hypothesis. This hypothesis is supported by the observation that some patients are minimally or only for a very short time responding to treatment. Residual disease after surgery is the strongest prognostic factor for survival despite a partial or complete clinical response to first-line platinum-based chemotherapy, which also supports the concept of intrinsically resistant subclones being already present. Alternatively, it cannot be excluded that acquired resistant cells arise during treatment as a result of the high genomic instability in HGSOC. Assuming that requiring resistance mutations is a stochastic event, more residual disease implies a higher tumour cell load and will thus increase the chances on subclones that have acquired resistance.

Mechanisms underlying resistance to platinum-based chemotherapy have been extensively studied. Several mechanisms were identified, including reduced intracellular cisplatin accumulation due to changes in

transmembrane transport, activation of cell growth-promoting and DNA damage repair pathways, aberrant DNA methylation, enhanced epithelial to mesenchymal transition and reduced endocytosis of cisplatin (29-31). However, this has not resulted in translational relevance for the clinical setting. A better understanding of factors determining response to therapy in ovarian cancer patients requires multiple approaches, with more representative experimental models being an important step in improving bench-to-bedside translation of results (32). Favourable responses have consistently been reported for a small subset of patients, emphasizing the need for upfront selection of eligible patients. In this respect, patient-tailored therapy, based on tumour characteristics of each individual patient, is widely considered as the next step in improving outcome, decreasing side effects and overcoming resistance (33-34).

In the past decades much progress has been made in developing representative ovarian cancer models using both *in vitro* and *in vivo* approaches that mimic ovarian cancer pathogenesis, tumour microenvironment, angiogenesis and therapy response (35). In this review we will focus on recently developed models and platforms suitable for investigating response to platinum-based chemotherapy and biomarker-based personalised strategies to overcome resistance. Finally, integration of several of these models in current preclinical studies on Poly(ADP)ribose polymerase (PARP) inhibitors in BRCA1/2-mutation carriers and sporadic ovarian cancer patients will be discussed.

Modelling ovarian cancer in a petri dish

Current *in vitro* ovarian cancer research is hampered by the low number of well-defined cell lines (36). Investigating large panels of cell lines may help in finding novel biomarkers of response towards certain (targeted) therapies (37-38). However, in the Broad-Novartis Cancer Cell Line Encyclopaedia

(CCLE) only 60 ovarian cancer cell lines are described, of which only a few are depicted as serous (37). Most of them were established over a decade ago and were often derived from ascites instead of primary tumour. Comparison of these CCLE ovarian cancer cell lines with primary ovarian cancer samples from the TCGA database identified distinct differences, with more mutations in the cell lines than expected on the basis of their claimed high-grade serous histology in literature (36). Alarming, the cell lines SKOV3 and A2780 are least likely to be obtained from a HGSOC patient, while they are the most frequently used *in vitro* models for HGSOC (36, 39). Therefore, a shift in *in vitro* ovarian cancer research is warranted, restricting *in vitro* experiments to well-defined cell lines that have been characterised extensively (39-40).

With different ovarian cancer subtypes, harbouring distinct mutations and displaying different behaviour, attention is now being focused on understanding the pathogenesis in order to develop ovarian cancer models that better reflect the different histological subtypes (41). There is accumulating evidence suggesting the fallopian tube fimbriae as the site of origin for HGSOC (42). In an *ex vivo* primary human fallopian tube epithelium culture system, a delayed DNA damage repair response of fallopian tube secretory cells to DNA damaging agents was found when compared to fallopian tube ciliated epithelial cells, potentially making them more susceptible to mutagenic events (43). Immortalised human fallopian tube epithelial cells (FTECs) cell lines have been established using an *in vitro* approach by transducing primary FTECs with the *hTERT* gene, encoding the catalytic subunit of telomerase, the SV40 large-T and small-T antigens, targeting among others p53 and Rb1, and either the *HRAS* or *MYC* oncogenes (44). A slightly different approach by exposing FTECs to a cocktail of retroviral vectors also led to immortalised FTECs by p53 and Rb1 inactivation and c-Myc and *HRAS* overexpression (45). Injecting these immortalised FTECs in severe combined immunodeficiency (SCID) mice resulted in xenografts closely resembling HGSOC with respect

to histological characteristics and genomic instability, indicating that serous ovarian cancer may indeed be originating from FTECs (44-45). Clearly, these models seem well suited to gain mechanistic insight into the putative contributions of different genetic alterations to the early oncogenic events inducing malignant transformation of the fallopian tube epithelium. Whether FTECs can also be used in understanding the development of resistance and whether resistant subclones arise during oncogenesis of these FTECs has yet to be determined.

Identifying the genetic alterations and mutations acquired during cancer treatment is considered a key-factor in understanding the mechanisms that are responsible for treatment-resistant relapses. In order to obtain drug-resistant subclones, various ovarian cancer cell lines have been treated with chemotherapeutic drugs, leading for instance to the cisplatin-resistant C30 and CP70 and the paclitaxel-resistant PTX10 and PTX22 cell lines, all derived from the primary cell line A2780 (46-47). However, resistance mechanisms as seen in the clinic might not be well mimicked using this approach. Therefore, efforts have been made to expand the number of patient-derived ovarian cancer cell lines (48-49). In 1988, a series of cell lines were established at different time points during treatment of three patients (50). A non-linear relationship of genomic duplications was found between cells before and after developing cisplatin resistance, with more genomic complexity at presentation than at relapse (51). Sampling and (short-term) culturing of tumour cells derived from relapsed disease may offer a tool for identifying clinically relevant mechanisms underlying platinum resistance, or provide material to test personalised therapy combinations to overcome resistance. Feasibility of this approach was shown with cell lines derived from both ascites as well as primary tumour material that could be cultured up to 8 passages before going into senescence (52). A positive predictive value for resistance of approximately 90% was shown, using patient-derived cell lines in clonogenic

assays (53-54). It was shown that responsiveness to platinum compounds *in vitro*, using primary cultures of ovarian cancer patient samples, was an independent predictor of progression-free survival and overall survival (55-56). Furthermore, other effective anticancer compounds, such as etoposide and doxorubicin, could be identified *in vitro*, pointing towards possible treatment alternatives for tumours that were non-responsive to platinum (56). However, multiple sampling might be necessary as discrepancies in sensitivity between tumour cells derived from primary tumour sites and metastatic lesions have been reported (57).

In conclusion, long-established ovarian cancer cell lines have many drawbacks in their clinical relevance. However, HGSOC cell lines are now being characterised in depth and may be especially useful when analysed in larger panels for sensitivity assays to identify key players in platinum resistance. Furthermore, patient-derived short-term tumour cell cultures may be used as screening tools for the identification of effective drug treatments and as a screening tool for treatment alternatives when all other therapies have failed.

HGSOC: A 3-dimensional disease

Several 3-dimensional ovarian cancer models have been established and previously reviewed (35, 58). These models incorporate a tumour microenvironment, thus achieving better resemblance to the mixture of tumour, stromal, endothelial and immune cells ovarian cancers largely consist of. 3-Dimensional models are considered to be more predictive and reliable in investigating resistance mechanisms compared to their 2-dimensional counterparts (58). By culturing ovarian cancer cells in a 3-dimensional system using agarose hydrogel or an alternative scaffold, more proliferative and platinum-resistant phenotypes were seen as compared to 2-dimensional cultures (59-61). Whereas the EZH2 inhibitor GSK343 showed limited effects

on a monolayer culture, it was able to sensitise OVCAR10 and SKOV3 ovarian cancer cell lines to EZH2 methyltransferase inhibition in a 3-dimensional matrigel basement membrane extracellular matrix (62). Cell migration and invasion were enhanced and resistance to cisplatin was promoted, when ovarian cancer cells were co-cultured with mesenchymal cells (63). This observation is in line with *in vivo* observations that mesenchymal cells are capable of inducing chemoresistance in tumour cells by releasing fatty acids in response to platinum-based chemotherapy (64-65). These so-called platinum-induced fatty acids (PIFAs) activate macrophages to secrete polyunsaturated lysophosphatidylcholines, which induce resistance by altering the DNA damage response with a rapid decrease in the DNA damage marker γ H2AX and thus restoring cell cycle progression (65). Several other explanations for the enhanced platinum resistance have been proposed, for example passively by preventing drug penetration, and actively by mechanisms such as secreting protective cytokines and soluble factors promoting survival and growth or by stimulating epithelial-to-mesenchymal transition (66). Also adipocytes are known to be involved in enhanced ovarian cancer cell proliferation via the secretion of leptin and by actively acting as a source of energy (67-68), further underscoring that 3-dimensional co-culture mixtures of tumour, stromal, endothelial and immune cells better reflect the *in vivo* situation for ovarian cancer with regard to proliferation and drug sensitivity.

Another example of implementing the 3-dimensional tumour structure with a mixture of normal and tumour cells in an *ex vivo* setting is the use of whole tissue slices, using for instance the Krumdieck tissue slicer (69). Such tumour tissue slices can be kept viable *ex vivo* for over 96 hours, while they maintain the interactions between tumour cells and surrounding stroma (70-72). Even after more than 96 hours, the 3-dimensional structure is preserved as demonstrated by the presence of tumour cells, vessels and stromal and inflammatory cells. These fresh tumour samples are likely to be more

representative with respect to cellular response to cytotoxic drugs and were reported to display different, or even completely opposite, drug responses than those observed using *in vitro* cultured cells (70-72). However, this technique is quite laborious with complicated logistics, strongly depending on a good collaboration between clinic and laboratory, and can only be used to measure short-term cytotoxicity.

Recapitulating, although incorporating a 3-dimensional culture model is a laborious technique with a clear need for optimisation, it is worth the effort in order to obtain more relevant and translational results when it comes to drug sensitivity and resistance.

2

Genetically engineered ovarian cancer mouse models

The use of genetically engineered mouse models (GEMMs) to study ovarian cancer has been reviewed extensively (35, 73-74). Most of the ovarian cancer GEMMs described thus far have been used to study the development and pathogenesis of the disease (75-79). Similar GEMMs have been proven useful for investigating mechanisms of resistance to platinum therapy and response to targeted therapy in other cancer types, however not yet for ovarian cancer (80). Indeed, GEMMs are being implemented in so-called co-clinical trials for other cancer types, like lung cancer, aiming to identify drug responses and uncover resistance mechanisms as observed in the clinic (81-82).

Recently, several studies reported responses to platinum-based chemotherapy and possible resistance mechanisms in breast cancer GEMMs. Tumours that arose from mammary-tissue-specific inactivation of *Brca1* and *p53* displayed hypersensitivity to cisplatin (83). Disease recurrence was invariably observed, but remarkably, these relapsed tumours never developed resistance towards cisplatin with identical time to regrowth over time during concurrent cisplatin regimens (83-84). In these specific models, treatment resistance due to

Brca1/2 reading frame restoration could not develop due to the engineered large deletions in the *Brca1/2* genes. In more recently described models, cancer-relevant mutations have been introduced which could allow *Brca1/2* restoration through secondary mutations (85-87). Currently available GEMMs, however, do offer opportunities to study resistance mechanisms beyond *Brca1/2* reactivation. For instance, by developing a breast cancer GEMM with a large irreversible *Brca1* mutation, it was shown that inactivation of the p53-binding protein-1 (53BP1), and thereby restoration of HR, is a possible mechanism of resistance to PARP inhibition (88). This mechanism was previously proposed based on a large cohort of breast cancer patients in which reduced 53BP1 expression was observed in triple-negative and *BRCA*-associated breast cancers (89). Also Rif1 and Rev7 have recently been shown to be involved in partly rescuing checkpoint activation in *Brca1* deficient cells (90). Although these results were obtained in a breast cancer GEMM setting, results may also be relevant for ovarian cancer in respect of sensitivity and resistance observed in *BRCA1/2* mutation carriers.

Additionally, allograft mouse models, so-called syngeneic mouse models, are arising, allowing to study tumour microenvironment, immune cell infiltration and anti-tumour response. By orthotopic implantation of ovarian cancers derived from *Rb/p53* and *Rb/p53/Brca1/2*- mutant GEMMs in immunocompetent mice, it was shown that treatment with cisplatin and the PARP inhibitor olaparib was more efficient in *Brca1*-deficient cancers than in their *Brca1* wild-type counterparts (91-92).

In conclusion, GEMMs offer unique opportunities to study mechanisms of resistance in a clearly defined genetic background. Efficacy of treatment strategies also depends on the tumour microenvironment and immune responses and therefore GEMMs appears to represent the clinical situation more closely. A major drawback are the high costs of GEMMs. Furthermore,

GEMMs only reflect a specifically defined genetic route of tumourigenesis and therefore do not take into account the intertumoural heterogeneity observed in human tumours. Moreover, species-specific differences with regard to proteins expressed, signalling pathways, and responses to (species-specific) drugs have to be considered as well.

Patient-derived ovarian cancer xenografts models

Patient-derived xenograft (PDX) models can take into account the intertumoural and intratumoural heterogeneity, as well as possible resistant subpopulations using multiple engraftments of tumour tissue from a single patient. The use, benefits and drawbacks of these models in studying cancer pathogenesis and drug resistance have been reviewed extensively (93-97). However, the concord between clinical response to treatment and the *in vivo* response in the PDX counterparts is unequivocal (54, 98). For some solid tumours that harbour targetable mutations, an upfront selection of certain therapies is very well possible, for example in lung cancers and melanomas with *EGFR* and *BRAF* oncogenes, respectively (99). Mechanisms of intrinsic resistance to the EGFR-antibody cetuximab have already been discovered for colorectal cancer using a panel of PDXs (100). Besides identification of predictive biomarkers in order to select the right patient for a certain drug, other approaches are arising in which PDXs can be useful. By implanting tumour specimens obtained by laparoscopy or biopsy into mice, a personalised *in vivo* drug screening can be performed, either using tumour material taken at initial presentation or at relapse (101-102). This way, PDXs can be used as a drug-testing platform, as well as a model to investigate resistance mechanisms. However, tumour tissue should be taken from the platinum-resistant relapse, as this tumour probably harbours a majority of resistant subclones not, or limitedly, present in the primary tumour or the metastasis at presentation. Furthermore, this methodology requires rapid establishment and expansion of a panel of PDXs in order to find the right

therapy for the individual patient on time.

For HGSOC, with no clear targetable driver mutations and a lack of biomarkers, choosing the right drug remains challenging. Although the first PDXs were established already more than a decade ago (103-104), these models only recently emerged as potential drug-testing platforms and developed models hitherto have been described extensively (35, 41, 105-106). There are several studies on therapy responses in ovarian cancer PDXs worth elaborating on. Validation of PDXs as models for predicting response to therapy in the individual patient requires a response to conventional chemotherapeutic agents comparable to the clinical response. Vidal *et al.* developed an ovarian cancer PDX model by engrafting treatment naïve tumour samples directly onto the ovarian surface and induced resistance to cisplatin using repetitive cycles of cisplatin (107). In this study, lurbinectidin, a novel DNA minor groove covalent binder, was potent in inhibiting tumour growth in both the cisplatin-sensitive as well as their cisplatin-resistant counterparts, with a synergistic effect when combined with cisplatin (107). As secondary resistance to carboplatin/paclitaxel after primary good response is the most important reason for the poor survival rates of HGSOC, the PDX model by Vidal *et al.* appears to be clinically relevant to identify mechanisms that underlie the development of resistance and to study drugs that can overcome resistance. One of the first studies to investigate treatment with conventional therapies, showed similar chemosensitivity towards cisplatin in 15 subcutaneous ovarian cancer PDX models (48%) when compared to average response rates in ovarian cancer patients (40%) (108). However, no direct link between the PDX and the patients from whom they were derived was made. A recent study by Topp *et al.* studied the responses of 12 HGSOC PDXs to 3 cycles of cisplatin treatment and found variable responses varying from complete responses up to more than 150 days to complete refractory tumours (109). Observed responses were stable over generations. When mice with recurrent tumours were re-treated, they developed cisplatin-refractory disease as

observed in the clinical setting. All PDX tumours refractory to treatment, had overexpression at baseline by amplification of one or more oncogenes that have been linked to platinum resistance before, like CCNE1, BCL-2 and members of the MYCN-pathway. In contrast, the cisplatin-sensitive PDXs overexpressed none of these genes. More importantly, *in vivo* responses of PDXs were linked to clinical responses. Patients with a progression free survival longer than 6 months all had sensitive PDX models, while the majority of patients with a progression free survival shorter than 6 months had refractory PDXs (109). Recently, Weroha *et al.* published a study on a biobank encompassing 168 ovarian cancer PDX models together with clinical data and follow-up from corresponding patients (110). In a small subset of 4 resistant and 4 sensitive patients, it was shown that the PDXs displayed the same pattern of sensitivity to carboplatin/paclitaxel as their corresponding patients. The similar pattern of sensitivity towards carboplatin/paclitaxel was again shown in another study, also comparing response of ovarian cancer PDXs with the response of the patients they were derived from (111). A key point that needs further investigation is whether the stochastic process of resistance development in PDXs after repetitive platinum exposure is representative for resistance mechanisms observed in patients. Pinpointing these mechanisms of resistance will then also allow further investigation of the origin of resistance, i.e. acquired or intrinsic.

One of the biggest concerns regarding PDX models is the gradual replacement of human by murine stroma and vasculature, shown to occur already in the first generation within 3-9 weeks (110-112). Although human tumour-associated lymphocytes and fibroblasts remained present in established ovarian tumours after engraftment, these human stromal cells were mostly lost after several passages (113). By measuring production of human interferon-gamma, the responsiveness to human IL-12 injection of functional tumour-associated T-lymphocytes was detectable at least 100 days after engraftment,

indicating that the human tumour-associated T-lymphocytes stay viable and responsive despite changes in their micro-environment (113). However, the functionality of tumour-associated T-lymphocytes in further PDX generations was not studied. It is well accepted that tumour progression, response to chemotherapy and platinum resistance are significantly influenced by the ovarian tumour microenvironment (114-115). Therefore, observed responses to therapy in later PDX generations could be influenced by a lack of human stromal factors and could therefore be less representative. On the other hand, many growth factors are not species-specific, with mouse ligands able to bind human receptors and vice versa, possibly reducing this drawback. The lack of an intact immune system in these severe immune compromised mice (e.g. NOD/SCID-gamma, BALB/C nude mice) is considered to be another major drawback in studying the biological features of tumours and their response to therapy in the context of a chemotherapy-induced anti-tumour immune response or immunotherapy (73). However, it might be possible to study the anti-tumour activity of the patient's own immune system, by co-engrafting autologous T-cells matched to the primary ovarian tumour in the PDX model. By retrovirally transducing patient T-cells with artificial T-cell receptors, so-called chimeric antigen receptors (CARs), tumour-associated antigens can be targeted (116). These model systems could well be used for studying recently developed antibodies against co-factors like PD-L1 (117), but have not been applied in ovarian cancer models yet.

Amongst other reasons, PDX models are also considered to be more representative of the human tumour than cell line models, as they exhibit increased tumour heterogeneity because of the implantation of larger tumour pieces. However, it is likely that the complete heterogeneous spectrum of one ovarian tumour will never be fully represented by a single PDX model. A study by Hoogstraat *et al.* showed that treatment-naïve ovarian cancers display diverse genomic rearrangements and heterogeneous gene

expression profiles, present in only certain subsets of a tumour from a single patient (21). Using unsupervised hierarchical clustering, it was shown that the primary tumour at the ovary site displayed a completely different cluster of genomic rearrangements when compared to lesions from the omentum or peritoneum (21). This finding, together with the possible presence of a minority of primary resistant subclones in the chemo-naïve primary tumour, possesses significant challenges to use the PDX model as a representative model for the individual patient. Studying the attribution of heterogeneity to platinum resistance would require multiple sampling and the generation of multiple PDX models derived from different tumour sub-populations of the same patient. Despite the fact that PDXs might not cover the full heterogeneity of the parental tumour, observed responses were found to be grossly translational and therefore relevant. Humanisation of the immune system will be an important step to further advance PDXs models.

Platforms for genomic profiling and serial blood sampling in HGSOC.

The above-mentioned models are all used to select the most effective drugs, to study mechanisms of drug resistance and to test strategies to overcome drug resistance. However, it is obvious that one model will never recapitulate the full spectrum of ovarian cancer patients, if even subgroups. Therefore, patients' tumours and their models need to be carefully characterised at the genomic level. Genomic profiles of models can then be related to treatment sensitivity and resistance and compared to genomic profiles of patients. This could allow upfront selection of individual patients that will benefit from a specific treatment, based on the genomic profile of a patient's tumour.

In search for identifying subgroups of ovarian cancer patients with a different survival pattern or response to platinum-based chemotherapy, several studies on gene expression profiling have been published (10, 26, 118-121). It

is likely that changes in expression of gene sets belonging to different cellular pathways have more relevance in response than changes in single genes as identified by such arrays. Therefore, pathway analysis is indispensable (122). However, these studies are also criticised for their limited overlap with respect to individual genes, lack of reproducibility of predictive and prognostic gene profiles, low clinical use and high amount of experimental noise (123-124).

Not only gene expression arrays can be used to identify subgroups, but also mapping of recurrent amplification/deletions. For this purpose array-CGH can be used, although the number of publically available ovarian cancer samples is limited. As an alternative method, amplifications and deletions can be inferred using 'Functional Genomic mRNA (FGM) profiling', which removes the large majority of variation in gene expression and is a good proxy of copy number variation (125). By applying FGM profiling to the publicly available microarray gene expression data from 16,172 solid tumours, recurrent copy number alterations were identified in genomically unstable cancers such as HGSOC (n=1,255). Genes that were negatively associated with genomic instability included well-known genes such as *TP53*, *CDKN2A*, *RB1*, *BRCA1*, *BRCA2* and *ATM*, which are frequently inactivated in serous ovarian cancers. Genes positively associated with genomic instability were, among others, *MYC*, *CCNE1*, *PIK3CA* and *BIRC5* and are often found to be amplified in these cancers. Using this platform, high levels of genomic instability were related to a better progression free survival of HGSOC patients (125).

Identification of secondary genetic changes leading to platinum resistance is paramount for the selection of appropriate second-line therapy. However, in ovarian cancer patients, serial tumour sampling during treatment is invasive, entails complication risks, and so far has not been shown so far to contribute to better care. Therefore, this tool can only be used within the protection of clinical trials on identification of driving factors in resistance.

A less invasive method to analyse tumour characteristics with no need for serial biopsies, is exome sequencing of circulating cell-free tumour DNA (ctDNA) during treatment (126). In two cases of breast and ovarian cancer, copy number aberrations and mutations detected in ctDNA were shown to be largely representative for genomic alterations found in the metastases. For instance, during the course of treatment, in one patient, an increase in abundance of a truncating mutation in the *RB1* tumour-suppressor gene was found in both ctDNA and the biopsy taken from the metastatic tumour (126). Another promising possibility is the isolation of circulating tumour cells (CTCs) from patients peripheral blood (127). A high number of CTCs has already been shown to be an adverse prognostic marker in several solid cancers (128-130), while its prognostic value in ovarian cancer remains to be defined (131-135). Recently however, it has been shown that ovarian cancer spreads preferentially through haematogenous metastases to the omentum, emphasizing that CTCs are important cells to study and are pathogenic in ovarian cancer metastasis (136). By analysing ERCC1 mRNA expression in isolated CTCs, a subgroup with unfavourable prognosis and platinum resistant disease could be identified, which was not picked up by analysing ERCC1 expression in the primary tumour (132). Now that it has become possible to sequence the genome of a tumour up to the level of a single cell (137), CTCs could be used as a replacement not only for diagnosis and selecting therapeutic targets for certain mutations, but also for following mutational status over time during treatment, follow-up and relapse. Furthermore, these isolated CTCs can be cultured and later used to establish so-called CTC-derived xenografts (CDXs), which can serve as a platform for studying therapy response and therapy decision making, as has been shown in small-cell lung cancer and breast cancer (138-139).

To fully understand the mechanism of resistance, the occurrence of resistance in time has to be monitored in patients as well as in *in vivo* models. This

requires repeated sampling and thus more easily accessible tumour material, in which circulating tumour DNA or circulating tumour cells in blood might be of great help.

DNA repair capacity as a personalised response predictive marker

The identification of the synthetic lethal interaction between mutated *BRCA1/2* and the inhibition of PARP led to the clinical development of a novel type of drugs, namely PARP inhibitors (PARPi) (140-141). By exploiting the intrinsic error in the HR repair pathway, PARPi have already shown promising results in *BRCA1/BRCA2* mutation carriers (142-143). Furthermore, a 'BRCAness' phenotype is present in approximately half of the HGSOC cases, with defects in the HR pathway caused by other mechanisms than *BRCA1/2* mutations. The fact that *BRCA1/2* mutation carriers but also *BRCA1/2* wild-type patients can potentially benefit from PARPi, highlights a possible therapeutic gain in a potentially large group of ovarian cancer patients (144-145).

Treatment with PARPi has resulted in favourable responses in *BRCA1/BRCA2* mutation carriers, which led to the registration of olaparib (Lynparza) for this group of patients. Clinical trials with PARPi have shown effectiveness, improving progression-free survival not only in *BRCA1/2* mutation carriers (146), but also in platinum sensitive sporadic HGSOCS (147). Unfortunately, overall survival did not significantly differ when PARPi was given as maintenance therapy (147). These findings underscore the need for biomarkers to select patients that will benefit from a PARPi treatment (148). In future trials, HGSOC patients will receive platinum-based chemotherapy combined with PARPi as a first line treatment. However, only patients with HR-deficient tumours are likely to benefit and adequate patient selection is therefore warranted.

Selecting tumours with inactivated *BRCA1* could theoretically be done using immunohistochemistry, which would detect both germline, somatic as well as epigenetic mechanisms of *BRCA1* inactivation (149). However, immunohistochemical analysis of *BRCA1* does not correlate with either *in vitro* determined HR-status or response to therapy in patients (148, 150). As most commercially available antibodies against *BRCA1* are raised against the N-terminal part of the protein, truncating mutations in the *BRCA1* gene, conflicting with the function of the *BRCA1* protein, may not necessarily be picked up by immunohistochemistry. In addition, BRCAness can be caused by mutations in genes other than *BRCA1*, such as *BRCA2*, *PALB2* and others, and therefore a different methodology is needed that takes into account also other abnormalities in the HR pathway as well.

Rad51 foci formation in response to irradiation has been shown to be a reliable read-out for HR function, both in primary patient-derived epithelial ovarian cancer cells and PDXs. Importantly, the inability to form irradiation-induced Rad51 foci correlated with responses to PARPi *in vitro* (151-152). Furthermore, by treating *ex vivo* tumour slices with PARPi and determining their HR function by Rad51 foci formation, patients with HR-deficient tumours were shown to have a better response to platinum-based chemotherapy and a better progression-free and overall survival (150). This *ex vivo* approach offers a diagnostic assay for upfront selection of HGSOc patients with HR deficiency that may benefit from PARPi and/or platinum-based chemotherapy as first-line chemotherapy (Figure 1). Integration of this assay in future clinical trials investigating PARPi efficacy will hopefully allow a proper assessment of its predictive value.

Another approach to predict HR-deficiency is the development of a BRCAness gene expression profile associated with responsiveness to platinum and PARPi (153). First validated on a subset of tumours of known *BRCA1/2* germline

Table 1. Benefits and drawbacks of current models and platforms for investigating platinum response and resistance in ovarian cancer

Model	Possibilities	Drawbacks
Cell lines	<ul style="list-style-type: none"> • Low costs. • Very homogeneous. • Large panels of cell lines can be used to identify pathways of interest. • Short term culturing of primary ovarian cancer cell lines for cytotoxicity assays. • Many cell lines can be used as xenografts in mice 	<ul style="list-style-type: none"> • Lack of well defined ovarian cancer cell lines. • Lack of heterogeneity. • High passage numbers for many years in non-physiological conditions. • Frequently used cell lines are probably not derived from primary HGSOC. • Clinical predictive value of xenografts is low.
3D models	<ul style="list-style-type: none"> • Ovarian cancer co-cultures show other chemo- resistant phenotypes than normal cultured cell lines. • Cell migration and invasion are enhanced. • 3D tissue slices can be used for short-term cytotoxicity assays with incorporation of the microenvironment. 	<ul style="list-style-type: none"> • Complex and laborious culturing techniques requiring optimization. • <i>Ex vivo</i> slices depend on clinical collaboration. • Slices suitable for short-term assays only.
GEMMs	<ul style="list-style-type: none"> • An immunocompetent host in which tumour-host interactions can be studied. • Specific mutations can be introduced to study resistance mechanisms beyond this mutational status. • Orthotopic growth. 	<ul style="list-style-type: none"> • Long latency time until tumour growth. • Requires significant funding resources. • Orthotopic growth requires more complex visualization/measurement of the tumour. • Not fully reflecting heterogeneity and complexity of tumours. • Species-specific characteristics. • Multiple GEMMs are required to represent the spectrum of genetic tumour background that is observed in patients.
PDXs	<ul style="list-style-type: none"> • Mimic clinical response to platinum/taxol treatment. • Microenvironment is co-transplanted in the first generation. • Characteristics of the primary tumour are maintained over generations, including histology, gene expression, copy number changes and mutational status. 	<ul style="list-style-type: none"> • Heterogeneity is limited to amount of biopsies implanted. • Possible clonal selection during propagation. • Murine take-over of microenvironment and vasculature after the first passaging. • Requirement of significant funding resources.

Table 1. Benefits and drawbacks of current models and platforms for investigating platinum response and resistance in ovarian cancer

Model	Possibilities	Drawbacks
PDXs	<ul style="list-style-type: none"> • By sampling metastatic sites or relapsed tumours, factors involved in resistance can be investigated. 	<ul style="list-style-type: none"> • Requires a good cooperation between clinicians and researchers for obtaining primary material. • Immunocompromised mice will never fully recapitulate the complex crosstalk between the human immune system and the tumour. • Orthotopic implantation requires skilled technicians and more complex follow-up of the tumour.
Circulating tumour DNA and tumour cells (CTCs)	<ul style="list-style-type: none"> • Serial sampling over time makes it possible to track changes in the tumour during treatment. • CTCs are shown to be clinically relevant in the hematological spread of HGSOC. • Serial sampling over time makes it possible to track changes in the tumour during treatment. • Single cell sequencing makes it possible to investigate genetic changes over time during treatment. 	<ul style="list-style-type: none"> • No complete genetic and epigenetic profiles of the tumour can be obtained with this technique. • Numbers of CTCs are low • Current methods are not yielding all CTCs.
Genomic profiling	<ul style="list-style-type: none"> • Large amounts of microarray gene expression data are publicly available. 	<ul style="list-style-type: none"> • Pathway analysis is indispensable. • Lack of reproducibility and limited overlap of prognostic profiles. • Low clinical use. • High amount of experimental noise.

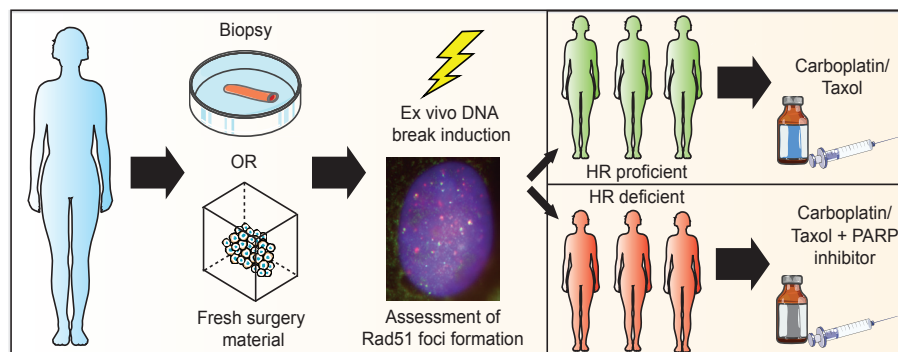


Figure 1. *Ex vivo* Rad51 foci formation in primary patient-derived epithelial ovarian tumours is a reliable readout for HR status and allows upfront selection of HGSOC patients with HR deficiency that will possibly benefit from PARPi treatment.

mutation carriers, this profile was able to distinguish a subgroup of sporadic HGSOC patients with a *BRCA-like* profile and a significantly longer disease-free and overall survival from sporadic HGSOC patients with a non-*BRCA-like* profile (153). Whether such a profile allows prediction for sensitivity to PARPi, and thus proper selection of eligible patients, must be elucidated prospectively in clinical trials.

Resistance to platinum-based chemotherapy and / or PARPi is clinically of great significance with several mechanisms proposed (154-155). One of the mechanisms of acquired resistance to platinum-based chemotherapy in patients with hereditary ovarian cancer encompasses the reversion of *BRCA1* and *BRCA2* mutations. Restoration of *BRCA2*-function as a mechanism of resistance first has been identified in CAPAN1 pancreatic cancer cells harbouring a *BRCA2* frameshift mutation. After clones of CAPAN1-cells resistant to cisplatin or PARP inhibition were obtained, it was found that cells had acquired secondary mutations restoring the open reading frame of *BRCA2* (156-157). Similar observations were made in *BRCA2* and *BRCA1*-mutated cancers in patients (156-158). By acquiring secondary mutations that restore the reading frame for *BRCA1* and *BRCA2*, these recurrent tumours had acquired resistance after first line carboplatin, in contrast to platinum

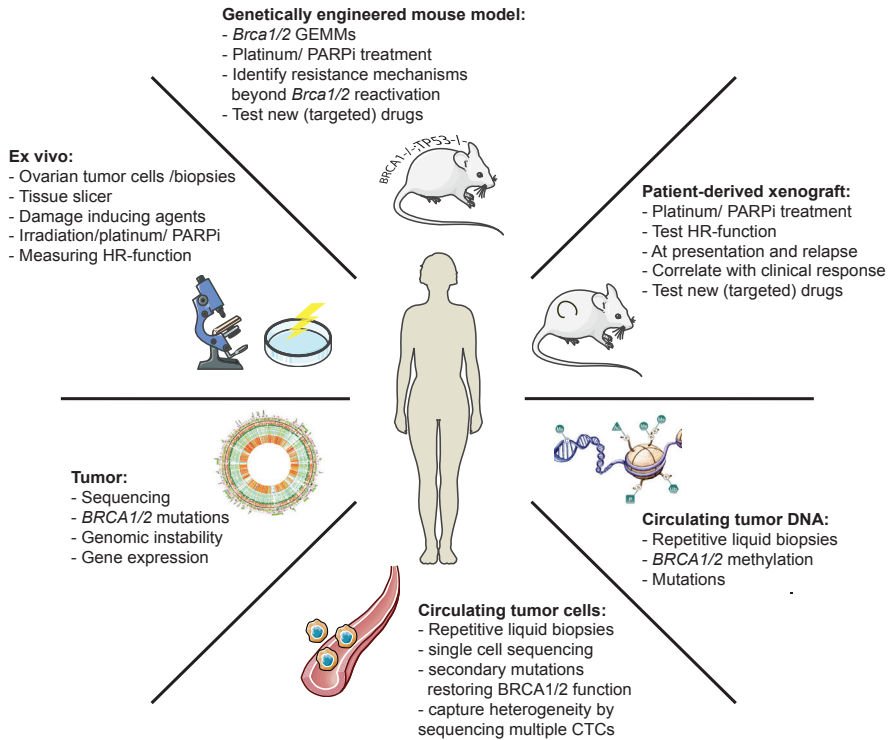
sensitive recurrences showing no secondary mutations in *BRCA1/2* (156-158). Furthermore, in a small subset of *BRCA1/2*-mutated patients, a secondary mutation at relapse appeared to be predictive for PARPi resistance in second line treatment (159). With only 6.3% of the *BRCA1/2* mutated ovarian cancers being primary resistant to platinum-based chemotherapy, and only one case of a secondary mutation restoring the open reading frame of *BRCA1*, restoration of *BRCA1/2* mutations seems to be rather rare and more involved in acquired resistance rather than in intrinsic resistance (159). Whether or not secondary mutations with restoration of *BRCA1/2* function also play a role in sporadic BRCAness ovarian cancer patients in developing platinum resistance remains to be elucidated.

Conclusions and future perspectives

Platinum resistance in HGSOc has been studied for decades without great impact on clinical outcome for these patients. Since the introduction of platinum-based chemotherapeutic regimens in the eighties and nineties of the last century, overall survival has been stable around 40%. Studying the mechanisms of acquired resistance requires multiple approaches combining pre-clinical *in vitro* experiments together with clinically obtained patient samples for *in vivo* and *ex vivo* analysis. In table 1, the pros and cons of the aforementioned models are summarised (Table 1). Recapitulating, *in vitro* research would benefit from the use of newly developed, better-defined ovarian cancer cell lines, which should represent different histological subtypes other than current widely used cell lines, such as A2780 and SKOV3. Furthermore, using *in vitro* 3-dimensional structures and incorporating micro-environmental factors may allow a better representation of patient responses towards platinum-based chemotherapy, which needs to be proven yet. To obtain a good reflection of the stochastic process of resistance as seen in the clinic, multiple and sequential sampling before and during treatment and at time of recurrence is necessary. However, in practice, this

is hard to incorporate due to ethical and practical reasons and will always have limitations because of sampling errors due to the heterogeneity in both primary tumours and metastatic sites in the individual patient. Resistance induction can be simulated in PDXs and in depth analysed at the molecular level. In the coming years, with the help of newly developed techniques to isolate circulating tumour cells and by analysing the mutational status of single cells or ctDNA with next generation sequencing, the appearance of resistant CTCs or mutations in ctDNA can be monitored in blood from PDXs and patients, allowing further investigation of the predictive value of PDXs. GEMMs can be used to selectively study mechanisms by patient-specific mutations of genes known to be involved in platinum sensitivity or resistance.

The incorporation of several recently developed models and platforms allows prediction of sensitivity towards platinum-based chemotherapy and the identification of subgroups among ovarian cancer patients that could potentially benefit from PARPi (Figure 2). In the future, the focus should be on identifying more subgroups with associated appropriate treatment strategies. By using an integrated approach of *in vitro* and *ex vivo* investigations, PDXs and GEMMs, and CTC- and ctDNA-based biomarker development, upfront selection of eligible patients will be possible.



2

Figure 2. Incorporation of recently developed models and platforms for investigating sensitivity towards platinum-based chemotherapy and PARP inhibition.

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