RNA splicing alteration in the response to platinum chemotherapy in ovarian cancer: A possible biomarker and therapeutic target

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Abstract
Since its discovery, alternative splicing has been recognized as a powerful way for a cell to amplify the genetic information and for a living organism to adapt, evolve, and survive. We now know that a very high number of genes are regulated by alternative splicing and that alterations of splicing have been observed in different types of human diseases, including cancer. Here, we review the accumulating knowledge that links the regulation of alternative splicing to the response to chemotherapy, focusing our attention on ovarian cancer and platinum-based treatments. Moreover, we discuss how expanding information could be exploited to identify new possible biomarkers of platinum response, to better select patients, and/or to design new therapies able to overcome platinum resistance.

Keywords
alternative splicing, clinical trials, DNA repair, drug uptake and detoxification, ovarian cancer, peritoneal dissemination, platinum chemotherapy

Abbreviations: ABCC, ATP binding cassette transporter family; AS, alternative splicing; ASO, antisense oligonucleotides; AR, androgen receptor; BARD1, BRCA1-associated RING domain protein; BEV, anti-VEGFA antibody Bevacizumab; BRCA1-IRIS, in-frame reading of BRCA1 intron 11 splice variant; CRPC, castration-resistant prostate cancer; EOC, epithelial ovarian cancer; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; FA, Fanconi anemia; GST, glutathione S-transferase; HE4, human epididymys protein 4; hnRNPs, heterogeneous nuclear ribonucleoproteins; HR, homologous recombination; HRD, homologous recombination deficiency; ISE, intronic splicing enhancer; ISS, intronic splicing silencer; NER, nucleotide excision repair; NHEJ, nonhomologous end joining; PARPi, poly-ADP-ribose polymerase inhibitor; pADPr, poly-ADP-ribosylation; PK, pyruvate kinase; PRMT5, protein arginine N-methyltransferase 5; PSF, PTB-associated splicing factor; PT, platinum; PTB, polypyrimidine tract binding proteins; SF, splicing factor; SFPQ, splicing factor proline-glutamine-rich; SMA, spinal muscular atrophy; SOCS-OSA, sodium channel splicing in obstructive sleep apnea; SR, serine-rich; SRPK, SR-rich protein specific kinase; SSOs, splicing-switching oligonucleotides.
1 | INTRODUCTION

In the late 70s, it was firstly reported that viral pre-mRNAs and, soon after, the ones of the kappa chain of immunoglobulins, underwent a process called alternative splicing (AS). From the very beginning, AS was proposed as a powerful mechanism to amplify the genetic information of an essentially linear genome. Indeed, we now know that AS regulates the expression of more than 90% of human genes and that an altered control of AS has been linked, in a direct or indirect way, to many pathological human conditions.

Several evidences connect the regulation of AS to the onset and progression of human tumors and to anticancer treatment response, including chemotherapy.

Among the different chemotherapeutic agents, platinum-based chemotherapy is widely used in the treatment of patients with different cancer types, including lung, ovarian, colon, breast, head and neck, and testicular cancers. In almost all cancer types, platinum-based chemotherapy is generally active as first-line treatment, but it is frequently followed by the appearance of platinum-resistant recurrences.

The molecular mechanisms underlying acquired and/or de novo platinum resistance have been excellently recently reviewed. Platinum mechanism of action includes drug internalization and nuclear import, followed by DNA binding and inter and intrastrand crosslink formation. Each one of these steps could be involved in resistance outcomes, essentially through (1) altered drug intake or expulsion, (2) increased drug inactivation, and (3) altered DNA damage response. Since the normal outcome for platinum-treated cells is the induction of apoptosis, it is conceivable that also (4) deregulation of pathways controlling cell death and survival can be considered bonafide mechanisms of resistance to platinum.

Here, we review the literature regarding the role of AS in the response to platinum-based chemotherapy. In particular, we will focus on the possible role of AS in ovarian cancer, a tumor in which platinum-based chemotherapy represents the standard of care and for which the response to platinum not only predicts the prognosis, but also dictates the subsequent lines of treatment.

We discuss evidences that describe, on one side, how platinum treatment regulates AS and, on the other, how alterations in AS modify the DNA damage response induced by platinum.

Next, we outline the main splicing events described in ovarian cancer and comment on how they could be connected to the response to platinum-based chemotherapy.

Finally, we comment on potential ways of intervention, reporting the clinical trials that are currently testing if targeting the AS is a feasible and an effective approach to fight cancer.

2 | BRIEF NOTES ON ALTERNATIVE SPLICING REGULATION AND ITS DEREGERULATION IN CANCER

AS is a highly regulated process that expands the potentiality and the complexity of the transcriptome, proteome, and signal transduction networks, by creating several different proteins from a single precursor mRNA. The complex molecular machinery that controls splicing events has been exhaustively reviewed elsewhere.

In this paragraph, we will only provide a brief description of which are the actors playing a role in the AS events, focusing on examples with clear involvement in cancer.

The regulation and selection of the splice sites are operated by the splicing regulators. These proteins represent activator and repressor proteins that bind to specific sequences of the pre-mRNA, known as intronic/exonic splicing enhancers (ISEs and ESEs) and intronic/exonic splicing silencers (ISSs and ESSs). The recognition of splice sites occurs at the spliceosome, an RNA–protein complex that eventually determines which intron end will be cut out and which exon end will be retained. ISS and ESS sites are bound by heterogeneous nuclear ribonucleoproteins (hnRNPs) that act as splicing repressor proteins and reduce the probability that a nearby site will be used as a splice junction. ISE and ESE are the binding sites for splicing enhancers, usually members of the SR
(serine-rich) protein family, that act as activator proteins and increase the probability that a nearby site will be used as a splice junction.\textsuperscript{17}

Approximately 95% of human protein-coding genes produce multiple mRNA isoforms through AS.\textsuperscript{18} This process seems to be particularly relevant in cancer, since tumors display up to 30% more splicing events than normal tissues.\textsuperscript{19}

Different AS events have been identified (Figure 1) and many of them, if not all, have been associated with cancer.

The use of alternative promoters (Figure 1A) regulates and increases the transcriptional and translational potential of many genes.\textsuperscript{20} Well-known tumor suppressor genes exploit alternative promoters. For instance, CDKN2C (p18\textsuperscript{INK4c}) mRNA has multiple promoters whose usage leads to the production of the same protein with different 5′-UTRs. When the shorter UTR is present, even if the mRNA level remains unchanged, p18\textsuperscript{INK4c} protein expression is increased by ∼50-fold contributing to cell cycle arrest and differentiation.\textsuperscript{21} Also the tumor suppressor TP73 (p73), a member of the TP53 (p53) family proteins\textsuperscript{22} is largely regulated by AS and, in particular, by the use of alternative promoters. Of particular interest, ΔNp73 protein, originating from a different promoter, has an opposite function to the full-length protein and represents an endogenous dominant-negative regulator of both p73 and p53.\textsuperscript{20,22} Another peculiar example is the regulation of CDKN2A gene locus, in which the use of alternative promoters results in the transcription of either exon 1α or exon 1β, that are then translated with two alternative reading frames. This leads to the generation of two different tumor suppressor proteins: p16\textsuperscript{INK4A}, an inhibitor of the cyclin-dependent kinases CDK4 and CDK6, and p19\textsuperscript{ARF}, an inhibitor of MDM2 activity. Thanks to the use of alternative promoters, the CDKN2A gene encodes two proteins that can fully control both pRB and p53 pathways.\textsuperscript{23–26} Not surprisingly CDKN2A is one of the most frequently inactivated locus in human cancers.\textsuperscript{23}

Exon inclusion or exclusion (Figure 1B) is regulated by the presence of ESE or ESS sequences.\textsuperscript{6} Interestingly, in human cancer, synonymous mutations frequently reside within the 30 base pairs of exon–intron junctions, leading to increased exon exclusion/inclusion events.\textsuperscript{27,28} One notable example regards the HER2 oncogene, frequently overexpressed in breast, gastro-esophageal and colorectal cancers.\textsuperscript{29–31} The AS of ERBB2 gene (encoding for HER2) can induce the exclusion of exon 16, leading to the production and coexpression with the full-length HER2 of the so-called Δ16HER2 isoform.\textsuperscript{32,33} Δ16HER2 displays higher receptor dimerization, increased resistance to the most common anti-HER2 therapy, trastuzumab, in vitro, and increased transforming and metastatic ability in vivo.\textsuperscript{32,34–36} It is worth noting also the exon 6 inclusion of the MDM4 that results in MDM4 overexpression and p53 degradation in cancer since this AS has been targeted with specific antisense oligonucleotides (ASOs) to reduce cancer cell growth, both in vitro and in vivo.\textsuperscript{37}

The use of mutually exclusive exons (Figure 1C) is a rare form of AS, which affects only ∼1% of transcripts. This AS mechanism, thoroughly reviewed by others,\textsuperscript{38} has not been fully studied in cancer, although some evidences suggest that it could participate in the control of cancer cell metabolism, by regulating the expression of pyruvate kinase (PK) isoforms.\textsuperscript{39} PK has four isoforms, produced from two distinct genes, PKLR and PKM. The PKM gene encodes for two isoforms, PKM1 and PKM2, produced through different exon usage. Most adult tissues express PKM1 that promotes oxidative phosphorylation, while PKM2 that promotes aerobic glycolysis is more abundantly expressed during embryonic development.\textsuperscript{39} Several data suggest that human tumors exclusively express the PKM2 isoform.\textsuperscript{39,40} The choice to include exon 9 (PKM1) or exon 10 (PKM2) is due to the expression of hnrNP positively regulated by oncogenic transcription factors, such as c-Myc,\textsuperscript{41} ultimately connecting an oncogene expression to the tumor metabolic phenotype.

Another important mechanism of AS is intron retention (Figure 1D), observed in human, animal, and plant cells and related to several human diseases, including cancer.\textsuperscript{32,42} Intron retention is physiologically observed during differentiation and development in immune cells and neurons and its use facilitates the dynamic cell response to biological stimuli. About 20% of all somatic exonic SNVs that impair AS cause intron retention or exon skipping.\textsuperscript{42} However, the full understanding of all biological functions played by intron retention remains quite challenging.\textsuperscript{43} In cancer, intron retention is enriched in tumor suppressor genes, often generating a premature termination codon with consequent loss of function.\textsuperscript{44} Not surprisingly, the highest rate of intron retention was observed in the TP53 tumor suppressor gene.\textsuperscript{44}
FIGURE 1  (Continued)
Finally, the use of alternative splice-acceptor-site selection (Figure 1E) induces the formation of proteins with partially different sequences and functions. One notable example in cancer is the exon 8 of the VEGFA gene. This AS event generates specific forms of VEGFA, named VEGFA\textsubscript{xxxab}, that differ from the VEGFA\textsubscript{xxxxa} isoform only for the last six amino acids but display antiangiogenic rather than proangiogenic activities, possibly resulting in different tumor growth, spreading, and response to therapies.

It is worth noting that intron retention, mutually exclusive exons, exon inclusion, and exclusion, could all result in alternative RNA polyadenylation. This RNA processing mechanism generates distinct 3’ termini and represents a powerful mechanism to control gene expression in a tissue- and context-specific manner. Although we will not go into the details of the regulation of alternative polyadenylation regulation, recent evidences clearly show that its deregulation can profoundly affect tumor growth and/or their response to treatments, especially in ovarian cancer models.

Overall, current literature converges on the possibility that AS represents a dynamic way that cancer cells exploit to grow, adapt, metastasize, and survive, even under the pressure of therapies. Accordingly, newly released data collected from 2658 cancer whole genomes and complemented by 1188 transcriptomes (the PCAWG data sets), strongly support the possibility that specific cancer driver alterations "manifest themselves via changes in RNA rather than DNA sequence mutations." Integrative pathway and network analysis evaluating coding and noncoding driver mutations annotated in the PCAWG data sets, pointed to splicing as one of the most perturbed networks in cancer. Further, since splicing is mainly affected by noncoding mutation (i.e., mutations in enhancers, promoters, and 3’-UTR regions) and most of the studies evaluating gene mutations have focused on coding regions, it is highly predictable that the overall perturbation of AS in cancer has been so far largely underestimated.

3 | ALTERNATIVE SPLICING AND THE RESPONSE TO PLATINUM

Among anticancer therapies, platinum-based chemotherapy is one of the most widely used in the clinic, since it is included in the treatment regimens of many different cancer patients. Understanding if AS impacts on the cellular response to platinum and/or if platinum itself may affect AS could represent an important step toward the full comprehension of platinum mechanism of action and, therefore, toward the optimization of its administration.

In this paragraph, we will review what is known on the interplay between AS and the cellular response to platinum. We will discuss the evidences suggesting that platinum may regulate AS, and the ones indicating that alteration of AS could modify the response to platinum.

3.1 | Platinum affects the alternative splicing

Original evidences in vitro demonstrated that cis-platinum and its clinically active derivatives (hereafter referred to globally as PT) were able to inhibit the formation of the spliceosome and the splicing activity, supporting the
possibility that PT may directly impact on AS regulation. These data, collected using nuclear extracts from HeLa cells and analyzing the human β-globin pre-mRNA, are in accord with subsequent proteomic studies that demonstrated the ability of PT to modify the expression of a substantial number of proteins implicated in the regulation of mRNA splicing.\textsuperscript{53,54}

More recently, Lambert et al. demonstrated that PT induced alteration in 717 splicing events in breast cancer cells. Among these, 34% were exon inclusion/exclusion and 34% alternative 5’ or 3’ splice site selection events. Other relevant AS events involved alternative promoter and alternative terminations (20% and 11%, respectively).\textsuperscript{55}

Overall, these studies support the possibility that PT treatment directly modifies the regulation of AS. It is therefore conceivable that AS regulation could contribute to the response of cancer cells to PT-based chemotherapy.\textsuperscript{56} The picture emerging from the current literature suggests that each chemotherapeutic drug specifically induces AS of selected genes with only partial overlap with other drugs.\textsuperscript{56} For instance, splicing alterations involving apoptotic genes induced by the Topoisomerase I inhibitor Camptothecin mainly regard caspase-2,\textsuperscript{57,58} while PT induces the AS of caspase-8, favoring the expression of its proapoptotic form.\textsuperscript{59}

Although it is now quite established that PT can impact on AS in cancer cells, the understanding of how this happens is still unclear (Figure 2). Selective redistribution of specific splicing factors has been observed in cells treated with PT and this relocalization could contribute to changes in AS of specific genes.\textsuperscript{60} This evidence is in accord with the above-mentioned proteomic analyses, showing an alteration of splicing factor expression in the nucleus of PT-treated cells.\textsuperscript{53,54} How PT can regulate the expression and localization of the splicing factors is unclear. One possibility is that PT-induced DNA damage activates intracellular pathways that in turn affect the expression, localization, and activity of specific splicing factor(s).\textsuperscript{61} Accordingly, it has been reported that PT regulates the expression and function of two kinases that phosphorylate splicing factor members of the SR protein family, namely SRPK1 and SRPK2. SR phosphorylation modifies their splicing activity and, thereby, the sensitivity of tumor cells to PT-induced death.\textsuperscript{59,62} Similarly, the splicing factor proline-glutamine-rich (SFPQ; a.k.a., PTB-associated splicing factor, PSF), which is also regulated by phosphorylation for its RNA binding and splicing activities,\textsuperscript{63,64} has been associated with PT-resistance when expressed at high levels in liver and ovarian cancer.\textsuperscript{65,66} However, since SFPQ is a multifunctional protein it is still unclear whether its role in PT resistance is specifically linked to its splicing activity.

Other evidences suggest that PT treatment leads to splicing factor acetylation. It has been shown that in PT-treated cells SRSF2 accumulates in the nucleus in a hypo-acetylated form. This is due to the deacetylation of

![FIGURE 2](image_url)

**FIGURE 2** Effects of platinum on alternative splicing regulation. Platinum (PT) treatment can induce several types of alteration in splicing factors (SFs), including alteration of their expression and localization and/or induction of specific posttranslational modification, such as phosphorylation, acetylation, parylation, etc. It is still unclear whether the pressure of PT treatment can also induce the appearance of novel mutations in SF.
SRSF2 by HDAC6 that leads to protein stabilization, allowing cells to survive from PT-induced apoptosis. More recently, it has been shown that PT also induced the acetylation of the serine-arginine protein kinase 1 (SRPK1). SRPK1 acetylation was associated with its cytoplasmic localization and PT sensitivity. Conversely, in PT-resistant breast cancer cells, PT treatment reduced SRPK1 acetylation favoring the expression of antiapoptotic splicing variants of several genes. These evidences are in good accord with the general knowledge that HDACs interact with the spliceosome and participate in the control of the acetylation states of splicing factors.

Thus, converging evidences suggest that PT-treatment directly regulates acetylation and phosphorylation of SRSF2 and SRPK1 which had opposite effects on their protein stability and subcellular localization. In this way PT could directly regulate the splicing activity.

It is well known that PT induces wide poly-ADP-ribosylation (pADPr or parylation) and that PARP1 participates to the PT-induced DNA damage response. Interestingly, PARP1 regulates the parylation of hnRNPs and this modification inhibits their RNA-binding ability. Besides, PARP1 could directly regulate the binding of specific splicing factors (e.g., SF3B1) to nascent pre-mRNA, thereby playing a direct role in the regulation of pre-mRNA splicing. Yet, the possibility that PT-induced parylation could modify the splicing of selected genes will need to be formally demonstrated in the future.

Another intriguing observation that links PT to AS has been recently reported on the SRSF2 gene, analyzed in 130 epithelial ovarian cancer (EOC) samples. An enrichment in SRSF2 mutations was observed in samples from PT-treated patients, compared to the untreated ones. Although the number of PT-treated patients was relatively low ($n = 14$), the percentage of SRSF2 mutations was significantly higher compared to PT-naïve ones (2/14, 14.3% vs. 3/116, 2.6%, respectively).

Overall, literature data support the possibility that PT could impact on the regulation of AS in several ways and that more than one molecular mechanism contributes to PT ability to modify RNA splicing. However, more studies will be needed to clarify which of these mechanisms is predominant in each type of cancer.

### 3.2 Alternative splicing in PT-induced DNA repair

It is known that cellular treatment with PT essentially induces three main types of DNA damage, namely intrastrand adducts, interstrand crosslinking, and double-strand break. Each type of DNA damage is preferentially restored by one specific mechanism of DNA repair, as reviewed by Rocha et al. and O’Connor and schematically depicted in Figure 3. Here, we will provide only some examples related to genes belonging to each one of these repair pathways and for which AS could change the response to PT.

Homologous recombination (HR) is the most common DNA repair pathway activated by PT-treated cells. A central role in HR and in Fanconi anemia (FA) DNA repair pathway is played by the BRCA1 and BRCA2 genes, whose ablation or mutation results in HR deficiency. Accordingly, breast and ovarian cancers carrying BRCA1/2 alterations are particularly sensitive to PT-based chemotherapy. BRCA1 is a gene that undergoes massive AS and it is known that different expression of naturally occurring BRCA1 splicing isoforms affects PT-sensitivity. For instance, the expression of p150 BRCA1-IRIS (in-frame reading of BRCA1 intron 11 splice variant) has been associated with PT-resistance in ovarian cancer cells. Of note, PT-treatment itself can induce the expression of p150 BRCA1-IRIS spliced form. Recent evidences also suggest that cancer cells could use AS as a strategy to remove deleterious germline BRCA1 mutations under the pressure of PT treatment. Similar observation has also been made for BRCA2.

The activity of BRCA1 is tightly regulated by its binding partner BARD1 (BRCA1-associated RING domain protein). As BRCA1, also BARD1 heavily undergoes AS, thereby generating several splicing variants that can display increased or decreased sensitivity to PT treatment, at least in vitro.

More recently, it has been reported that many HR genes, including ATM, BRCA2, and FANCD2, have multiple intrinsic polyadenylation sites that could lead to alternative polyadenylation and, consequently, to lower levels of
This process, activated by CDK12 inhibition/dysfunction, could be mediated by AS regulation and results in the acquisition of homologous recombination deficiency (HRD). This biological condition clinically phenocopies the loss of BRCA1/2 and results in increased sensitivity to PT and to PARP inhibitors.\(^\text{77,78}\)

The FA pathway plays a central role in the repair of DNA interstrand crosslink induced by PT. The first step for the FA pathway activation is represented by the localization of the FANCD2–FANCI complex at the site of damage, where FANCD2 is monoubiquitinated allowing the assembly of the repair complex.\(^\text{74}\)

FANCD2 monoubiquitination is tightly controlled and its deubiquitination is necessary to delocalize FANCD2 from the chromatin and allow the DNA repair pathway to proceed.\(^\text{84}\) It has been proposed that AS deregulation may interfere with the regulation of this post-translational modification. USP1 is the deubiquitinase that removes a ubiquitin moiety from the monoubiquitinated FANCD2.\(^\text{84}\) High USP1 expression leads to increased PT-resistance in different tumor types\(^\text{85,86}\) and increased expression of USP1 in PT-resistant cells may be achieved by the usage of alternative promoters.\(^\text{85}\)

Another mechanism of DNA damage repair is the nucleotide excision repair (NER). After the recognition of DNA adducts, the activation of NER eliminates DNA lesions by sequential cut and patch reactions. The short single-stranded DNA segment containing the lesion is removed and the undamaged single-stranded DNA is used by DNA polymerase as a template, to synthesize a short complementary sequence.\(^\text{87}\) Interestingly, AS of the endonuclease ERCC1, which catalyzes the excision reaction, correlates with a reduction in the cellular capability to repair PT-induced DNA adducts.\(^\text{88}\) Also XPG (ERCC5) and XPF (ERCC4) endonucleases can be transcribed in several splicing variants, differently expressed in human tissues and displaying different repair abilities following exposure to PT.\(^\text{89,90}\)

Nonhomologous end joining (NHEJ) is the DNA repair pathway involved in the resolution of double-strand breaks in cells that are mainly in the G1 phase of the cell cycle. NHEJ requires the activity of the DNA-PK complex, which recognizes the DNA break and catalyzes the end processing, then ligated by the activity of XRCC4 and DNA ligase IV.\(^\text{91}\) Two splice variants of the catalytic subunit of DNA-PK (DNA-PKcs) are known to be NHEJ-defective and can impair the cell response to radiation.\(^\text{92}\) These splicing variants are conserved along the

![Image of Figure 3](image)

**FIGURE 3** Impact of alternative splicing on DNA repair pathways. Platinum (PT)-induced DNA damages (intra or interstrand adducts and double-strand breaks) are repaired mainly by four DNA repair pathways, including nucleotide excision repair (NER), Fanconi anemia (FA), homologous recombination (HR), and nonhomologous end joining (NHEJ). Alternative splicing of the indicated genes, belonging to the indicated pathway, can alter their DNA repair activity and, as a consequence, PT-sensitivity. Relevant references are reported on the right.
evolution and expressed in normal tissues and cancer cells. Whether they are also involved in the response to PT is still to be investigated.

Although nonexhaustive, these examples clearly show that AS of genes involved in DNA repair pathways may actually influence the response to PT, indicating that AS could play a central role in dictating not only the type of DNA damage response but also the survival of cancer cells.

3.3 Alternative splicing in the regulation of PT-induced apoptosis

The fact that AS strongly regulates apoptosis is well established and has been recently reviewed.\textsuperscript{93,94} It is known that DNA damage, including the ones induced by PT, mainly activates the intrinsic apoptotic pathway, initiated by mitochondrial permeabilization and leading to caspase activation, principally through the activity of caspase-8 and -9.\textsuperscript{59,95} Like most conventional chemotherapeutic agents, PT elicits mitochondrial permeabilization by increasing the concentration of pro-apoptotic second messengers, such as p53 and Bcl-2-like proteins, and/or altering the redox balance.\textsuperscript{95}

p53 and its family members p73 and p63 undergo extensive splicing regulation and play a central role in PT-induced cell death. The p73\textsuperscript{Deltaexon2} isoform of p73 reduces the ability of p53 to promote apoptosis, by competing with p53 and acting as a dominant-negative form of it.\textsuperscript{96} Interestingly, endogenous p73\textsuperscript{Deltaexon2} expression is downregulated by PT in p53 wild-type cancer cells,\textsuperscript{97} supporting the existence of a positive regulation loop. High expression of the p53 splicing isoform Delta40p53 has been associated with PT-resistance.\textsuperscript{8} Delta40p53 (also known as p47 and DeltaNp53) is an N-terminal truncation of full-length p53 that lacks the first transcriptional activation domain (TAD1). Initial studies reported that Delta40p53 acts as a dominant-negative regulator of p53, but its functions seem to be more complex and cellular context-dependent.\textsuperscript{98}

It is largely accepted that PT treatment induces apoptosis via activation of caspases.\textsuperscript{95} Only a few reports have addressed the role of caspase splicing in the response to PT. As mentioned above, some evidences suggest that PT can impact on caspase splicing, ultimately determining whether the pro or antiapoptotic forms will be expressed.\textsuperscript{59,99,100} Yet, with the exception of a proved antiapoptotic role for the caspase-2 short form (caspase-2S) and caspase-9b spliced forms, no other data formally demonstrate a specific role for AS of caspases in the response to PT. Recently, we observed that PT treatment in EOC cells induces a rapid increase of caspase-9 proapoptotic form that paralleled the induction of DNA damage and preceded the appearance of cell death, suggesting that it played a causative role in PT-induced death.\textsuperscript{66} Accordingly, the use of a specific caspase-9 inhibitor protected cells from PT-induced apoptosis.\textsuperscript{66} This is, of course, a field that calls for further investigations.

3.4 Alternative splicing in uptake, detoxification, and expulsion of PT

A pivotal role in the response to PT is played by the pathways governing the uptake, detoxification, and expulsion of the drug.\textsuperscript{7,74} PT can enter the cells by passive diffusion or by active transport, principally mediated by the copper transporter CTR1 (SLC31A1). Possible additional roles in PT-uptake have been proposed for two other copper transporters, namely CTR2 (SLC31A2) and OCT3 (SLC22A3). To our knowledge, no data regarding the AS of these genes in the response to PT have been reported so far, although different splicing isoforms of CTR2 have been described.

PT expulsion is mainly due to anion transporters ATP7A, ATP7B, and ABCC1 (MRP1). The ATP7A gene encodes for the ATPase copper transporting Alpha that is able to expulse PT and its overexpression has been linked to PT-resistance in vitro and in vivo.\textsuperscript{101,102} Albeit no study has investigated whether AS of ATP7A may play a role in the response to PT, it is interesting to note that alterations in the ATP7A gene are the cause of Menkes disease, an X-linked, multisystemic lethal disorder leading to altered metabolism and copper deficiency. Among the
different ATP7A gene alterations observed in patients with Menkes disease, splice site mutations account for 22% of the cases.\textsuperscript{103,104} This evidence suggests that splicing of ATP7A could also contribute to the response to PT and/or the onset of PT-resistance, a possibility that certainly merits to be better investigated. Similar evidences are present for structurally and functionally highly homologous copper-transporting ATPase ATP7B, whose mutations, including those at splice sites, are the cause of Wilson disease, another copper metabolism disorder leading to an excess of copper stored in tissues.\textsuperscript{105,106} ATP7B has been also associated with PT resistance, although the role of AS in this function of ATP7B has not been determined yet.\textsuperscript{107} The ABCC1 (MRP1) gene, a prototypical member of the ATP binding cassette transporter family (ABCC), has been associated with the resistance to several drugs, including PT.\textsuperscript{108} ABCC1 undergoes AS, and in ovarian tumors, the expression of spliced forms seems to be higher than in matched normal tissues.\textsuperscript{109} Their effects on cell sensitivity to PT have not been tested yet but, interestingly, some of these splice variants confer resistance to doxorubicin.\textsuperscript{109} PT can be inactivated by intracellular thiol-containing molecules, such as glutathione S-Transferase (GST) and metallothioneins.\textsuperscript{9,74,110} However, the role of GST or metallothioneins AS, in response to PT, has not been described yet.

Overall, some available evidences suggest that AS might be implicated in the regulation of PT uptake/inactivation pathways, particularly in drug-efflux, but more studies will be necessary to confirm these hypotheses.

4 | ALTERNATIVE SPLICING IN EPITHELIAL OVARIAN CANCER

EOC is a relatively rare but highly lethal disease, representing the fifth cause of cancer death in women in developed countries.\textsuperscript{13} The high mortality-to-incidence ratio is mainly due to late diagnosis, to the frequent appearance of chemoresistance, and to the peculiar and deceitful way this cancer disseminates.\textsuperscript{13} In this paragraph, we will briefly outline the evidence linking alteration of AS with the main aspects that represent clinical unmet needs for EOC patients’ management (Figure 4), with a final consideration about the possible role of splicing in contributing to the observed racial/ethnic differences. Indeed, both diagnosis and treatment of EOC patients are influenced by racial/ethnic differences. For instance, although the incidence of EOC is higher among Caucasian women, African American women display worse prognosis compared to other racial/ethnic groups.\textsuperscript{111} This worse prognosis seems however more likely linked to unequal access to care and receipt of treatment, later diagnosis, higher residual disease after surgery, and presence of comorbidities (e.g., obesity) than to specific identified molecular factors.\textsuperscript{111,112}

4.1 | Alternative splicing in EOC late diagnosis

At the time of diagnosis, the large majority of ovarian cancer patients present with already disseminated disease (>70%).\textsuperscript{13} Late diagnosis is mainly due to the absence of symptoms until the disease is largely disseminated in the abdomen and to the fact that these symptoms are generic. Considerable efforts have been made to implement screening of the general population for early EOC detection, but no feasible strategy has been identified so far.\textsuperscript{13} Serum evaluation of CA-125 and human epididymis protein 4 (HE4), two glycoproteins secreted by EOC cells, is very useful to follow the disease evolution in response to therapies\textsuperscript{13} but has failed in the setting of early diagnosis, either alone or in combination with an echography.

CA-125 is a high-molecular-weight glycoprotein expressed by the endometrium while HE4 is a glycoprotein primarily expressed in the reproductive and respiratory tracts.\textsuperscript{113} Both glycoproteins are overexpressed in ovarian cancer and their serum dosages have proved of clinical utility in the follow-up of EOC patients.\textsuperscript{114}

Intriguingly, both MUC16 (the gene encoding for CA-125) and HE4 undergo AS and it has been proposed that AS of these genes may not only enhance their complexity but also impact their ability to function as
In particular, it has been reported that antibodies recognizing different spliced regions of HE4 may display different levels of sensitivity in detecting the tissue and serum protein in different patients. These observations suggest that evaluating the expression of circulating CA-125 and/or HE4 specific spliced forms could help to define a better assay for the early EOC detection. More studies are needed to evaluate this possibility.

Another hint suggesting the potential role of AS in early detection strategies comes from the integrated analyses of normal and ovarian cancer via RNASeq revealed that cancer-specific mRNA isoforms exist and could be used for improving diagnosis and/or select new targeted therapies. In contrast to what seen by gene expression profiling studies, the authors did not observe AS profile similarities between breast basal-like tumors and serous EOC. The possibility to use cancer-specific mRNA spliced isoforms for a more specific ovarian cancer diagnosis has been also previously proposed, using specific qRT-PCR analyses on 600 cancer-associated genes. Therefore, this diagnostic approach could be further explored for a possible transferability in the clinical setting.

**FIGURE 4** Key cellular pathways altered by alternative splicing in ovarian cancer. Misregulation and differential expression of splicing isoforms can contribute to ovarian cancer progression. Key cellular pathways, influenced by alternative splicing, are depicted (examples of genes in each pathway are indicated). Alterations in drug intake/expulsion, DNA damage response, apoptosis, and adhesion processes can lead to the main clinical issues currently encountered in EOC patients’ management, that is, chemo (PT)-resistance and metastatic dissemination. EOC, epithelial ovarian cancer.
The evaluation of splicing events in selected genes was also used to better predict the prognosis of EOC patients.\textsuperscript{119} Using the top 20 survival-associated splicing events, it was demonstrated that they can predict patients’ survival with an AUC of 0.93 in ROC analyses.\textsuperscript{119} Similarly, AS events have been used to predict chemoresistance in ovarian cancer,\textsuperscript{120} overall suggesting that AS deregulation could be explored as a biomarker at different stages of EOC disease. One possible limitation of these large scale studies is that they were almost all based on RNASeq analyses of high-grade serous ovarian cancer samples, performed by the TCGA consortium.\textsuperscript{121} Therefore, it would be important to expand the analyses to other histotypes and to different cohorts of samples possibly, from patients included in clinical trials with translational endpoints.

### 4.2 Alternative splicing in EOC chemoresistance

In EOC patients, the response to first-line therapy divides the patients in PT-sensitive and PT-resistant and is a strong indicator of prognosis, dictating the subsequent lines of therapy. EOC patients are usually sensitive to first-line PT-based chemotherapy. Yet, more than 75% of PT-sensitive EOC patients later develop a PT-resistant recurrent disease.\textsuperscript{13} The extent of AS implication in the development of acquired or de novo PT-resistance is still unclear. However, AS alterations in genes involved in DNA damage response have been frequently observed in EOC, suggesting a possible role for AS in the acquisition of PT-resistance. This field certainly deserves a deeper investigation in the future.

Using an unbiased shRNA screening, we recently identified the splicing factor SFPQ as a critical molecule for the survival of EOC cells after PT-induced apoptosis.\textsuperscript{66} Consistently, SFPQ was overexpressed in EOC tumors and cell lines that were resistant to PT.\textsuperscript{66} Moreover, we observed that SFPQ regulated the timing and the extent of caspase-9 AS, in concert with the splicing factor SRSF2, further linking splicing regulation to the onset of PT-resistance in EOC.\textsuperscript{66}

ERCC1 is one of the first identified genes with an unusual presence of spliced variants in ovarian cancer.\textsuperscript{122} In vitro data, using ovarian cancer cells, demonstrated an association between AS of ERCC1 and the ability to repair cisplatin-DNA adducts and, thus, PT sensitivity. However, further analyses in ovarian cancer samples highlighted that only total ERCC1 mRNA, but not the spliced ones, correlated with PT-resistance.\textsuperscript{122–124} This observation, quite unexpected based on the notion that ERCC1 AS correlates with a reduction in cellular ability to repair PT-induced DNA adducts,\textsuperscript{88} might be due to the low number of patient analyzed (i.e., 28) or to the evaluation of relative and not absolute ERCC1 spliced form expression.\textsuperscript{122} Thus, larger studies are needed to confirm this evidence.

As mentioned above, BRCA1 and BRCA2 genes undergo very complex AS and their splicing variants could represent a way for cancer cells to bypass the presence of inactivating mutations and promote resistance to chemo- or targeted-therapies.\textsuperscript{81,82} Moreover, AS of BRCA1 and BRCA2 genes could be associated with an increased risk of ovarian cancer onset. A systematic analysis of the AS of BRCA1 locus has provided a model in which most non-mutually exclusive AS events are randomly combined into individual mRNA molecules, to produce hundreds of different BRCA1 isoforms.\textsuperscript{125} In hereditary breast and ovarian cancers, a high fraction of BRCA1/2 DNA variants present splicing aberrations and these variants might have functional consequences on BRCA1/2 activities and, then, on cancer development and progression.\textsuperscript{126} A careful examination of some BRCA1/2 splicing variants suggested that until they allow for a 20%–30% residual tumor suppressor activity, they should not markedly increase the BRCA-associated cancer risk.\textsuperscript{127} Conversely, the activation of cryptic splice sites, resulting in a clear pathogenic role for some mutations (e.g., c.213-14C>G in BRCA1 and c.7618-2A>G and c.7806-2A>G in BRCA2), has been recently reported and, at least in some instances, might be associated with a lower cancer risk compared to classical pathogenic BRCA gene variants.\textsuperscript{128} Although these splicing variants apparently maintain some tumor suppressor activity, probably because they maintain the synthesis of an at least partially functional protein, they could nonetheless represent the rationale for the treatment with PARP inhibitors when expressed in EOC patients, in view of the potential beneficial effect of this therapy.\textsuperscript{128}
Specific combinations of BARD1 alternative spliced forms, generated by differential splicing and by alternative transcription initiation, were identified in gynecologic cancer cell lines and in primary ovarian cancers. Among these, the expression of an NH2-terminally truncated BARD1 correlated with advanced-stage ovarian cancer. Moreover, splicing alteration in BARD1 seems to be associated with increased ovarian cancer risk.

Another central player of the BRCA1 pathway, FANCD2, has also been recently reported to undergo AS in ovarian cancer cells and tissues. In particular, the retention of intron 43 leads to the formation of a FANCD2 variant with lower tumor suppressor activity, which has been found to be expressed at higher levels in tumors than in normal tissues and associated with higher grade and stage in ovarian cancer samples.

Finally, the fact that the splicing factor SRSF2 was found more frequently mutated in PT-treated recurrent/resistant EOC, further suggests that AS might contribute to the onset of acquired PT-resistance. This evidence will need confirmation on a larger cohort of samples, both from naïve and PT-treated EOC patients.

Overall, these data support the possibility that, at least in EOC, AS frequently causes the alteration of genes involved in DNA damage response, potentially leading, on one side, to disease progression but, on the other, to the higher sensitivity to first-line PT-based therapies, typical of these tumors (Figure 4).

4.3 Alternative splicing in EOC metastatic dissemination

EOC display a very typical pattern of metastasis, mainly spread within the peritoneal cavity. The mechanisms exploited for this dissemination hardly ever involve the hematogenous route but rather the exfoliation of cellular aggregates or spheroids from the primary tumor, followed by the passive transport through the peritoneal fluid and the metastatic colonization of the peritoneal organs. These cellular spheroids are slow proliferating, resistant to anoikis, and other survival-threatening insults, overall sharing many features of the so-called cancer stem-like cells.

CD44 is a type I transmembrane glycoprotein that varies in molecular size depending on glycosylation status and AS modifications. CD44 not only acts as an adhesion molecule but has also been associated with the cancer-initiating properties of tumor cells. For its ability to bind hyaluronic acid, collagen, and laminins, it is thought that CD44 is deeply involved in the ability of EOC spheroids to adhere to the peritoneum. The expression of the CD44v6 splicing variant has been recently proposed as an important predictor of poor prognosis and distant metastasis.

L1CAM, also known as CD171, is a surface glycoprotein that mediates cell adhesion and is also regulated by AS. Typically, while neurons express the full-length variant of L1CAM, non-neural cell types express a shorter isoform lacking exon 2 and exon 27. L1CAM is overexpressed in ovarian cancer where it is associated with poor prognosis and advanced stages. Recently, it has been demonstrated that the vascular endothelium in ovarian cancer expresses a newly identified alternative spliced form of L1CAM that lacks exon 25, not expressed in normal endothelium. The generation of this L1CAM splice variant is mediated by the expression of the NOVA2 splicing factor, whose expression predicts poor prognosis in EOC patients.

We have recently reported that the deubiquitinase USP1 links PT resistance to the metastatic dissemination of ovarian cancer via the stabilization of the mesenchymal transcription factor Snail. It is known that USP1 is overexpressed in PT-resistant cells and that alternative promoter usage leads to USP1 overexpression, suggesting that USP1 AS could at least in part contribute to ovarian cancer dissemination.

Overall, these data support the possibility that the deregulation of AS could also mediate some of the known EOC abilities to disseminate within the abdominal-pelvic cavity (Figure 4).

4.4 Alternative splicing in race/ethnicity-related differences of EOC prognosis

There is a clear association between ethnicity and diagnosis, treatment, and prognosis of EOC. Whether these differences are also linked to different AS regulation is still unknown. Yet, increasing evidences support the
existence of relevant race-related differences in AS regulation in cancer. For instance, racial-specific AS events have been reported as critical drivers of prostate cancer aggressiveness and therapeutic resistance in African American men.

Looking at the incidence and survival of EOC patients of different ethnicities, a recent large multiethnic cohort study has confirmed that African Americans and Asian Americans had a lower EOC risk compared to the Caucasian population. Native Hawaiians display a slightly higher risk while Hispanic women showed no significant incidence of EOC compared to Caucasian women. Despite having similar lower EOC risk when compared to Caucasian, Asian women tended to be diagnosed at an earlier age than African American women, who were more likely to be diagnosed with advanced disease. These differences result in longer survival of Asian and shorter survival of African American women. Regarding the EOC subtypes and comparing them with Caucasian women, while African Americans more often are diagnosed with a serious EOC, Asian women more frequently with a clear cell or mucinous tumor. Since earlier data suggest that subtype-specific AS difference might exist in EOC, it would be interesting to verify if these AS differences are also due to the different ethnicities.

Many studies tried to link the mutational status of BRCA1/2 genes with diverse ethnicities. A recent study of about 30,000 families of BRCA1 and BRCA2 carriers, conducted worldwide, evaluated the breast cancer and the ovarian cancer risk in Caucasian, African American, Asian and Hispanic women. BRCA1 carriers among Asian and Hispanic women displayed a higher risk for ovarian cancer and a lower risk of breast cancer compared to Caucasian. Conversely, African American women displayed the lowest BRCA1-associated ovarian cancer risk, overall suggesting that BRCA1 mutation-associated cancer risks may vary by race/ethnicity. Interestingly, African American women have somatic mutations in BRCA1 more frequently associated with cis-acting splicing elements than women of other ethnicities. Whether, the different splicing of BRCA1, could explain the lower incidence of BRCA1 associated EOC in African American women is something to be further explored.

Altogether, our survey of the literature indicates that more studies are needed to understand which, if any, is the impact of AS regulation on racial disparities in EOC. In turn, identification of AS deregulation may help in mitigating cancer disparities among the diverse ethnicities, by identifying new possible diagnostic tools and/or therapeutic targets for precision medicines of specific racial groups.

5 | TARGETING THE ALTERNATIVE SPLICING PATHWAY IN HUMAN CANCER AND OTHER DISEASES

Thanks to the increasing awareness of the complex mechanisms underlying the process of AS and their deregulation in human diseases, the development of AS targeting drugs has also initiated. Many different approaches have been exploited to develop compounds that can affect the splicing, resulting in potential novel therapies. The detailed description of these approaches has been excellently reviewed very recently. Here, we will focus on the modalities that have been adopted to transfer the study of splicing regulation to clinical trials involving cancer patients (Table 1) and patients with other diseases (Table 2).

5.1 | Clinical trials currently targeting or evaluating alternative splicing

To get a complete picture of the ongoing clinical trials dealing with splicing regulation and targeting in human cancer, we interrogated the ClinicalTrials.gov database (https://clinicaltrials.gov/) and annotated the ongoing, completed, or forthcoming clinical trials in any type of cancer, using the word “splicing” as a search term. From the curated analysis of the 22 items that were retrieved, we extrapolated the presence of 13 clinical trials that are investigating or targeting the regulation of AS in cancer patients, of which 4 were interventional and 9 observational (Table 1). Among the interventional group, NCT02841540 is a Phase I first-in-human trial directly testing, in
| NCT number | Title                                                                 | Status | Tumor type     | Interventions                      | Note                                                                                                                                 |
|------------|------------------------------------------------------------------------|--------|----------------|-----------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|
| NCT 03424213 | Race-related alternative splicing: novel targets in prostate cancer   | R      | Prostate cancer | No intervention retrospective study | Observational study that aims to identify race-related splice variants using the NimbleGen SeqCap Target Enrichment and SeqCap RNA System |
| NCT 03447431 | Aberrant splicing due to microsatellite instability (MSI) in colorectal cancer: physiopathological and clinical impact | R      | Colorectal cancer | No intervention retrospective study | Observational study that aims to identify clinically relevant MSI-associated splicing aberrations due to mutations in long noncoding repeats located in splice acceptor sites |
| NCT 00550563 | DNA changes that affect vitamin D metabolism in patients with colorectal cancer receiving vitamin D supplements | C      | Colorectal cancer | Dietary supplement cholecalciferol | Interventional study that aims to determine the relationship, if any, between serum cholecalciferol pharmacokinetic parameters and CYP24 SNPs, splicing variants, and enzyme activity |
| NCT 03000764 | RNA and heat shock protein biomarkers in radiation-induced fibrosis in breast cancer (SPLICI-Rad) | C      | Breast carcinoma fibrosis | Skin biopsies blood samples | Interventional study that aims to find a molecular signature of pathological radiation-induced fibrosis, based on the response of skin fibroblasts after irradiation, looking at the overall splicing profile of heat shock proteins |
| NCT 02922218 | PROSENZA: prospective multi-center study of prognostic factors in mCRPC patients treated with enzalutamide (PROSENZA) | R      | Prostate cancer | Pre and posttherapy blood collection | Prospective observational study in metastatic castration-resistant prostate cancer (mCRPC), exploring prognostic biomarkers in patients treated with enzalutamide and assessing the prognostic value of androgen receptor splicing variant 7 (AR-V7) and/or AR amplification |
| NCT 02844491 | Study of T specific immune response against delta-CD20 peptide in hematological malignancies B | U      | Hematological B malignancies | Blood and tissue samples | Prospective observational study that aims to detect the presence of a specific memory response to delta-CD20 peptides spliced form, in patients with lymphoproliferative B malignancies |
| NCT number   | Title                                           | Status | Tumor type              | Interventions                                | Note                                                                                                                                 |
|-------------|-------------------------------------------------|--------|-------------------------|----------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| NCT02229565 | Molecular mechanisms underlying prostate cancer disparities | R      | Prostate cancer         | Tissue samples                              | Prospective observational study that aims to elucidate the molecular mechanisms underlying prostate cancer disparities. Altered expression and alternative splicing in selected deregulated genes will be assessed by targeted RNA sequencing of genetic factors specific to African American prostate cancer patients |
| NCT02823184 | Endoplasmic reticulum stress and resistance to treatments in Ph-negative myeloproliferative neoplasms (PhiNESS) | R      | Polycythemia vera essential thrombocythemia | No intervention retrospective study          | Observational retrospective study that aims to evaluate endoplasmic reticulum stress markers (defined as splicing of XBP1 above 30%) as predictors for the response to hydroxyurea in patients with polycythemia vera and essential thrombocythemia |
| NCT02928432 | SWITCH: study of the prednisone to dexamethasone change in mCRPC patients treated with abiraterone | C      | Prostate cancer         | Steroids switch                             | Phase II pilot study of the prednisone to dexamethasone switch in metastatic castration-resistant prostate cancer (mCRPC) patients with asymptomatic biochemical and/or limited radiological progression on abiraterone and prednisone. Archival tissue for IHC and FISH, and peripheral blood to perform androgen receptor amplification studies and determination of AR alternative splicing transcripts from exosomes will be used |
| NCT03156933 | Alternative splicing and leukemia initiating cells (ASLIC) | U      | Acute myeloid leukemia  | No intervention retrospective study          | Observational retrospective study that aims to determine the splice variants on AML initiator cells and define a splicing pattern |
| NCT02841540 | A Phase 1 study to evaluate H3B-8800 in participants with myelodysplastic syndromes (MDS), acute myeloid leukemia (AML), and chronic myelomonocytic leukemia (CML) | NYR    | MDS-AML-CML             | Drug: H3B-8800                              | Phase I, open-label, first-in-human (FIH) study designed to evaluate the safety, tolerability, pharmacokinetics (PK), pharmacodynamics (PD), and preliminary activity of the splicing modulator drug H3B-8800 |

(Continues)
| NCT number  | Title                                                                 | Status | Tumor type                          | Interventions            | Note                                                                                                                                                                                                 |
|------------|------------------------------------------------------------------------|--------|-------------------------------------|--------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| NCT03770429 | AZD6738 for patients with progressive MDS or CMML                    | R      | Leukemia myelodysplastic syndrome   | Drug: AZD6738            | Phase Ib clinical trial evaluating the ATR inhibitor AZD6738 as a possible treatment for myelodysplastic syndrome or chronic myelomonocytic leukemia carrying or not mutation in splicing factor genes |
| NCT03904251 | CPX-351 and gemtuzumab ozogamicin in treating patients with relapsed acute myeloid leukemia | R      | Relapsed acute myelogenous leukemia | Drug: gemtuzumab ozogamicin CPX-351 | Phase Ib trial to select the best dose of gemtuzumab ozogamicin (Anti CD33 Ab) when given together with CPX-351 (liposome-encapsulated daunorubicin-cytarabine) in treating patients with relapsed acute myeloid leukemia. Secondary objective will be to evaluate if there is a difference in remission rate based on CD33 splicing or single-nucleotide polymorphism genotype |

Note: Data refer to the information available in March 2020 through the www.clinicaltrials.gov web site.

Abbreviations: C, completed; NYR, not yet recruiting; R, recruiting; U, unknown status.
| NCT number     | Title                                                                 | Status | Conditions                        | Interventions                                                                                       | Note                                                                                                                                 |
|---------------|-----------------------------------------------------------------------|--------|-----------------------------------|------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| NCT03040635   | A study to investigate the safety, tolerability, pharmacokinetics, and pharmacodynamics of risdiplam (RO7034067) in healthy Japanese participants | C      | Healthy volunteers                | Drug: risdiplam selective modulator of SMN2 gene splicing                                           | Randomized, placebo-controlled study to investigate the safety, tolerability, PK, and PD of a single oral dose of risdiplam in healthy Japanese volunteers. Risdiplam (RO7034067) is a selective and orally active small molecule able to modify the splicing pattern SMN2 gene to treat spinal muscular atrophy (SMA) |
| NCT03032172   | A study of risdiplam (RO7034067) in adult and pediatric participants with spinal muscular atrophy (jewelfish) | R      | Spinal muscular atrophy           | Drug: risdiplam selective modulator of SMN2 gene splicing                                           | Multi-center, exploratory, non-comparative, and open-label study that aims to investigate the safety, tolerability, PK, and PK/PD relationship of risdiplam in adults, children, and infants with spinal muscular atrophy previously enrolled in study BP29420 (moonfish) with the splicing modifier RO6885247 or previously treated with nusinersen, olesoxime, or AVXS-101 |
| NCT02633709   | A study to investigate the safety, tolerability, pharmacokinetics, and pharmacodynamics of risdiplam (RO7034067) given by mouth in healthy volunteers | C      | Healthy volunteers                | Drug: risdiplam selective modulator of SMN2 gene splicing                                           | This study aims to assess the safety and tolerability of risdiplam in healthy people. The study will assess what the body does to risdiplam (RO7034067) and what risdiplam does to the body. Risdiplam will be given by mouth in gradually increasing doses |
| NCT02240355   | A study of RO6885247 in adult and pediatric patients with spinal muscular atrophy (moonfish) | T      | Muscular atrophy and spinal       | RO6885247                                                                                            | Phase I study terminated for unexpected toxicology findings observed in the 39-week monkey study. RO6885247 is a selective SMN2 splicing modifier |
| NCT02725632   | Sodium channel splicing in obstructive sleep apnea (SOCS-OSA)         | C      | Sleep apnea syndromes            | Continuous positive airway pressure (CPAP)                                                          | This study is designed to test if SCNSA mRNA processing is altered in OSA patients, which may contribute to their increased arrhythmic risk, and if the processing of SCNSA mRNA is modulated by CPAP treatment by comparing sodium channel splicing variants in mild, moderate, or severe OSA patients at baseline and in 1 month after CPAP treatment |

(Continues)
| NCT number   | Title                                                                 | Status | Conditions                                    | Interventions            | Note                                                                                                                                 |
|-------------|----------------------------------------------------------------------|--------|----------------------------------------------|--------------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| NCT02274051 | The safety and tolerability of kinetin, in patients with familial dysautonomia | C      | Familial dysautonomia (Riley-Day syndrome)   | Kinetin                  | The objective of this Phase I study is to assess the safety and tolerability of administering kinetin in patients with familial dysautonomia (FD). FD is a rare fatal autosomal recessive disease due to a point mutation in the intron 20 of the IKAP gene that causes exon 20 exclusion. The mutated mRNA produces a short unstable, quickly degraded IKAP protein. Kinetin (nutritional supplement) corrects the splicing defect and increases the production of normal IKAP protein. Enrolled patients will then proceed to steady-state long-term phase at a maximum individual dose of kinetin for 3 years |
| NCT01349387 | Effect of treatment with metformin in type 2 diabetes patients on alternative genes splicing (metforgene) | C      | Type 2 diabetes                              | Metformin                | This study aims to test if treatment with metformin (1400 mg/day) in patients with type 2 diabetes has an effect on alternative splicing of the insulin receptor |
| NCT00889434 | Efficacy and safety study of EGCG/tocotrienol in 18 patients with splicing-mutation-mediated cystic fibrosis (CF) | C      | Cystic fibrosis                              | EGCG and tocotrienol     | This study aims to determine in patients with CF if oral administration of EpiGalloCatechin Gallate (EGCG) and tocotrienol modify splicing of CFTR, as evaluated by improved transepithelial potential difference (TEPD) assessment of chloride secretion. In vitro treatment with EGCG and/or tocotrienol of cells harboring splicing mutations in the CFTR gene augment production of the full-length transcripts |

Note: Data refer to the information available in March 2020, through the www.clinicaltrials.gov web site.

Abbreviations: C, completed; R, recruiting; T, terminated.
hematological malignancies, the tolerability of the splicing modifier H3B-8800, an orally available molecule that modulates the SF3B1 splicing complex. With promising results from previous in vitro and in vivo preclinical models, H3B-8800 preferentially targets spliceosome-mutant cancer cells. In the future, it will be interesting to evaluate if targeting the mutant spliceosome represents a feasible and promising strategy, at least for a well-defined group of patients, such as those carrying mutation of the splicing factors SF3B1, U2AF1, and SRSF2.

Differently from the above, the other three interventional trials aimed at investigating whether drugs not directly targeting the AS could be more active in splicing defective cancer patients or if they are able to alter the splicing of defined genes. NCT03770429 is a Phase Ib clinical trial evaluating the toxicity of the ATR inhibitor in patients with hematological malignancies, carrying or not mutations in splicing factors genes. NCT02928432 is a Phase II trial testing the efficacy of switching the steroid therapy in castration-resistant prostate cancer (CRPC) patients at the early phase of progression. A secondary aim of the study will be to verify if this therapy switch also impacts the splicing of androgen receptor (AR). Indeed, the splicing of AR exon 7 has been proved to predict poor prognosis in CRPC patients. It will be interesting to evaluate whether this splicing variant could influence the efficacy of the therapy-switch or if, vice versa, switching the steroid therapy could alter the expression of this worse prognostic factor. Similarly, NCT03904251 is a Phase Ib trial, aimed at selecting the best dose of anti-CD33 Mab to be administered together with CPX-351 (liposome-encapsulated daunorubicin-cytarabine), to treat patients with relapsed acute myeloid leukemia. The secondary objective will be to evaluate if there is a difference in remission rate based on the splicing of CD33.

With regard to the nine observational trials, they will investigate some specific AS events in selected types of cancer patients, as specified in Table 1.

Next, we similarly interrogated the ClinicalTrials.gov database for ongoing, completed, or forthcoming interventional clinical trials in any type of disease. Although not related to cancer, these ongoing clinical research could be inspirational to design new trials also for cancer patients.

By this survey, excluding the above-mentioned NCT02841540, we retrieved eight curated relevant results (Table 2). Four of these trials (NCT03040635, NCT03032172, NCT02633709, and NCT02240355) deal with the possibility to treat spinal muscular atrophy (SMA) patients with the SMN2 splicing modulator Risdiplam. SMA could be caused by an AS alteration of the SMN2 gene, which results in the formation of a truncated protein (SMNΔ7). These trials test if risdiplam is safe in healthy volunteers and SMA patients. NCT02633709, which was also testing an SMN2 splicing modulator, has been terminated for unexpected toxicities during the experimentation in monkeys. Other ongoing interventional clinical trials include: (i) treatment of sodium channel splicing in obstructive sleep apnea (SOCS-OSA) with continuous positive airway pressure that might impact on the AS of SCN5A (NCT02725632); (ii) treatment of patients with familial dysautonomia with the nutritional supplement kinetin that seems to correct the splicing defect of the IKAP gene (NCT02274051); (iii) treatment of type 2 diabetes patients with metformin that has an effect on the AS of the insulin receptor (NCT01349387); and (iv) treatment of patients with cystic fibrosis caused by a splicing-mutation of CFTR gene with the EpiGalloCatechin Gallate in combination with tocotrienol that should impact on the altered AS of CFTR.

5.2 | Novel approaches for the targeting of alternative splicing

Overall, the above-reported data demonstrate that targeting AS is feasible and could represent a promising way to treat diseases that rely on splicing alteration. Many efforts are currently dedicated to the identification of novel ways to target AS and several small molecules, emerged from in vitro studies, have been screened and modified for a future application in patients.

The small molecule H3B-8800 is being evaluated in the Phase I clinical trial NCT02841540. H3B-8800 is an orally available synthetic derivative of the natural compound pladienolide B that exhibits potent antitumor effects.
with very low toxicity. H3B-8800 modulates the SF3B complex and induces lethality preferentially in spliceosome-mutated cancers. Indeed, the mutation in SF3B1 and SRSF2 would confer sensitivity to H3B-8800.\textsuperscript{151} Other synthetic derivatives (E7107 and FR901464) of natural compounds (pladienolides, spliceostatin, meayamycins, and sudemycins) targeting the spliceosome have demonstrated a potent and selective antitumor activity. E7107 has entered Phase I clinical testing in 2007 (NCT00499499 and NCT00459823) in patients with advanced-stage solid tumors, not responding to approved therapies. Despite the promising effects shown in some patients, the clinical Phase I trial was terminated due to the high visual toxic effects.\textsuperscript{152} However, the recent observation that loss of BCL2L1 sensitized cells to E7107 suggests that BCL2L1 expression could be used as a predictive biomarker to select patients.\textsuperscript{153} Hematopoietic and lymphoid malignancies, harboring mutations in splicing factors, are particularly sensitive to clinically tested sulfonamides (E7820, indisulam, and tasisulam), which target the U2AF-related splicing factor RBM39 (RNA binding motif protein 39) and lead to its ubiquitin-mediated degradation.\textsuperscript{154} This notion, along with the discovery and development of proteolysis targeting chimeras (PROTACs, heterobifunctional compounds which simultaneously bind a target protein, and its E3 ubiquitin ligase), has suggested novel opportunities to target the splicing pathway.\textsuperscript{154} In addition to interfering directly with core spliceosome components, the development of inhibitory molecules, targeting protein kinases involved in splicing, is another emerging field now being tested in preclinical models. For instance, inhibition of the SR-rich protein-specific kinases (SRPKs) by the covalent inhibitor SRPKIN-1, efficiently reduced SR protein phosphorylation. This in turn resulted in the conversion of the proangiogenic splicing isoform of VEGFA (VEGFA\textsubscript{165a}) into the antiangiogenic VEGFA\textsubscript{165b} isoform, thereby inhibiting neovascularization in a murine retinal model.\textsuperscript{152,155} Other compounds that indirectly interfere with the splicing complex also exhibited antitumor effects. Among these, it is worth mentioning the pharmacological inhibition of protein arginine N-methyltransferase 5 (PRMT5) that has been investigated in several clinical trials. PRMT5 activity is required for spliceosome assembly, since it dimethylates arginines in several proteins, including histones and spliceosome-associated proteins. The orally available PRMT5 inhibitor, GSK3235025, suppressed mantle-cell lymphoma cell growth, both in vitro and in vivo and is now being tested in two different clinical trials (NCT03614728 and NCT02783300).\textsuperscript{152,156} A completely different approach to target the AS is based on the newly developed approach called “RNA therapeutics” that allows the direct targeting of specific individual splicing events with ASOs.\textsuperscript{152,154} Despite many challenges, such as the efficient delivery to particular tissues and the management of toxic side effects, this approach has the great advantage of high selectivity, provided by the recognition of specific target sequences. Oligonucleotide-based therapies seem very promising in the treatment of specific pathologic splicing events, particularly in noncancer monogenic disorders (see Table 2). Similarly, ASOs splice-switching oligonucleotides (SSOs) have been used to modulate splicing. SSOs are synthetic antisense composed of 15–30 modified nucleotides and they are designed to base pair and create steric hindrance to the binding of splicing factors to the pre-mRNA at the splice site, without inducing RNA degradation. SSOs can target and modulate RNA splicing in different ways, changing exon splicing and generating a new alternatively spliced protein isoform.\textsuperscript{149} Given the success of several FDA-approved SSOs to treat monogenic disorders, "oligonucleotide therapy" has become attractive also for therapeutic applications in cancer and several promising preclinical studies have been already reported. SSOs can modulate cancer-associated alternative splicing, inducing apoptosis, cell cycle arrest in cancer cell lines, and tumor regression in xenografts.\textsuperscript{37,152} For instance, targeting with SSOs the Bcl-X gene (which undergoes AS to express the anti (Bcl-X\textsubscript{L}) or pro (Bcl-X\textsubscript{S}) apoptotic isoforms) redirects the splicing favoring the expression of the proapoptotic Bcl-X\textsubscript{L} isoform. This approach has proved efficacy in preclinical in vitro and in vivo models.\textsuperscript{156,157} Finally, the notion that altered tumor glucose metabolism could be due to the mutually exclusive exon usage of the PKM gene,\textsuperscript{40} as mentioned in Section 2, led to the development of SSOs targeting the PKM2 isoform, promoting aerobic glycolysis, expressed in cancer. In vitro results demonstrated that SSOs blocking PKM2 expression induce apoptosis in glioblastoma cell lines.\textsuperscript{152}
5.3 | Design new trials to overcome platinum resistance in EOC

No trial, either observational or interventional, specifically dealing with AS in ovarian cancer is currently ongoing. This could be due, at least in part, to the fact that ovarian cancers are quite rare and it is objectively difficult to design this type of translational studies in rare diseases.

In the future, we expect that the study of AS will provide useful information for the management of EOC patients to cover the clinical unmet needs that deserve to be addressed. As mentioned, we still do not have good noninvasive biomarkers for EOC early diagnosis, and there is no doubt that anticipating the diagnosis of EOC would change the clinical history of this disease. Since altered AS might generate new and cancer-specific transcripts, possibly also linked to tumor initiation, exploring AS in the initial stages of EOC could lead to the identification of valuable early biomarkers. However, the low prevalence of EOC will necessitate a very high number of healthy donors to have a potentially validated test. As a consequence, this type of trial is difficult to implement and very expensive often resulting not cost-effective.

More expectations rely on the study of AS for the clinical management of EOC patients, especially in the context of PT-resistance. First, it would be important to evaluate if selected splicing forms are specific for PT-resistant cells/tumors. This could be done using both retrospective studies and available cellular models. The use of data from patients already included in translational clinical trials would be of utmost importance.

Current options for the treatment of EOC patients in first line include the use of poly-ADP-ribose polymerase inhibitor (PARPi) and/or the anti-VEGFA antibody Bevacizumab (BEV). Although BRCA1/2 mutations represent a clear and validated biomarker for PARPi activity, it is still unclear which BRCA1/2 wild-type tumors will respond to this maintenance therapy. The fact that AS regulates not only BRCA1/2 but also many of the genes involved in HR DNA repair pathway supports the possibility that a “splicing signature” might be able to better identify homologous recombination deficiency (HRD) and PARPi-sensitive patients, in a better way than current available HRD tests do. On the other hand, we do know that a small percentage of BRCA1/2 wild-type HRD-proficient high-risk EOC patients respond to BEV, used as maintenance therapy. Recent evidences in colorectal cancer suggest that the expression of the antiangiogenic isoform VEGFA145b mRNA may predict resistance to BEV. Whether the same is true for EOC patients treated with BEV is unknown and certainly represents something worth to be evaluated. If this will be the case, the use of ASO or SSO, or other methods, to favor the expression of VEGFAXXXb mRNA isoforms could be an experimental approach to improve BEV efficacy.

In the case of PT-resistant disease, the therapeutic options for EOC patients are very scarce and the prognosis is worse. The results collected over the past years and here reported suggest that AS plays a role in the response to PT. Thus, new splicing modulators should be tested for their ability to improve PT efficacy in EOC patients. Moreover, since SRSF2 mutations increase in PT-treated patients and the small molecule H3B-8800 is particularly active in SRSF2-mutated cancers, it will be important to re-evaluate the mutational spectrum of SRSF2 (and possibly other splicing factors) on tumor biopsies obtained from patients with acquired PT-resistance.

To date only a few samples from patients with acquired PT-resistant disease have been sequenced thus we do not have a clear picture of the differences, if any, between acquired and de novo resistance in EOC. Since spatial and temporal tumor heterogeneity seems to play a central role in the progression of EOC and in limiting the efficacy of the therapy, having the possibility to reevaluate the mutational landscape and the AS of tumor with acquired resistance to PT, would be of primary clinical relevance.

Finally, immunotherapy that has been successfully used to treat diverse solid tumors, demonstrated a controversial efficacy in EOC. One of the possible reasons for this partial failure could be related to the peculiar way of dissemination of EOC and the consequent tumor-microenvironment interplay. Since the alteration of AS can produce neo-antigens, not only in cancer cells but also in the local microenvironment, as demonstrated using microdissected samples, it will be important to verify if AS alteration correlates with the efficacy of immunotherapy and if targeting the splicing machinery could improve its efficacy.
CONCLUSION AND FUTURE PERSPECTIVE

Very solid evidences suggest that alteration in AS plays a pivotal role in the progression and response to therapies of human cancer. Increasing data points to AS deregulation as a possible mechanism underlying PT resistance in ovarian cancer patients. Here, by focusing on PT chemotherapy and ovarian cancer, we highlighted how AS deregulation could determine the sensitivity of cancer cells to drugs and how AS alteration could be implicated in all clinical unmet needs of ovarian cancer patients.

The deeper molecular understanding of the spliceosome has allowed the design of new splicing modulators that have recently reached the clinic and opened new therapeutic possibilities, to at least some defined groups of patients. It is conceivable that, in the near future, the updating of the notions on how these compounds are tolerated and work in vivo will translate into new therapeutic opportunities also for cancer patients. Up to now, the road ahead is still long and to successfully transfer these new strategies to the clinical practice, the identification of less toxic AS modulators seems to be the priority.

Finally, and most importantly, assessing if and how this new class of drugs could be combined with chemo-, targeted- and/or immune-therapy and, finally, identifying clinically validated biomarkers able to select cancer patients that could benefit from the use of AS modulators, will be crucial for the success of these new therapeutic approaches. Further implementation of clinical-translational studies in the context of multicentered and multidisciplinary research groups will be a critical step to achieve these goals.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Gustavo Baldassarre collected the related literature and drafted the manuscript; Gustavo Baldassarre, Ilenia Pellarin, and Barbara Belletti discussed and wrote the manuscript. Gustavo Baldassarre, Ilenia Pellarin, and Barbara Belletti reviewed and edited the manuscript. Ilenia Pellarin and Barbara Belletti designed the figures. All authors read and approved the final manuscript.

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Ilenia Pellarin's education and training were fulfilled at the University of Trieste, achieving the Bachelor's degree in Biological Sciences (2006) and Master's degree in Functional Genomics (2008), with projects aimed to construct a phage display expression cDNA library to identify new autoantigens in multiple sclerosis. Soon after she obtained PhD in Molecular Biomedicine (2014) studying architectural chromatin proteins (HMGA) involved in epithelial to mesenchymal transition and tumor progression in breast cancer. Since 2015, she got a postdoctoral fellowship to study ovarian cancer progression and chemoresistance at the IRCCS CRO Aviano. In particular, she is involved in projects aimed to understand the possible role of alternative splicing deregulation in epithelial ovarian cancer progression and how chemotherapy could influence or alter these mechanisms.

Barbara Belletti has graduated in Biological Sciences at the University of Bologna, Italy, in 1992. After concluding her PhD in Cell Biology at the University of Bologna, Italy, in 1996, she moved to Naples at the National Cancer Institute, IRCCS "Fondazione Pascale" and in 2000 in Philadelphia, USA, at Kimmel Cancer Center, Thomas Jefferson University. In 2002, she moved back to Italy, at Centro di Riferimento Oncologico di Aviano (CRO), NCI, IRCCS, where she first was awarded a 2-year fellowship from European Molecular Biology Organization (EMBO) in 2003 and then became a staff scientist and principal investigator, position held till today (2005–now). She has been working on signal transduction, cell cycle, cell transformation, and in vivo models of tumor progression, cell motility, and invasion, all during her PhD and postdoctoral training. As a group leader, she has investigated the crosstalk between mammary carcinoma cells and tumor microenvironment, in vivo models of tumor progression, cell cycle and tumor onset and progression, and the role of miR in ovarian and breast cancer models. She carries out a translational research project focused on the investigation of the molecular determinants of aggressiveness of breast cancer, involving the use of patients' specimens to perform molecular and genetic profiling, and patient-derived organoids and xenografts.
Gustavo Baldassarre has graduated in Medicine and Surgery (1991) and obtained a specialization degree in Clinical Oncology (1996) at the University of Naples "Federico II". Along with the clinical activity he has cultivated research studies in molecular oncology, working at the International Institute of Genetics and Biophysics of Naples (1993–1995) and the National Cancer Institute "Fondazione G Pascale" of Naples (1995–2000). In 2000, he moved to the Thomas Jefferson University of Philadelphia, USA, at the Kimmel Cancer Center. Since 2002, he is a group leader at the National Cancer Institute, CRO of Aviano where, since 2014, he serves as director of the Molecular Oncology unit. As a group leader, his research interests have focused on the control of cell cycle progression and cell proliferation, motility, and drug resistance in cancer always following a highly translational approach. He has built at CRO translational research groups for breast, ovarian, and head and neck cancers, involving all the specialists dealing with the management and cure of these tumors, with the aim of finding new reliable therapeutic targets and biomarkers. He is a founder member of the Multicenter Italian Trials in Ovarian Cancer (MITO) translational research group and an active member of AACR, SIC, EACR, and EUTROC. Since 1992, he published 116 articles in the field of oncology in peer-reviewed journals.

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