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SPECIAL ARTICLE

Recommendations for biomarker testing in epithelial ovarian cancer: a National Consensus Statement by the Spanish Society of Pathology and the Spanish Society of Medical Oncology

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Abstract Because of advances in the understanding of histological and molecular characteristics in ovarian cancer, it is now possible to recognize the existence of five subtypes, which in turn has allowed a more refined therapeutic approach and better design of clinical trials. Each of these five subtypes has specific histological features and a particular biomarker expression, as well as mutations in different genes, some of which have prognostic and predictive value. CA125 and HE4 are examples of ovarian cancer biomarkers used in the diagnosis and follow-up of these malignancies. Currently, somatic or germinal mutations on *BRCA1* and *BRCA2* genes are the most important biomarkers in epithelial ovarian cancer having prognostic and predictive value. This article will review the histological and molecular characteristics of the five subtypes of ovarian cancer, describing the most important biomarkers and mutations that can guide in diagnosis, screening and tailored treatment strategy.

Keywords Screening · Mutations · Prognosis · Diagnosis · BRCA

Introduction

In the past decade, the histological and molecular diversity of ovarian cancer has been recognised, and it is no longer regarded as a single entity. This accomplishment has permitted more refined management and better clinical trial design. Since the dualistic model was proposed 10 years ago by Kurman et al., due to current massive sequencing techniques, a deeper understanding has been gained of not only the carcinogenesis of the various types of ovarian cancer but also their molecular features [1]. This more in-depth analysis has revealed the existence of five different types of ovarian carcinoma, termed high-grade serous ovarian carcinoma (HGSOC), endometrioid carcinoma (EC), clear-cell

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carcinoma (CCC), low-grade serous ovarian carcinoma (LGSOC) and mucinous carcinoma (MC) [2].

HGSOC accounts for 75% of ovarian carcinomas. In a substantial proportion of cases, it develops from a precursor lesion in the fallopian tube, called a serous tubal intraepithelial carcinoma (STIC) [3]. From the molecular point of view, the presence of mutations in the *TP53* gene is regarded as a virtually invariable feature of HGSOC and STIC. Except for these mutations, and others in the *BRCA1/2* genes, point mutations in oncogenes or tumour suppressor genes are relatively uncommon in HGSOC. Its characteristic feature is chromosomal instability. Approximately 50% of HGSOCs have deficiencies in the homologous recombination (HR) pathway. Most of these deficiencies are due to germline, somatic or epigenetic mutations in the *BRCA1/2* genes and, to a lesser extent, in other components of this pathway. HR deficiencies are a key feature when testing for sensitivity to platinum and new drugs that inhibit the enzyme poly(ADP-ribose) polymerase (PARP). For all these reasons, *BRCA1/2* mutations are now considered an important biomarker in ovarian cancer [4].

Endometrioid and clear-cell carcinomas, besides their association with endometriosis, are characterised by the presence of mutations in genes that rarely feature in other types of ovarian cancer, such as *CTNNB1* (which encodes beta-catenin), *ARID1A* (AT-rich interaction domain 1A), *PTEN* (phosphatase and tensin homologue) and *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α). Gene mutations in *KRAS* (Kirsten rat sarcoma viral oncogene homologue) and *BRAF* (B-Raf proto-oncogene, serine/threonine kinase) are often described in low-grade serous carcinoma, but have also been reported in mucinous carcinoma [2].

These guidelines review the key morphological and molecular features for diagnosis of the various types of ovarian cancer. The role of current and future biomarker is discussed.

Histopathological classification of ovarian cancer

High-grade serous ovarian carcinoma

Histologically, HGSOC most commonly presents as a proliferation of markedly atypical cells adopting a papillary, glandular or cribriform pattern, with frequent necrosis (Table 1). It was recently suggested that, compared with forms displaying the usual classical pattern, tumours that show a solid, endometrioid or transitional growth pattern, either alone or in combination (the SET pattern) [5], contain more tumour-infiltrating lymphocytes, have a higher mitotic index, are more often associated with *BRCA1* mutations, occur in younger women, respond better to chemotherapy and PARP inhibitors and, all in all, seem to have a better prognosis than conventional HGSOC [1, 2].

The molecular alterations most consistently described in HGSOC include *TP53* mutations [>96% of cases according to data from The Cancer Genome Atlas (TCGA) Research Network]. Because these mutations induce chromosomal instability, they are associated with DNA copy-number aberrations. Other molecular alterations commonly found in these tumours are *CCNE1* amplification, somatic and germline mutations in *BRCA1/2* and abnormalities in pathways that regulate DNA repair mechanisms involving homologous recombination [2]. Recently, four molecular subtypes were described within HGSOC, termed immunoreactive, immunomodulatory, proliferative and mesenchymal [3]. These molecular subtypes vary in their prognoses, with the immunoreactive subtype identified as having the best. This finding is consistent with the presence of more tumour-infiltrating lymphocytes in these carcinomas. It has been suggested that these molecular subtypes may reflect different patterns of oncogene activation [4].

As regards their origin, it is now thought that a significant number of HGSOCs develop from precursor tubal lesions, known as STICs, present in 50–60% of cases, especially in the distal portion of the fallopian tube [4]. It was recently suggested that HGSOC may develop by two different pathways, with conventional HGSOC arising from classical STIC, whereas the SET variant may derive from another STIC variant or an as yet unidentified precursor tubal lesion [3].

Endometrioid carcinoma

Histologically, most ECs are formed by adjacent glands, with a confluent (cribriform) or villoglandular pattern. The glands are round, oval or tubular, lined with stratified epithelium. They sometimes exhibit squamous morules, mucinous differentiation and secretory, fusiform, ciliated, oxyphilic or clear cells. They may be of the neuroendocrine or transitional type, with a sex cord-like or squamous carcinoma-like pattern. Three grades of differentiation have been established for this type of carcinoma: G1 (less than 5% with a non-squamous solid pattern), G2 (6–50% solid pattern) and G3 (over 50% solid pattern). The 2015 Fifth Ovarian Cancer Consensus Conference in Tokyo proposed a binary classification [5]: low-grade carcinoma (G1 EC) and high-grade carcinoma (G2 and G3 EC). This classification requires validation, since some earlier studies suggested the existence of more molecular similarities between G1 and G2 ECs than between G2 and G3 ECs [6]. Ten per cent of ovarian ECs are associated with an endometrial EC, which may be synchronous or metastatic.

Table 1 Histopathological classification of ovarian cancer

Features	Type I	Type II
Histological type	Endometrioid carcinoma; clear-cell carcinoma; mucinous carcinoma; low-grade serous carcinoma; seromucinous carcinoma	High-grade serous carcinoma (conventional and SET type); carcinosarcoma; undifferentiated carcinoma
Stage at diagnosis	Often initial	Almost always advanced
Ascites	Uncommon	Usual
Response to chemotherapy	Poor	Good (relapse common)
Five-year survival	Approximately 55%	Approximately 30%
Tumour grade	Low[a,b]	High
Proliferative activity	Generally low	High
Risk factors	Endometriosis	Uninterrupted ovulatory cycles, germline *BRCA* mutations
Origin	Endometrium (endometrioid carcinoma, clear-cell carcinoma, seromucinous carcinoma); tubal-peritoneal junction/ovary (mucinous carcinoma); fallopian tube (low-grade serous carcinoma)	Fallopian tube in most cases
Precursor	Atypical proliferative tumour (borderline)	STIC in most cases
Chromosomal instability	Low	High
Characteristic molecular alterations	Endometrioid carcinoma: activation of the Wnt/β-catenin pathway; microsatellite instability; inactivating mutations in *PTEN*; activating mutations in *PIK3CA*; inactivating mutations in *ARID1A* Clear-cell carcinoma: inactivating mutations in *ARID1A*; inactivating mutations in *PTEN*; activating mutations in *PIK3CA* Mucinous carcinoma: activation of *KRAS*, *MEK*, *ERBB2* Low-grade serous carcinoma: activation of *KRAS*/*BRAF*/*MEK*	*TP53* mutation (almost invariably) Defective homologous recombination repair mechanisms *CCNE1* amplification *NOTCH1* activation Inactivation of *Rb*, *NF1*

[a] Clear-cell carcinoma is not graded but regarded as a high-grade carcinoma

[b] Can sometimes progress to high grade

ARID1A AT-rich interaction domain 1A, *BRAF* B-Raf proto-oncogene, serine/threonine kinase, *ERBB2* erb-b2 receptor tyrosine kinase 2, *KRAS* Kirsten rat sarcoma viral oncogene homologue; *NF1* neurofibromatosis-1, *PIK3CA* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α, *PTEN* phosphatase and tensin homologue; *Rb* retinoblastoma, *SET* solid, pseudoendometrioid, transitional, *STIC* serous tubal intraepithelial carcinoma

Recent preliminary genetic studies suggest a clonal relationship between the two lesions. Given the non-aggressive course seen in many patients, this might mean that malignant tissue arises in the endometrium in these cases and reaches the ovary by transtubal retrograde flow [7]. Immunohistochemically, ovarian ECs express cytokeratin 7 (CK7), paired box 8 (PAX8), estrogen receptors (ERs) and progesterone receptors (PRs).

In molecular terms, ECs have: mutations in *CTNNB1* (16–38%), generally associated with squamous differentiation, low grade (G1), and a favourable prognosis [8]; mutations in *ARID1A* (30%), *PTEN* (14–21%) and *PIK3CA* (20%); microsatellite instability (13–20%); and mutations in *KRAS* and *BRAF* (fewer than 7%).

Clear-cell carcinoma

CCC is the ovarian tumour most often associated with endometriosis (50–70%). It tends to present as a unilateral ovarian mass and is diagnosed at early stages in most cases (FIGO I/II). Histologically, it displays different architectural patterns: tubulocystic, papillary or solid. Conventional CCC is composed of polygonal cells containing clear cytoplasm, in a "hobnail" arrangement, with an enlarged nucleus and prominent nucleolus. However, pleomorphism and mitotic index tend to be low. Immunohistochemically, CCC is characterised by PAX8 expression and no expression of WT1 (Wilms' tumour 1), ERs or PRs. Both the napsin A protein (NAPSA) and HNF-1β (hepatocyte nuclear factor 1 beta) have shown high sensitivity and specificity as CCC markers and are very useful in differential diagnosis from HGSOC [9, 10].

The most common molecular alterations in CCC are inactivating mutations in the *ARID1A* gene (46–57%), activating mutations in *PIK3CA* (40%), overexpression of hypoxia-inducible factor 1 beta (HIF-1β) and inactivating mutations in *PTEN* (8.3%). Recent studies have demonstrated that *ARID1A* inactivation alone is not enough to

generate CCC. Instead, production of these tumours requires co-deletion of *ARID1A* and *PIK3CA* [2]. It has also been suggested that CCCs have a unique methylation profile, with increased methylation of the promoter for multiple genes in the estrogen receptor alpha (ERα) pathway, and loss of methylation of the promoter for genes in the HNF-1 pathway [2].

Low-grade serous ovarian carcinoma

The clinical behaviour of LGSOC is often indolent, with high rates of disease-free survival. It has an immediate precursor of intermediate malignancy, known as serous borderline tumour (SBLT) or atypical proliferative serous tumour. Some authors have suggested that tumours with micropapillary architecture may represent an intermediate stage between SBLT and LGSOC [1, 11]. Histologically, LGSOC consists of a uniform population of cells with mild or moderate atypia, with little mitotic activity, and frequent psammoma bodies. It displays different architectural patterns, with a large or small papillary component. The immunohistochemical profile is characterised by expression of CK, WT1 and PAX8, as well as ERs and PRs [4].

The molecular alterations characteristic of LGSOC relate to activation of the MAPK (mitogen-activated protein kinase) pathway, based on *KRAS* and *BRAF* mutations. Mutations in *BRAF* and *KRAS* are mutually exclusive and occur at early stages in the development of these tumours. The *BRAF* mutation rate is lower in carcinomas than in SBLTs (2–6 versus 23–48%), so it has been suggested that this mutation may protect against the development of LGSOC from SBLT. In contrast, the *KRAS* mutation rate is similar in LGSOC and SBLT [1, 12, 13]. Compared with HGSOC, LGSOC has lower *TP53* (<10%) and *BRCA1/2* (3%) mutation rates [1, 3].

Mucinous carcinoma

Most MCs are well differentiated. Moderately and poorly differentiated tumours are relatively uncommon. Two variants of MC are described. The most common is the intestinal variant, composed of cells resembling the gastrointestinal epithelium, which possess intracytoplasmic mucin and adopt a wide range of morphological patterns. They also include areas of cystadenoma and atypical proliferative tumour (borderline tumour). MC displays two patterns of invasion: expansile and infiltrative. The expansile pattern is characterised by glandular confluence and a stroma attaining at least 5 mm in one dimension. The infiltrative pattern is characterised by the presence of glands, nests or individual cells, often goblet cells, with cytological atypia in a desmoplastic stroma. Sometimes it also exhibits elements of anaplastic carcinoma (rhabdoid, sarcoma-like or pleomorphic mural nodules). Differential diagnosis mainly concerns metastases of other mucinous tumours. Immunohistochemically, MC is characterised by diffuse CK7 expression, CK20 expression, variable CDX2 expression and PAX8 expression (50–60%). The most typical molecular alterations are somatic mutations in *KRAS* [14] and *HER2* amplification (15–20%) [15].

The endocervical variant (seromucinous carcinoma) is often associated with endometriosis. Histologically, it is characterised by a papillary pattern with stratification resembling serous carcinoma. It consists of serous and endocervical-type cells, and may even show squamous, endometrioid differentiation and clear cells. Growth is usually expansile, with a low mitotic index. Immunohistochemically, expression of CK7, ERs, PRs and PAX8 is observed. Thirty per cent of cases have *ARID1A* mutations [16]. The clinical, morphological and molecular features of these tumours link them more closely to endometrioid and clear-cell carcinomas than to intestinal-type mucinous carcinomas, and the name "mixed müllerian carcinoma" has been proposed for them [17].

Diagnostic algorithm

With appropriate training in the use of current morphological criteria, reproducibility between pathologists in assigning the histological types of ovarian cancer is as high as 85–94% ($\kappa = 0.9$). However, it has been suggested that concordance is lower in routine practice ($\kappa = 0.6$). An algorithm based on the use of four immunohistochemical markers (WT1, p53, NAPSA and PRs) was recently proposed as an aid to morphological diagnosis (Table 2).

WT1 expression is typical of serous carcinomas. However, this marker does not have 100% sensitivity and specificity (Table 2). Expression of p53 makes it possible to distinguish between HGSOC and LGSOC (Table 3). Both overexpression (>70% of tumour cells) and complete absence of expression in tumour cells ("null pattern") with focal expression in the stroma are regarded as abnormal patterns of expression indicative of *TP53* mutation ("mutated pattern"), characteristic of HGSOC. A different pattern of p53 expression is considered normal ("wild-type or native *TP53*"). In cases in which the p53 expression pattern is dubious, diffuse p16 staining supports a diagnosis of HGSOC.

In the case of WT1− ovarian carcinomas, NAPSA has been shown to be a highly sensitive and specific marker for CCC, which has a WT1−/NAPSA+ phenotype in 91% of cases. NAPSA expression can be weak and focal in some tumours, and its expression may occasionally be detected in other histological types. In ECs that express NAPSA, the possibility of a diagnosis of mixed carcinoma (EC/CCC) should also be considered, if supported by the morphology.

Table 2 Percentage expression of immunohistochemical markers in the various histological types of ovarian carcinoma

	HGSOC %	LGSOC %	CCC %	EC %	MC %
WT1+	97	98	1	10	1
TP53 "mutated pattern"	94	0	12	15	61
NAPSA	2	0	92	8	3
PR	40	60	7	85	4

CCC clear-cell carcinoma, *EC* endometrioid carcinoma, *HGSOC* high-grade serous ovarian carcinoma, *LGSOC* low-grade serous ovarian carcinoma, *MC* mucinous carcinoma, *NAPSA* napsin A, *PR* progesterone receptor, *WT1* Wilms' tumour 1

Table 3 Frequency of characteristic immunohistochemical patterns in the various histological types of ovarian carcinoma

	HGSOC %	LGSOC %	CCC %	EC %	MC %
WT1 +/p53 ("mutated pattern")	92	0	<1	2	1
WT1−/p53 ("non-mutated pattern")	5	99	<1	8	0
WT1−/NAPSA+	<1	0	91	8	3
WT1−/NAPSA−/PR+	1	0	1	71	1
WT1−/NAPSA−/PR−	2	1	7	11	95

CCC clear-cell carcinoma, *EC* endometrioid carcinoma, *HGSOC* high-grade serous ovarian carcinoma, *LGSOC* low-grade serous ovarian carcinoma, *MC* mucinous carcinoma, *NAPSA* napsin A, *PR* progesterone receptor, *WT1* Wilms' tumour 1

Most ECs and MCs are WT1−/NAPSA−. When their differential diagnosis is dubious, expression of PRs (and ERs) supports a diagnosis of EC. In contrast, 95% of MCs have a WT1−/NAPSA−/PR− profile, compared with 12% of ECs.

Serum biomarkers

Ca125

CA125 (cancer antigen 125) is a glycoprotein encoded by the *MUC16* gene on chromosome 19. Elevated levels of it are found in 85% of serous ovarian carcinomas and 65% of ECs, with rates dropping to 40, 36 and 12%, respectively, in CCCs, undifferentiated tumours and MCs [18]. Its specificity is affected by false positives, because its levels also rise in non-malignant gynaecological conditions, such as endometriosis, uterine fibroids and even pregnancy, and in non-gynaecological diseases, such as cirrhosis of the liver, hepatitis, pancreatitis and congenital heart disease [18–21]. CA125 tests have various applications in clinical practice.

Screening

The role of CA125 plus transvaginal ultrasound as a population screening method has been evaluated in two randomised studies, the Prostate, Lung, Colorectal and Ovarian (PLCO) trial [22] and the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) [23]. Results were negative in both cases.

The PLCO trial randomised 78,216 women aged 55–74 years to annual CA125 testing and transvaginal ultrasound versus no intervention. One hundred and eighteen ovarian cancer deaths (3.1 per 100,000) occurred in the intervention group versus 100 deaths (2.6 per 100,000) in the control group. The RR was 1.18 (95% CI 0.82–1.71) and differences were not significant.

The UKCTOCS trial recruited 202,638 women, who were randomised to annual multimodal screening (MMS) versus observation. MMS involved testing for CA125 levels over time, interpreting the results with the aid of the Risk of Ovarian Cancer Algorithm (ROCA). If the patient had intermediate risk by ROCA a second CA125 test was done after 3 months, whereas if the ROCA risk was elevated the CA125 test was repeated and transvaginal ultrasound performed after 6 weeks. In this trial, screening also failed to demonstrate any significant reduction in mortality. Based on the results of the above studies, screening for the early detection of ovarian cancer is not recommended in the general population.

More recently, results were published from a joint analysis of two studies by the Cancer Genetics Network (CGN) and the Gynaecologic Oncology Group (GOG). This study included 3692 women with a strong family history of breast/ovarian cancer or *BRCA1/2* mutations. They underwent CA125 screening using the ROCA algorithm on a three-monthly basis, and annual transvaginal ultrasound. The results were compared against CA125

screening of the general population every 6–12 months and historical controls. ROCA screening every 3 months showed greater sensitivity (92%) with high specificity in this high-risk population [24].

The UK Familial Ovarian Cancer Screening Study was performed in women with a familial estimated high risk (lifetime risk >10%). Enrolled women were submitted to ROCA-based screening. A total of 4348 women were included and ROCA screening achieved a significant staging shift, as only 36.8% of cancers diagnosed during screening were stage III–IV compared to 94.4% of stages III–IV after screening ended [25].

Diagnosing pelvic masses

Initial-stage ovarian cancer, presenting as adnexal masses, typically has either vague symptoms or non-specific symptoms in most cases. When confronted with an adnexal mass, the main aim is to determine whether the lesion is benign or malignant. Clinical exam, tumour marker CA125 tests and ultrasound and/or computed tomography (CT) imaging have been the standard basis for deciding whether an adnexal mass is likely to be malignant.

Tumour marker CA125 is elevated in fewer than 50% of initial-stage epithelial ovarian cancer cases, and proves negative in 20% of all these cancers, which substantially reduces its sensitivity [18].

Monitoring and diagnosing ovarian cancer relapse

Elevated CA125 levels have also proved useful in the diagnosis of relapsed ovarian cancer (Table 4). Rustin et al. found that a doubling of CA125 levels (twice the upper limit of normal) had 86% sensitivity and 91% specificity for detecting disease progression [26].

According to the Gynaecological Cancer Intergroup, relapsed ovarian cancer is defined in terms of CA125 by one of the following criteria being met: (1) in patients whose CA125 level was elevated before treatment and returned to normal, CA125 must be elevated above twice the upper limit of normal; (2) in patients whose CA125 level was elevated before treatment but did not return to normal afterwards, CA125 must show evidence of being elevated to twice the nadir or above; or (3) patients whose CA125 is within the normal range must show a rise to twice the upper limit of normal or above. In all cases, elevated CA125 must be confirmed on at least two occasions at least a week apart [27]. A second confirmatory value reduces the false negative rate to just 2% [28]. An elevation in previously increased CA125 levels is an accurate tool for diagnosing progression [29]. However, the monitoring of CA125 levels is controversial. The EORTC/MRC trial showed that initiating treatment on the sole basis of elevated CA125 did not increased overall survival compared with delaying the start of treatment until radiological and/or clinical progression had occurred [30].

Table 4 Serum biomarkers

Uses	CA125	HE4	OVA1
Screening	No	No	No
Diagnosing pelvic mass	Yes[a]	Yes[b]	Yes
Monitoring treatment response	Yes	Yes	No
Detecting relapse	Yes	Yes	No

[a] Also as part of the ROMA algorithm

[b] As part of the ROMA algorithm

HE4

HE4 (human epididymis protein 4) is a glycoprotein expressed in both respiratory and reproductive (male and female) epithelium. It belongs to a family of trypsin inhibitors involved in protective immunity. In contrast to CA125, HE4 levels are not normally elevated in benign conditions, endometriosis or pelvic inflammatory disease. However, an increase in its expression has been observed in many malignancies, especially of the lung and ovary [30], so it has been investigated as a tissue and serum biomarker [29].

Serum HE4 levels are tested by immunoreactivity (ELISA). Compared with levels seen in healthy women or those with benign conditions, ovarian carcinoma patients have HE4 levels of over 70 pmol/L. Combined detection of HE4 and CA125 significantly increases diagnostic sensitivity and specificity for distinguishing between benign and malignant disease [31]. HE4 is more specific than CA125 in premenopausal patients, whereas CA125 is more sensitive in this patient subgroup [32].

As well as being useful for diagnosing pelvic masses, testing for serum HE4 levels is beginning to acquire an important role as a potential biomarker for monitoring treatment and detecting relapse [33] (Table 4).

In a case–control study comparing ovarian cancer patients and healthy subjects, Hellström et al. showed how HE4 was able to identify ovarian cancer patients with 67% sensitivity and 96% specificity [30]. Also, Montagnana et al. tested serum HE4 and CA125 levels in healthy subjects and women with pelvic masses, revealing a significantly larger area under the curve for HE4 than for CA125 (0.99 versus 0.91), with sensitivity and specificity of 98 and 100%, respectively [34].

Combined detection of CA125 and HE4 has enabled development of the ROMA diagnostic algorithm (Risk of Ovarian Malignancy Algorithm). Using a mathematical formula incorporating logarithmic HE4 and CA125 values,

ROMA predicts the percentage risk of ovarian cancer. High risk is defined as a score of 12.5% or above in premenopausal women and 14.4% or above in postmenopausal women. ROMA has 94% sensitivity and 75% specificity [35].

Many attempts have been made to implement a diagnostic algorithm combining ultrasound and CA125 detection. One example is the RMI (Risk of Malignancy Index), obtained by multiplying U (ultrasound risk) by M (menopausal status) by the serum CA125 value (U/mL). A diagnosis of pelvic mass with an RMI of over 200 is regarded as high risk, with 87% sensitivity [36, 37].

OVA1 is a test for five markers, two of which are overexpressed (CA125 and β_2-microglobulin) and three underexpressed (apolipoprotein A1, prealbumin and transferrin). The test is approved by the Food and Drug Administration (FDA). The American Society of Gynaecologic Oncology (SGO) considers it potentially useful for identifying patients who should undergo surgery for a pelvic mass and require assessment by a gynaecological oncologist in order to increase the malignancy detection rate [38].

BRCA and other markers of deficient homologous recombination

Fifty per cent of HGSOCs have faulty DNA repair because of deficiencies in the HR pathway, a DNA damage response mechanism [39, 40]. When a double-strand break occurs in DNA, HR enables the damaged sequence to be exchanged for the same genetic sequence from the healthy homologous chromatid, repairing the break correctly. Causes of this deficiency include germline or somatic mutations in *BRCA1* and *BRCA2* (most commonly), and in genes such as *EMSY* (8%), *RAD51C* (0.41–2.9% of ovarian cancers), *RAD51D* (0.35–1.2%), *RAD51B* (0.065%), *RAD50* (0.2%), *RAD54L* (0.5%), *ATM* (0.8–0.86%), *BRIP1* (0.9–1.72%), *CHEK2* (0.4–1.6%), *FANCA* (0.5%), *FANCI* (0.5%), *NBN* (0.2–0.25%) and *PALB2* (0.2–0.5%). Also included are deletions or mutations in *PTEN* (7%), which are characteristic of EC and CCC.

Germline mutations in *BRCA1* and *BRCA2*

The genes *BRCA1* and *BRCA2* are located at positions 17q21 and 13q12.3. Deficiency in them leads to impaired HR, chromosomal instability, aneuploidy and centrosome amplification [41]. Between 10 and 13% of ovarian cancer patients harbour a germline mutation in *BRCA1/2*. A recent meta-analysis, which evaluated 11 studies involving 6218 patients, found the mean probability of having these germline mutations to be 14.5% for HGSOC patients, compared with 7.7% for EC, 4.9% for CCC, and 12.3% for other histological types [42]. The probability of finding a *BRCA1/2* mutation was highest in patients aged 40–50 years at diagnosis, but there was also a risk in patients diagnosed after the age of 60, especially for *BRCA2*. Moreover, 27–56% of patients harbouring these mutations had no family history. For the above reasons, it is recommended that all women with high-grade, non-mucinous, epithelial ovarian cancer be tested for germline *BRCA1/2* mutations, irrespective of age at diagnosis, family history or histological type.

In clinical practice, *BRCA1* and *BRCA2* mutation tests are performed by sequencing the coding regions and exon–intron junctions. This enables identification of single-nucleotide substitutions, as well as small insertions and deletions, which account for 90% of pathogenic variants. Pathogenic mutations of this type are traditionally determined by Sanger sequencing. As well as the above-mentioned genetic alterations, large rearrangements should be identified by MLPA (Multiplex Ligation Probe Assay) or massive sequencing techniques (NGS, Next-Generation Sequencing), because the Sanger method cannot detect large rearrangements [43]. Given the crucial nature of the result, it is important for *BRCA* mutation tests to be done in accredited laboratories, with internal and external quality-control systems [44].

Somatic mutations in *BRCA1* and *BRCA2*

Somatic mutations in *BRCA1* and *BRCA2* can also contribute to loss of function of these genes, with similar clinical significance. A review of several series identified somatic mutations in 5–7% of ovarian cancers [45]. Data from patients with somatic mutations enrolled in recent PARP inhibitor development studies reveal similar prognoses and treatment responses as in patients with germline mutations [46].

Deficient homologous recombination not involving *BRCA1/2* mutation

Various genetic and epigenetic alterations have been described that might entail loss of function of the *BRCA1* and *BRCA2* genes. These include hypermethylation of the *BRCA1* promoter and *EMSY* amplification, which would lead to loss of *BRCA2* function. *BRCA1* promoter hypermethylation was identified in 5–30% of ovarian carcinomas, mainly HGSOC [47]. However, its clinical significance is controversial. There is no firm evidence to date in support of any favourable prognostic value or response prediction for tumours with epigenetically altered *BRCA1* [45].

Thanks to massive sequencing techniques, the sequence of non-*BRCA* HR genes can be analysed, although their clinical significance has yet to be validated. A recent study tested 390 ovarian cancers for somatic and germline mutations in *BRCA1*, *BRCA2*, *ATM*, *BARD1*, *BRIP1*, *CHEK1*, *CHEK2*, *FAM175A*, *MRE11A*, *NBN*, *PALB2*, *RAD51C* and *RAR51D* [2]. Thirty-one per cent of the tumours showed germline (24%) and/or somatic (9%) mutations in one or more of these 13 genes, irrespective of histological type. The main challenge is interpreting the pathogenic significance of certain variants. Whereas some are clearly pathogenic, because they produce abnormal proteins, others have an unknown effect, as they do not cause major changes in the protein. It is, therefore, important for information about the pathogenic effects of these variants to be kept up to date. This will require bioinformatic support and consultation of properly updated public databases.

Strategies under investigation for identifying HR deficiency involve using massive NGS systems to analyse "scarring" produced in the tumour genome as a consequence of that deficiency. Three types of "scars" have been identified: (1) TAI (telomeric allelic imbalance: the number of subtelomeric regions with allelic imbalance, that start beyond the centromere and extend to the telomere); (2) LST (large-scale state transitions: the number of chromosomal breaks [translocations, inversions or deletions] between adjacent regions, of at least 10 Mb); and (3) LOH (loss of heterozygosity: the number of regions with LOH larger than 15 Mb in size, but smaller than the whole chromosome).

Two platforms capable of identifying these "scars" currently exist, having been developed together with the new PARP inhibitors. The ARIEL programme has validated a genetic test for quantifying heterozygosity in HR deficiency using NGS systems as a biomarker associated with rucaparib use. On the other hand, niraparib development has been accompanied by another diagnostic test that includes analysing HR deficiency (by adding the TAI, LST and LOH scores) and sequencing the *BRCA1/2* genes. An HR deficiency score of 42 can capture 95% of *BRCA* mutations, and is used to identify tumours with deficient HR but no *BRCA* mutations.

Clinical implications

A recent meta-analysis of 14 studies showed a more favourable prognosis for women with ovarian cancer associated with *BRCA1/2* mutations [48]. These women had greater overall survival [hazard ratio (HR) 0.76; 95% confidence interval (CI) 0.70–0.83 for *BRCA1*; HR 0.58; 95% CI 0.50–0.66 for *BRCA2*] and greater progression-free survival (HR 0.65; 95% CI 0.52–0.81 for *BRCA1*; HR 0.61; 95% CI 0.47–0.80 for *BRCA2*), irrespective of grade, tumour stage and histological subtype. The most plausible hypothesis envisages greater sensitivity to platinum-based chemotherapy, because HR deficiency means that chemotherapy-induced double-strand DNA breaks cannot be repaired.

Germline or somatic *BRCA1/2* mutations have also been associated with greater sensitivity to PARP inhibitors and currently constitute a validated biomarker for olaparib use [49]. This conclusion is based on an analysis of patients with *BRCA* mutations included in Study 19 [50]. That study recruited patients with relapsed HGSOC who had previously had at least two platinum-based regimens, with a platinum-free interval of more than 6 months, who experienced a sustained response after at least 4 cycles of platinum in the last regimen. These patients were randomised to receive olaparib maintenance treatment or placebo until disease progression. The study demonstrated a very significant impact on progression-free survival in patients treated with olaparib versus those given placebo, especially in patients with a germline or somatic *BRCA* mutation (11.2 versus 4.3 months; $p < 0.0001$).

Study 19 was not designed to demonstrate an increase in overall survival. Nevertheless, survival data of a merely descriptive nature were recently reported [51]. It is interesting to note that, after a median follow-up of 5.9 years, there are still 15 patients (11%) taking olaparib (8 with mutated *BRCA* and 7 wild-type) and one patient taking placebo, and no new safety data have been documented that were not already known [51]. These results have been confirmed in the phase III SOLO2 study [52], which included patients with high-grade endometrioid or serous carcinoma with a germline *BRCA* mutation. The PFS results were 19.1 vs 5.5 months (HR 0.30; $p < 0.0001$), and 30.2 vs 5.5 (HR 0.25; $p < 0.0001$) in the independent central review.

Data from the phase III NOVA niraparib maintenance study [53], which has a similar design to Study 19, have confirmed the significant progression-free survival benefit among patients with a germline mutation (21.0 vs 5.5 months), which resembles that obtained among patients with a somatic mutation (20.0 vs 11.0 months). Patients without a *BRCA* mutation were included and stratified according to whether the HRD test was positive. All subgroups obtained a significant treatment benefit. Although the magnitude of the benefit was greater in HRD-positive patients, the HRD test was inefficient at identifying patients in whom treatment provided no benefit.

ARIEL2 [54], a phase II study of patients with platinum-sensitive relapse, consisted of two parts. In the first, patients were categorised into three predefined subgroups on the basis of tumour mutational analysis: *BRCA* mutant (germline or somatic), *BRCA* wild-type with high LOH, or

BRCA wild-type with low LOH. Patients were treated with rucaparib monotherapy. Rucaparib was more active in the *BRCA* mutant patient group than in the *BRCA* wild-type, low LOH group. Median PFS was 12.8 months in the *BRCA* mutant group, and no differences were observed between germline and somatic mutations. Median PFS was similar in both the high and low LOH wild-type patient groups, (5.7 vs 5.2 months), although the duration of response and the response rate were greater in the high LOH group. These results are not regarded as especially relevant, which means the biomarker is not considered robust enough to distinguish, those patients without BRCA mutation will benefit from rucaparib treatment. Subsequently, another assessment of the results, with a change in the LOH cut-off value, showed greater concordance. This is being validated in the ARIEL3 study (NCT01968213) [55].

Recommendation

In conclusion, it is recommended that all patients with non-mucinous epithelial ovarian cancer undergo germline *BRCA1* and *BRCA2* mutational analysis in the first instance. In patients who test negative for germline mutation, analysis should be completed with somatic testing of tumour tissue. The gradual implementation of panels in next-generation sequencers is facilitating these assays. It is crucial to know *BRCA1/2* status, because of its importance for prognosis, and as a predictive biomarker for sensitivity not only to platinum-based chemotherapy, but especially to the PARP inhibitors available. PARP inhibitors are being developed in parallel with the validation of biomarker platforms (companion diagnostic) for predicting which patients will benefit most from this therapy. To date, none of the tests examined has shown enough accuracy to identify these groups of patients.

Biomarkers currently in development

Folate receptor

Folate is essential for nucleotide synthesis and DNA replication. It must be transported into the cell either by the reduced folate carrier or via its own receptor (FR). FR is a transmembrane glycoprotein that permits one-way transport of folate into the cell. It is extensively expressed on ovarian cancer cells (80%). Its overexpression has also been regarded as a poor prognostic factor associated with a sub-optimal response to chemotherapy. Accordingly, FR has been considered a target for new drug development [56].

Farletuzumab is a humanised monoclonal antibody (IgG) that binds to the folate receptor α subunit (FRα). Its anticancer activity is exerted through antibody-dependent cytotoxicity. Results of a phase III trial in platinum-sensitive relapsed ovarian cancer showed no significant increase in survival of groups treated with farletuzumab [57]. In subgroup analysis, however, patients with low CA125 levels showed an increase in both progression-free survival ($p = 0.0028$) and overall survival ($p = 0.0108$). Based on these results, farletuzumab is currently being used in a phase III study in patients in platinum-sensitive relapse with CA125 levels less than or equal to three times the upper limit of normal (NCT02289950).

FRα (≥25% of tumor cells with at least 2+ staining intensity) was a selection criteria for including patients in a phase I expansion study with the antibody–drug conjugate mirvetuximab soravtansine (IMGN853). This new compound showed a promising activity with an objective response rate of 26% in patients with platinum-resistant ovarian cancer and positive for FRα. Notably, in the subgroup of patients who had received three or fewer previous lines showed a response rate of 39%. Moreover, IMGN853 at a dose of 6.0 mg/kg showed a manageable safety profile [58]. Based on these findings, a new phase III trial, the FORWARD I trial, is recruiting platinum-resistant ovarian cancer patients expressing medium or high levels of FRα. Patients will be randomized to mirvetuximab soravtansine versus liposomal doxorubicin, weekly paclitaxel or topotecan. The primary endpoint is PFS that will be assessed in both the entire population and in the high levels of FRα subset (NCT02631876, clinical trials.gov).

p53

Mutations in the *TP53* gene, with overexpression or loss of protein expression, are highly prevalent in HGSOC (96–100%) and uncommon in other ovarian carcinoma subtypes, including LGSOC (<10%) [1, 3]. Identical p53 mutations have been described in HGSOC and its most direct precursor, STIC, suggesting that they are hypothetically useful for early detection of these tumours.

The prognostic value of p53 is still the subject of debate, although some literature evidence suggests that the presence of p53 mutations is related to a worse prognosis [59]. Other studies, however, link the presence of mutated p53 to a greater response to chemotherapy [60, 61]. Also, truncated isoforms of the protein have been described (Δ133p53 in mutated p53 or Δ40p53 in wild-type p53), associated with a better prognosis [62]. Detecting anti-p53 antibodies in serum has been proposed as a potential biomarker for the detection and prognosis of ovarian cancer, with very limited results to date [63].

It was recently suggested that detecting somatic *TP53* mutations in circulating tumour DNA (ctDNA) is a sensitive marker for early response to treatment [64].

Immunological biomarkers

Intratumoral T cells, PD-1 and PD-L1

The presence of intratumoral T cells is a predictive factor for better survival, whereas an increase in regulatory immunosuppressive T cells is associated with a worse prognosis [65]. This suggests a possible functional role for T cells in controlling the progression of ovarian cancer. Many immune checkpoints have been described, involving molecules associated with cytotoxic T cells, especially programmed cell death 1 (PD-1) and its ligand (PD-L1). PD-1 is a member of the B7 immunoglobulin superfamily involved in immunomodulation mechanisms. It is expressed on the surface of activated T cells, especially germinal centre-associated T cells and intratumoral T cells. Binding of PD-L1 to PD-1 induces effector T cell exhaustion, and immune escape by cancer cells. This adaptive process is triggered by the specific recognition of cancer cells by T-cells, which leads to the production of immune-activating cytokines, being interferon gamma (INF_{γ}) the most important, that triggers the expression of PD-L1 in both inflammatory and tumor cells. For Anti-PD-1/PD-L1 antibodies to be effective requires pre-existing $CD8^{+}$ T-cells that are negatively regulated by PD-1/PD-L1-mediated adaptive immune resistance. Recent clinical trials have demonstrated that monoclonal antibodies against PD-L1 or its receptor PD-1 prevent the inhibitory effects of the PD-1/PD-L1 pathway and improve T cell function, with encouraging results in various tumour types, such as melanoma, renal cell carcinoma, non-small-cell lung cancer and bladder carcinoma [66]. In general, patients with ovarian cancer and high levels of PD-L1 expression have lower overall survival [66]. A recent phase II clinical trial assessing the safety and anticancer activity of the anti-PD-1 antibody nivolumab, in patients with platinum-resistant ovarian carcinoma, showed a general response rate of 15% and a disease control rate of 45% [67]. Phase Ib clinical trials evaluating the efficacy of the antibodies pembrolizumab (anti-PD-1) and avelumab (anti-PD-L1) in the treatment of advanced ovarian cancer suggest that inhibiting these molecules may help to control the disease [68].

CXCL9 and CXCL10

CXCL9 and CXCL10 are two chemokines that facilitate the chemotactic recruitment and intratumoral accumulation of tumour-infiltrating T cells. In a recent study in HGSOC, high expression of CXCL9 and CXCL10 significantly increased patient survival (CXCL9 HR 0.41; $p = 0.006$; CXCL10 HR 0.46; $p = 0.010$) [69].

Angiogenesis-related biomarkers

The prognostic value of vascular endothelial growth factor (VEGF) expression, both in patients' serum and in tumours, has been extensively explored. Elevated VEGF levels are related to greater vascular permeability and increased tendency towards peritoneal progression and ascites [70]. A meta-analysis including over 1000 ovarian cancer patients confirmed that elevated serum VEGF levels were associated with shorter progression-free survival (HR 2.46; 95% CI 1.84–3.29) and lower overall survival (HR 2.21; 95% CI 1.57–3.13) compared with low levels. As regards tumour VEGF levels, the only evidence was that elevated VEGF levels (tVEGF) had a negative impact on patient survival at early stages [71]. More recent genomic signature studies, including multiple genes related to this pathway, confirm the adverse prognostic value of elevated VEGF expression levels [72].

To date, the predictive role of angiogenesis markers is still under discussion. Results from a retrospective analysis of markers in 283 tumour specimens from Scottish patients involved in the ICON7 trial, evaluating the role of bevacizumab (an anti-VEGF monoclonal antibody) added to chemotherapy, were reported in 2014. Transcriptome analysis identified three signatures. In two of them, angiogenesis was up-regulated, whereas in a third, immunogenic, group angiogenesis was down-regulated. The immune subgroup had better overall survival than the angiogenic subgroups (HR = 0.66; 0.46–0.94). However, the addition of bevacizumab in the experimental arm of ICON-7 trial was associated with worse survival than chemotherapy alone, in the immune subgroup (HR = 1.73; 1.12–2.68). In contrast, the pro-angiogenic subgroups showed a trend towards greater PFS with the incorporation of bevacizumab [73].

In 2015, the Cooperative Group GOG carried out a retrospective marker analysis in 1455 patients enrolled in the prospective GOG218 trial (78% of the trial sample size). Parameters analysed consisted of tVEGF levels and microvascular density (MVD), as measured by CD31 on tumour, among other assays. MVD was a prognostic and predictive factor for bevacizumab treatment benefit. In fact, the addition of bevacizumab was associated with greater impact on PFS in patients with MVD > Q3 (HR 0.38) versus those with MVD < Q3 (HR 0.68). Similar results were seen in terms of impact on OS. Levels of tVEGFA were also predictive of bevacizumab benefit [74].

Retrospective studies of angiogenic markers in ICON7 and GOG218, therefore, suggest a role in predicting

bevacizumab benefit; nevertheless, validation in prospective studies is required.

Conclusions

The approach to diagnosis and management of ovarian cancer has changed substantially in the past decade due to the ability to distinguish five tumour types with different morphological, immunophenotypic and molecular characteristics, in what was previously considered a single entity. This accomplishment was reached both by better knowledge of the histopathological features of ovarian cancer and by a deeper understanding of its carcinogenesis and molecular biology.

Given their implications for prognosis and therapy, analysis of the *BRCA1/2* genes in all women diagnosed with serous ovarian carcinoma is a target to be achieved in the next few years. In fact, these genes constitute a biomarker for response to PARP inhibitors. Likewise, it is essential to refine the new massive sequencing techniques, to enable more accurate identification of patients with wild-type *BRCA* genes, but with a deficient HR pathway, who also benefit from PARP inhibitor treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that, when writing and revising the text, they did not know the names of the pharmaceutical companies that provided financial support for this project, so this support has not influenced the content of this article.

Ethical statement The study has been performed in accordance with the ethical standards of the Declaration of Helsinki and its later amendments. This article does not contain any studies with human participants or animals performed by any of the authors.

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