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AQUAPORIN-1 EXPRESSION AS PREDICTIVE MARKER OF CHEMO-RESISTANCE IN OVARIAN HIGH-GRADE SEROUS CARCINOMA: A COMPARATIVE STUDY BETWEEN PREOPERATIVE PERITONEAL BIOPSIES AND SURGICAL SAMPLES

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INDEX

1. Epithelial ovarian cancer

Epithelial ovarian cancer (EOC) represent the most lethal gynaecological malignancy, being the fifth cause of female related cancer death, with an estimated total of 225,500 diagnosed each year [1,2]. Its incidence and mortality are constantly increasing, mainly because the majority of women are diagnosed in advanced stage [1, 2].

In fact, most EOC cases are detected at International Federation of Gynecology and Obstetrics (FIGO) stage IIIC or IV. Most patients relapse within the first 5 years of the initial treatment, and only 20%–25% patients are effectively cured [1-5].

The two main prognostic indicators are FIGO stage at diagnosis and size of residual disease after surgery. Poor survival rates are partly the result of late stage at presentation, many patients being stage III–IV at diagnosis [1-5].

From a pathological point of view, EOC are subdivided into serous, mucinous, endometrioid, clear cell, transitional cell, squamous, mixed epithelial and undifferentiated types. Except for squamous and undifferentiated tumors, there are benign, borderline, and malignant subcategories within all of these categories [6].

1.1. Epidemiology and risk factors

Like other epithelial malignancies, EOC is more frequently diagnosed more in older women, with a median age of diagnosis is at 63 years, therefore, as global life-expectancies continue to increase, the number of newly diagnosed cases is expected to improve [1,7].

The higher incidence rates are observed in Northern and Central/Eastern Europe; intermediate incidence rates are observed in North America, Western Europe and Australia; and lower incidence rates in Asia and Africa [1, 2, 7].

Regarding the most relevant risk factors, a heritable component related to genetic factors, has been shown to greatly influence the risk of developing EOC. In fact, a greater risk has been

observed in women with an affected first-degree mainly due to germline mutations in the tumour-suppressor genes BRCA1 and BRCA2, also related to increased risk of developing breast cancer in these same relatives [7,8]. Overall, 10–20% of EOCs arise as a consequence of these germline BRCA1 and BRCA2 mutations. BRCA-related EOCs, usually present at an earlier age and are usually high-grade serous carcinomas [7,8].

However, in BRCA1/2 wild-type population, many other genes play an important pathogenetic role, these include BRIP1, RAD1C, RAD1D BARD1, CHEK2, MRE11A, RAD50, PALB2 and ATM, all involved in the homologous recombination (HR)-mediated pathway of DNA repair [9-11].

Mutations in genes involved in DNA mismatch repair, mainly observed in women with Lynch syndrome are also related to an increased risk of EOCs, mainly clear-cell and endometrioid subtypes [1].

Regarding non-genetic related risk factors, endometriosis is the main clinical condition related to an increased risk of developing EOC, particularly clear-cell and endometrioid subtypes, which are known to arise from endometriotic cysts [1].

Other potential risk factors include the number of ovulatory cycles, nulliparity, obesity, diabetes, smoking and usage of perineal talc [1].

1.2. Pathogenesis and molecular subgroups

According to the most accepted pathogenetic model of ovarian cancer, the fallopian tube represents the primary site of origin for incidentally detected high-grade serous carcinomas (HGSCs), both in women with BRCA mutations or the general population [5-7]. Moreover, to fully understand the origin of EOC, the following pathogenetic concepts must be taken into consideration:

1. A subset of well to moderately differentiated tumors, mainly endometrioid, clear cell, mucinous, borderline and low-grade serous subtypes, are thought to arise from the ovarian cortex, probably from cortical inclusions or endometriosis [6, 12,13].

2. A second subset of tumors, which represent the most common subtypes, are regarded as high-grade müllerian carcinomas [1,6,12,13]. These neoplasms are all related to TP53 mutations; defects in DNA repair and germ-line mutation in either BRCA1 or BRCA2 also contribute to their pathogenesis. Tumors previously diagnosed as high-grade endometrioid and transitional carcinomas that contain TP53 mutations are now regarded as morphological variants of high-grade serous carcinoma (HGSC): SET variant (solid, endometrioid-like, or transitional).

The origin of "classic-type" HGSCs seem is closely related to the distal fallopian tube, since about 75% of cases show a concomitant serous tubal intraepithelial carcinoma (STIC).

On the other hand, the SET group is associated with an STIC in 25% of cases.

Other possible sites of origin include the ovarian parenchyma, endometriosis, adenofibromas, peritoneal or ovarian surface epithelium.

3. Peritoneal surface is thought to represent the site of origin of a small subset of EOCs, mainly borderline serous tumors and endometrioid carcinomas. The most probable sites of origin are represented by müllerian inclusions (endosalpingiosis or endometriosis) [1,6,12,13].

Regarding the molecular Pathogenesis of EOC, in recent years their molecular signatures have been increasingly refined, permitting investigators to uncover pathways and biomarkers that distinguish the individual groups and provide both pathogenetic information as well as potential diagnostic and therapeutic targets [1,13]. The most relevant molecular pathways in EOC are illustrated in Figure 1.

1.3 High-Grade Serous Carcinoma (HGSC)

HGSCs account for about 80% of ovarian carcinomas and are most commonly observed in the postmenopausal age groups, with a mean age at presentation of 56 years [1]. Their clinical manifestations are usually related to an ovarian mass or abdominal swelling. HGSCs account for almost 90% of stage III and IV ovarian tumors. In fact, only 16% of tumors are stage I, with 11% stage II, 55% stage III, and 18% stage IV [1,2].

1.3.1 Gross features

The macroscopic appearance of HGSCs is similar to other types of poorly differentiated ovarian tumors, ranging from cystic and papillary tumors to entirely solid masses, which involve both ovaries in about two-thirds of all cases [1,6]. On rare occasions the tumor is entirely exophytic presenting with patches or hard plaques on the ovarian surface.

1.3.2 Histopathology

On microscopic examination, HGSC typically display a solid growth of cells with slit-like fenestrations. Other frequently encountered growth patterns include papillary (fibrous papillae and micropapillae), nested, glandular, cribiform and single cells pattern [1,6,13]. In some areas, the tumor architecture is closely reminiscent of the surface epithelium of the fallopian tube. These patterns are often admixed and accompanied by areas of extensive necrosis. HGSCs which simulate the appearance of endometrioid or transitional cell carcinoma are now referred as SET (“Solid, pseudo-Endometrioid and/or Transitional cell carcinoma-like”) tumours [1,6,13]. It has been shown that SET tumours are frequently associated with BRCA1 mutations and contain an increased number of tumour-infiltrating lymphocytes compared to classical HGSC [1,6,13].

The neoplastic cells of HGSC are characterized by high-grade nuclear atypia with large, hyperchromatic and pleomorphic nuclei, often multinucleated and prominent eosinophilic

nucleoli [1,6,13]. The mitotic index is often elevated, exceeding 12 mitoses/10 high power fields. Stromal concentric calcifications referred as psammoma bodies, are also frequently encountered.

The most reliable immunohistochemical markers to support the diagnosis of HGSOC are represented by PAX8, a marker indicative of Müllerian origin, WT1, P16 and Cytokeratin 7 [1,6,13]. Most cases also show nuclear stain for estrogen and progesterone receptors. Missense mutations in TP53, typical of HGSOC, are usually correlated with positive immunohistochemical staining for p53, which is diffuse and present in >75%of neoplastic cells. If, the gene contains a nonsense mutation, the resultant staining pattern would be almost totally negative and is referred as "null-type" pattern [14].

1.3.3 Neoplastic dissemination

Rather than metastasizing through blood or lymphatic drainage, HGSC typically spreads by direct extension in the peritoneal cavity involving all organs and structures it encounters [1]. Moreover, neoplastic cells have the ability to exfoliate from the primary tumour, either singly or in clusters, and spread in the peritoneal fluid implanting to distant organs or tissues, which develop secondary neoplastic nodules [1].

The omentum represents the most common site of neoplastic dissemination, although every organ or structure within the peritoneal cavity may be involved [1].

Despite neoplastic spread outside the peritoneal cavity is uncommon, certain extra-peritoneal sites are frequently involved in advanced stages, these include pelvic and/or para-aortic lymph nodes, liver, spleen, and the diaphragmatic barrier including the pleural space [1].

Advanced stage patients frequently develop neoplastic ascites secondary to obstruction of the lymphatic drainage by tumor growth or to the secretion of vasoactive and angiogenic factors by tumor cells [1].

1.3.4 Clinical presentation, Diagnosis and Staging

The majority of HGSC cases are diagnosed with late stage disease since there are no effective screening strategies for the diagnosis of ovarian cancer at early stage [1].

Genetic screenings are useful to detect BRCA mutations in patients with a family history of breast and ovarian cancer. In these patients, a prophylactic surgery such as bilateral salpingo-oophorectomy, may be an effective strategy to significantly reduce the risk of developing EOC [1,11].

The clinical presentation of HGSC is often nonspecific; the most common symptoms include abdominal pain, nausea, constipation, anorexia, diarrhoea and acid reflux [1]. Other symptoms include fatigue, back pain, tenesmus, as well as elevated urinary frequency [1].

If case of suspected EOC, the patient will undergo a pelvic and rectovaginal examination along with transvaginal or abdominal ultrasonography, CT or MRI and measurement of CA125 blood levels [1].

Peritoneal carcinomatosis with the accumulation of large volumes of ascites is usually observed in advanced disease. In these cases, laparoscopic surgery can be performed to obtain a bioptic tumour sample essential for the diagnosis and the staging of the disease [1].

In fact, the 2014 FIGO staging system is based on the degree of disease dissemination at diagnosis. At stage I, the cancer is confined to the ovaries or fallopian tubes; stage II disease includes neoplastic spread to other pelvic organs including the uterus; stage III involves tumor spread within the peritoneal cavity beyond the pelvis; stage IV disease includes tumor spread beyond the peritoneal cavity (inguinal and other extra-abdominal lymph nodes, spleen, liver or lung) [15].

1.3.5 Treatment strategies

Primary debulking surgery (PDS) with adjuvant taxane-platinum chemotherapy represents the standard treatment for advanced ovarian cancer [1,3-5]. Complete gross tumor resection is the main goal of PDS and represents the most reliable predictor of clinical outcome.

The chemotherapy protocol, based on the use of platinating agents, is referred as "platinum-based therapy". This represents the therapeutic standard of care for ovarian cancer patients and is the same irrespective of the EOC subtype involved.

In detail, the actual treatment protocol consists of 75 mg/m2 cisplatin intravenous infusion, plus 135 mg/m2 paclitaxel infused over 24 h every 3 weeks for a total of 6 cycles [1,3-5].

Neoadjuvant chemotherapy (NACT) with platinum-containing agents followed by interval debulking surgery (IDS) represents an alternative treatment strategy, especially for patients that are too ill to undergo surgical cytoreduction or for patients with advanced disease for whom complete resection is impossible. In these cases, patients undergo 3 cycles of chemotherapy, followed by surgical cytoreduction and lastly 3 cycles of chemotherapy [1,3-5].

NACT has been increasingly used to treat women with advanced stage EOC given the favourable results observed in two randomized controlled phase III trials which demonstrated similar progression-free survival (PFS) and overall survival (OS) after neoadjuvant chemotherapy and interval debulking surgery compared with surgery alone [1,3-5].

In detail, The European Organization for Research and Treatment of Cancer-National Cancer Institute of Canada Clinical Trials Group (EORTC-NCIC) randomized trial reported a median progression-free survival (PFS) and OS of 12 and 29 months for the PDS group and 12 and 30 months for the NACT group, respectively [4]. Similarly, the CHORUS trial, reported a median PFS and OS of 11 and 23 months for the PDS group and 12 and 24 months for the NAC group [5].

1.3.6 Pathological chemotherapy response score (CRS)

NACT followed by IDS provides an opportunity to establish a pathological tumor response score to chemotherapy [1,3-5]. Several validated scoring systems have been reported in solid tumors including breast, esophageal, gastric and rectal cancers and are essential to guide treatment decisions after surgery [16-19].

In EOC, it is well known that the histopathological assessment of NACT response represents the most important prognostic tool to establish the rate of complete citoreductive surgery and to predict patient outcome [20-27].

Currently, the chemotherapy response score (CRS) system proposed by Böhm et al., is considered the most reliable histopathological grading system for assessing NACT response in OC [20-27].

Specifically, it consists of a three-tier CRS based on the evaluation of omental residual disease, which shows a good correlation with progression-free survival and overall survival: Score 1: No or minimal tumor response; Score 2: Partial tumor response; Score 3: Complete or near-complete response [23].

This scoring system was recently included into the International Collaboration on Cancer Reporting (ICCR) and the College of American Pathologists (CAP) guidelines for histopathologic reporting of ovarian carcinoma [24]. Moreover, several studies have validated the CRS system in external cohorts of EOC patients [20-27].

In a recent study conducted by our group on a large series of EOC patients, we confirmed that CRS represents a reliable surrogate to early predict patient survival and risk of early relapses [22].

Moreover, for the first time, we demonstrated the prognostic significance of adnexal CRS, originally considered by Böhm et al. as less reproducible to score and with no prognostic significance [22].

1.4. Aquaporin 1 (AQP1) role in carcinogenesis, tumor progression and metastasis

Despite the considerable advances in the pre-operative diagnosis and treatment strategies, the majority of OC still recur and develop chemoresistance with poor 5-year survival [1,2,28]. Therefore, novel prognostic biomarkers are needed to predict the biologic behaviour and therapeutic response, improving the OC patient's clinical outcomes. In this field, some previous studies highlighted the potential role in carcinogenesis, tumor progression and metastasis development of different cancers by Aquaporin 1 (AQP1), a small trans-membrane water channel protein [29-42]. Aquaporin (*AQP*) gene was first identified in 1992 as a water transport channel. Since 1992, 13 AQP genes have been discovered to be widely expressed in numerous human tissues [29-42]. AQPs play an important role in fluid homeostasis since their main function is to facilitate passive water transport across the plasma cell membrane. Several studies have demonstrated that AQPs are also expressed in a variety of tumor types and are strictly related to different tumor biological functions [29-42].

In this regard, in a recent study performed by our group, we demonstrated the immunohistochemical expression of AQP1 in a series of malignant pleural mesotheliomas related to fluoroedenite fibers exposure [33]. In this study, a significantly longer OS was found in the group with AQP1 overexpression, with delayed recurrences and death for the disease. By contrast, earlier recurrences and the worst prognoses were encountered in patients who showed a low immunohistochemical expression of AQP1 [33]. In another study, we also demonstrated that AQP1 immunoistochemistry can also be performed on pleural effusions cytological samples where we observed similar prognostic differences in terms of OS and PFS when comparing AQP1+ and AQP- cases [34].

At the same time, several studies have demonstrated the prognostic role of AQP1 in different solid tumors including breast cancer, brain tumors, prostate adenocarcinoma, lung adenocarcinoma and carcinomas of the gastrointestinal tract [29-32, 34-37].

However, only few scientific papers have investigated the prognostic role of AQP1 expression in EOC [38-43]. In detail, when ovarian carcinomas were divided by histological types, low AQP1 expression correlated with poorer prognosis in clear cell variant, while high AQP1 content has been related to poorer prognosis in mucinous and endometrioid carcinomas [43].

2. Aims of the study

Consequently, the aim of the present study is to investigate the AQP1 immunohistochemical expression in a series of advanced stage high-grade serous ovarian carcinoma. We expect to clarify its potential relationship with response to chemotherapy and with patient's prognosis in order to verify if AQP1 may be considered an additional useful biomarker in OC patients.

3. Materials and Methods

The study complied with the Ethical Principles for Medical Research Involving Human Subjects according to the World Medical Association Declaration of Helsinki; the non-interventional, retrospective nature of our study did not require any informed consent, even if a written informed consent has been obtained from each patient before surgical procedures. The clinical information had been retrieved from the patients' medical records and pathology reports. Patients' initials or other personal identifiers did not appear in any image. Finally, all samples were anonymized before histology and immunohistochemistry; therefore, no further ethical approval was necessary to perform the retrospective study.

3.1 Patient Selection and Clinical Data

A cohort of 32 patients presenting with peritoneal carcinosis documented by diagnostic peritoneal biopsies, which confirmed the histological diagnosis of high-grade serous ovarian carcinoma, were included in the study. All patients met the following additional inclusion criteria: International Federation of Gynecology and Obstetrics (FIGO) stage IIIC/IV, platinum-based NACT, and complete clinical response after neoadjuvant chemotherapy: score 0 according to the surgical scoring system for the IDS residual disease (0, no residual disease; 1, ≤ 1 cm residual disease; 2, >1 cm residual disease; 3, Unknown).

All selected patients, on the basis of clinical, serologic, instrumental data, and/ or surgical exploration were considered as non-eligible for primary debulking surgery. IDS was performed either by laparotomy or minimal invasive surgery according to pre-operative evaluation, preference and experience of surgeons. After surgical procedures, all patients were routinely evaluated with clinical visits and CT-scan examination after three cycles of NACT and the IDS was proposed after the third cycle, if there was any evidence of progressive disease. After concluded initial treatment, the follow-up was scheduled for all patients, every 3–4 months for 2–3 years and successively, every 6 months for the next 3 years.

3.2 Pathology Evaluation

The histological diagnosis of HGSC, which allowed us to include 32 peritoneal biopsies in our study, was rendered on the basis of the following histopathological and immonohistochemical criteria:

i) Neoplastic cells growing in solid, papillary, glandular structures with high grade nuclear atypia, large, hyperchromatic and pleomorphic nuclei

ii) Increased mitotic index (desirable: 12 mitoses/10 high power fields).

iii) Positive immunohistochemical stain for PAX8, WT1, P16 and cytokeratin 7 along with negativity for the following antibodies: cytokeratin 20, CDX2, GATA3.

iv) Complete absence or nuclear overexpression in >75% of tumor cells for p53; these immunohistochemical staining patterns are essential to support the diagnosis of HSOC and denote an underlying *TP53* mutation.

The histological CRS following IDS was determined in the omental sites according to the three-tiered CRS proposed by Böhm et al. [23]. All the omental and ovarian formalin-fixed paraffin embedded tissue blocks were sectioned at 4–5 μm intervals, stained with haematoxylin and eosin (H&E) and reviewed by a team of experienced pathologists (GFZ, AS, GT and GA), who were blind of clinical data and each other results to assign the CRS of 1–3 for the omental samples. Fleiss-Cohen weighted k statistics were used to assess the concordance rate of CRS in high grade OC. *k* values between 0 and 0.2 were regarded as no agreement, between 0.21 and 0.4 as fair agreement, between 0.41 and 0.6 as moderate agreement, between 0.61 and 0.8 as substantial agreement and between 0.81 and 1 as almost perfect agreement.

As previously reported, the histological CRS was determined as follows: Score 1: No or minimal tumor response (mainly viable tumor with no or minimal regression-associated fibro-inflammatory changes, limited to a few foci; cases in which it is difficult to decide between regression and tumor-associated desmoplasia or inflammatory cell infiltration); Score 2: partial tumor response (multifocal or diffuse regression associated fibro-inflammatory changes, with viable tumor ranging from diffuse sheets, streaks or nodules, to extensive regression with multifocal but easily identifiable residual tumor; Score 3: Complete or near-complete response (mainly regression, with few irregularly scattered individual tumor cells or cell groups, all measuring <2 mm, or no residual tumor identified) [16]. In case of

disagreement, slides were jointly discussed by using a double-headed microscope, until agreement was reached.

3.3 Immunohistochemistry

Additional sections from the most relevant histological samples were cut for immunohistochemistry.

Tumor tissue biopsies were obtained at first surgery for all cases. Tissue specimens were fixed in 10% neutral buffered formalin and were paraffin embedded according to standard procedures. 4-5-micrometer sections of representative blocks from each case were deparaffinized in xylene, rehydrated, and treated with 3% H2O2 in tris buffered saline (TBS) for 5 minutes to block endogenous peroxidase activity. Antigen retrieval procedure was performed by microwave oven heating in citrate buffer (pH 6).

AQP1 immunohistochemistry was evaluated in preoperative peritoneal diagnostic biopsies from all patients before they received chemotherapy. In this way, we ensured that our immunohistochemical results were not altered by drug-changes in tissue samples. Four-five μm thick sections were cut, mounted on xylane-coated slides (Dako, Glostrup, Denmark), stained with hematoxylin and eosin (H&E) and examined using a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) for a preliminary morphological evaluation, avoiding the presence of structural alterations. Moreover, on parallel sections, AQP1 (B-11, Santa Cruz Biotechnology, Santa Cruz, CA, USA; wd of 1:100) was applied using a Ventana Benchmark immunostainer (Ventana Medical Systems, Inc., Oro Valley, AZ, USA). The reaction was then visualised with 3-3' diaminobenzidine tetrahydrochloride, and the slides were counterstained with Mayer's haemalum. Only membrane labelling was considered specific, and this pattern of labelling was confirmed from 10 high-power (×400) fields (Figure 2A). Positive and negative controls for AQP1 were used to test the specificity of the immunoreaction. Vascular endothelial cells and non-neoplastic mesothelial cells served as positive internal controls (Figure 2B); in negative controls, the primary antiserum was omitted

and replaced by non-immune serum or phosphate buffered saline solution (pH 7.6). Finally, representative photomicrographs were captured using a digital camera (AxioCam MRc5, Carl Zeiss).

In addition, as elsewhere suggested [33,34], the percentage of immunostained cells was assessed by semi-quantitative optical analysis according to a four-tiered system (0 = negative; ≥ 1% to 24% positive cells = focal staining; ≥25 to <49% positive cells = not uniform staining; ≥50% positive cells = diffuse staining). Cases showing a value more than >1%, as the median of immunoreactive neoplastic cells, were considered positive for AQP1 expression.

Moreover, for diagnostic purposes, the following IHC stains, supporting the diagnosis of HGSC were performed: pankeratin cocktail (AE1/AE3, 1:50, Dako, Carpinteria, CA); PAX-8 (polyclonal, 1:100, Protein Tech, Chicago, IL); anti-ERα (Clone 1D5, Dako, ready to use); anti-PR (clone PgR 1294, Dako, dilution 1:50); WT1 (Clone 6F-H2, Dako, ready to use); p16 (E64H, 1:3 of pre-dilute, Ventana, Tuscon, AZ); anti-p53 (DO-7, pre-dilute, Dako), Ki67 (MIB, pre-dilute, Dako), CK7 (Clone OV-TL 12/30 pre-dilute, Dako); CK20 (Clone Ks20.8, pre-dilute, Dako); GATA3 (clone EP368 pre-dilute, Dako) and anti-Human CDX2 (EPR2764Y Rabbit Monoclonal, pre-dilute, Roche Ventana Medical System, Inc).

IHC stain results were assessed semiquantitatively as follows: negative (no cells stained), focal positive (≤ 10% cells stained), patchy (11% to 49% cells stained) and diffusely positive (≥ 50% of cells stained).

3.4 Statistical Analysis

To assess the predictive value of AQP1 for omental residual disease, Fisher exact test was performed using the SPPS Statistics 23 software (SPSS Inc, USA). Statistical analysis was carried out by chi-square test to analyze associations between high and low AQP-1 expression and clinico-pathological parameters such as age, stage, CRS, and outcome. A *P*

value less than 0.05 was considered statistically significant. The sample size was determined in order to achieve a power of 0.80, an alpha of 0.05 and the hazard ratio of 2 between the two groups. Cancer-specific survival analysis was performed using the Kaplan-Meier method, and for comparison of the survival curves, the Mantel-Cox log-rank test was used.

Progression-free survival was defined as the time elapsed between the date of diagnosis and evidence of recurrence, as assessed by imaging or clinically, or date of last follow-up. Overall survival was defined as the time elapsed between the date of diagnosis and death or date of last censored. Median follow-up was calculated according to the inverted Kaplan-Meier technique. Overall survival and progression-free survival curves were estimated by the Kaplan-Meier product limit method and compared by log-rank test. For progression-free survival and overall survival, Cox proportional hazards models were used to assess treatment effect at univariate and multivariate analyses.

4. Results

4.1 Patient Baseline Characteristics

A total of 32 women (mean age 62 years, age range 42–86 years) with advanced stage IIIC-IV ovarian high-grade serous carcinoma treated with neoadjuvant chemotherapy and interval debulking surgery were identified and included in the study. According to the surgical scoring system for the IDS residual disease, all patients were considered score 0.

Moreover, 27 patients had stage IIIC disease, and 5 had stage IV disease. In our study cohort, 10, 17, and 5 patients had omental CRS of 1, 2, and 3, respectively. The *k* value for the CRS in high grade OC among different observers was 0.87 (almost perfect agreement).

All clinico-pathological and immunohistochemical data are analytically summarized in Table 1.

4.2 AQP1 Immunohistochemistry

The immunohistochemical expression of AQP1 was documented by the linear (partial) and/or circumferential (complete) membranous staining, not exclusively lining the apical cellular portion of neoplastic elements (Figure 2A). Taking into consideration a cut-off of $\geq$ 1 % positive tumor cells, 20 (62.5%) cases showed positive AQP1 staining (AQP1+), while 12 (37.5%) cases were considered negative (AQP1-) (Figure 2C). In detail, positive cases were immunohistochemically scored as follows: diffuse (6 cases), not uniform (4) and focal (10).

4.3 AQP1 and omental chemotherapy response

In our study cohort, 10, 17, and 5 patients had omental CRS of 1, 2, and 3, respectively.

In the AQP1+ group, the statistical analysis (Fisher exact test) showed a significant association of AQP1 expression with poor chemotherapy response in omental tissues CRS1-2 ($p= 0.0039$). In fact, all positive cases showed an omental response score of 1 and 2 (Figure 3 A, B), while a complete response score (CRS3) was never observed (Table 2). By contrast, in the AQP1- group, 5 cases showed a complete pathological omental response (Figure 3 C,D), while 7 cases were considered as poor responders (CRS1-2).

4.4 AQP1 and clinico-pathological characteristics

The follow-up of patients ranged from 12 to 60 months (mean follow-up 33,65 months). During the follow-up observation period, nine patients died of the disease, while the remaining twenty-three patients were still alive at the end of the observation period.

No significant relationship emerged between AQP1 expression and other clinico-pathological variables; only a statistical trend has been observed for the patient's age. Among younger patients (<50aa) we more frequently noted loss of AQP1 expression. Finally, The Kaplan-Meier survival curves, documenting patient survival times stratified according to the AQP1 immunostaining showed a moderate difference in survival rates between positive and negative cases. In detail, starting from the initial pathological diagnosis, the AQP1- and AQP1+ groups showed a median survival time of 32 and 24 months, respectively (p = 0,1012) (Figure 4).

5. Discussion

AQP1 has been investigated in several neoplastic tissues, in which a significant association between its expression, tumor phenotype and survival outcomes has been documented [30-43]. In particular, the high AQP1 expression has been associated with poor prognosis in numerous cancers, including ovarian carcinoma, lung cancer, prostate adenocarcinoma, brain tumors and breast cancer [30-43]. By contrast, AQP1 high expression in mesotheliomas is associated with improved survival rates, as elsewhere by us reported [33-35].

In two previous studies performed by our group, we documented the immunohistochemical expression of AQP1 in a series of malignant pleural mesotheliomas related to fluoroedenite fibers exposure [33,34]. AQP1 showed positive immunostain in neoplastic mesothelioma cells from both histological and cytological samples from pleural effusions. We also demonstrated a statistically significant association between AQP1 overexpression patient's survival; in fact, we observed a mean OS of 26.3 months for patients with >50% AQP1 expression versus a mean OS of only 8.9 months for patients with <50% AQP1 expression. This relationship between higher levels of AQP1 in MPM tissues and a better prognosis was quite different to that reported in other tumors, including breast cancer, brain tumors, prostate adenocarcinoma,

lung adenocarcinoma and carcinomas of the gastrointestinal tract, for which increased levels of AQP1 are associated with a poorer prognosis [36]. However, similar results have been observed by other authors when analysing AQP1 expression in malignant mesotheliomas related to asbestos exposure [35].

Recently, in a gynaecological context, some Authors have immunohistochemically evaluated the expression of AQP1, 3, 5, and 9 in a total of 300 ovarian carcinomas using tissue microarrays, by demonstrating that AQPs can be considered useful prognostic markers in ovarian carcinoma [43]. However, the correlation with prognosis depends on the histological type of ovarian carcinoma; specifically, high AQP5 expression is related to poorer prognosis in serous carcinoma, while low AQP1 expression was evident in clear cell carcinomas with poorer prognosis [43]. Moreover, high AQP1 expression is associated with poorer prognosis in mucinous and endometrioid carcinomas [43].

Although controversial results are reported concerning AQP1 expression and tumor progression or metastasis development, only few data are available in the literature regarding the association between AQP1 and response to chemotherapy [44-46]. Recently, in patients with stage II–III colorectal cancer treated 5-FU-based adjuvant chemotherapy, positive AQP1 expression was associated with an increased DFS rate compared with that of AQP1-negative ones [44]; therefore, it has been suggested that AQP1 may be a candidate biomarker predictive of response to 5-fluorouracil-based adjuvant chemotherapy [44]. Furthermore, in prostatic adenocarcinoma cell lines, AQP1 was suppressed by ginsenoside Rg3, together with cell migration [45]. A down-regulation of AQP1 has been reported in lung cancer cell lines treated by combination therapy of celecoxib and afatinib [37]. Moreover, different subtypes of AQPs play different roles in ovarian cancer cell in vitro, suggesting thus AQPs might be associated with chemotherapy sensitivity [46]. In detail, the cisplatin effects were different between since the expression of AQP1 mRNA decreased significantly, while expression of AQP3 and AQP8 increased [46].

In the present study, we investigated the immunohistochemical expression of AQP1 in pre-operative peritoneal samples obtained from advanced stage serous OC. We have shown that a sub-group of these OC exhibited an evident immunohistochemical AQP1 expression in comparison to a negative one. Although no relationship between clinico-pathological parameters and AQP1 has been encountered in our cohort, we have thought to be of interest to verify if AQP1 expression is able to predict the chemotherapy response following NACT and IDS. In detail, evaluating the omental tissues chemotherapy response, a significant association was observed between AQP1 expression and poor chemotherapy response CRS1-2; in addition, a complete response score (CRS3) was never noted in AQP1+ patients. Consequently, it may be hypothesized that AQP1 could represent a useful predictive biomarker of tissue response to platinum-based chemotherapy in patients affected by high grade serous OC.

Moreover, accordingly to previous observations regarding the relationship between AQ1 and patient outcome in carcinomas of different sites, such as ovary, lung, prostate, brain and breast [31-43], we have documented a sensible trend for better survival in patients with negative AQP1 immunoexpression.

Finally, regarding the pathological assessment of omental residual disease, as expected, CRS revealed significant prognostic differences in terms of survival time. In detail, the mean OS of patients with CRS1, 2 and 3 was 26, 32, and 39 months, respectively.

Therefore, our study confirms that complete or near-complete pathologic response assessed in the omental samples of advanced-stage EOC patients after neoadjuvant chemotherapy is predictive of better survival. However, the prognostic role of CRS system has been already validated and confirmed by previous studies, also performed by our group [20-27].

In fact, in a previous study, we already documented significant differences in terms of OS and PFS according to CRS evaluated in omental samples [22]. In detail, in a cohort of 161 EOC patients, the median PFS of patients with CRS1-2 and 3 was 15, and 22 months, respectively,

the median OS was 41 and >50 months, respectively. Moreover, we have also found significant differences between ovarian CRS1 and ovarian CRS2 in terms of OS being the median OS for ovarian CRS1 patients 41 months vs. a median OS of >50 months observed for ovarian CRS2 patients. However, no significant differences were observed in terms of PFS between ovarian CRS1 and CRS2 groups.

Based on all the above-mentioned findings, the combined use of CRS scoring system and AQP1 immunohistochemistry has the potential to be a surrogate of a more precise prognostic classification of chemotherapy response, and patient's survival. In the near future, if our results will be validated on larger series, or if novel prognostic biomarkers will be discovered, the histological and immunohistochemical evaluation of peritoneal biopsies will allow clinicians to identify subgroups of patients with different outcomes, with a possible reduction of the effective number of surgical procedures for good responders.

6. Conclusions

Our data may stimulate future research in expanding the comprehension of platinum-resistance mechanisms in ovarian cancer, since we retain the water permeability regulation of AQP1 may play an important role in drug metabolism and drugs chemo-sensitivity as elsewhere previously reported [35-38].

According to our results, we have demonstrated that high grade serous OC could be classified in 2 predictive groups on the basis of AQP1 expression at the time of the pre-operative diagnostic peritoneal biopsy. The first group AQP1+ patients exhibited a poor pathological response in omental samples, indicating an eligibility for cytoreductive surgery rather than candidate for NACT. Nevertheless, the results from the present study need to be furtherly validated on larger cohorts to establish the biological role of AQP1 as well as its clinical utility in the therapeutic approach of serous high-grade OC patients.

7. Declaration of interest

The author has no proprietary, financial, professional or other personal interest of any nature in any product, service or company. The author alone is responsible for the content and writing of the paper.

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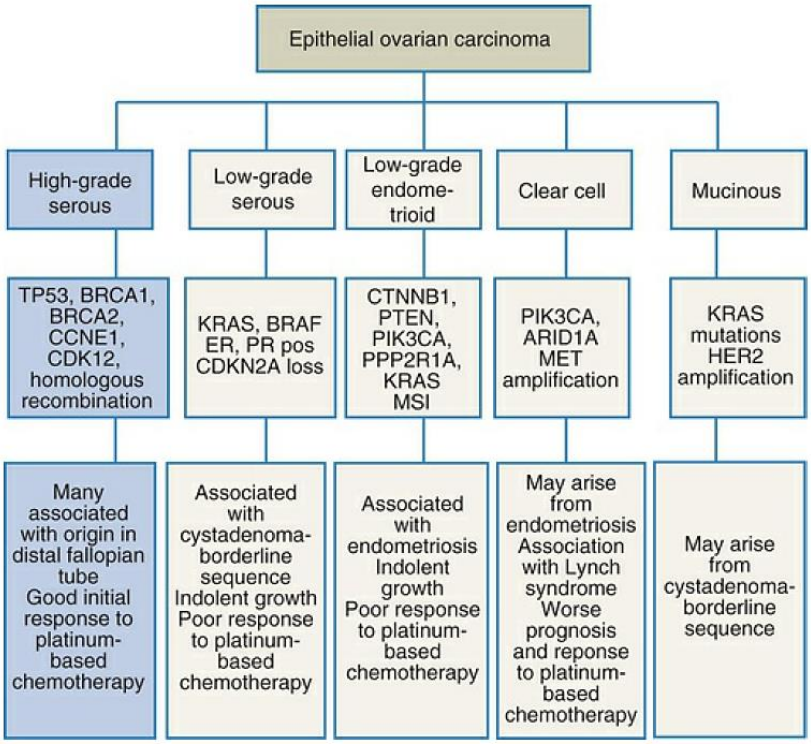

FIG. 1. Ovarian epithelial carcinomas, relevant genetic alterations and notes on origin. Data from Banerjee S, Kaye SB: New strategies in the treatment of ovarian cancer: current clinical perspectives and future potential. Clin Cancer Res 19[5]:961-968, 2013; Konstantinopoulos PA, Matulonis UA: Current status and evolution of preclinical drug development models of epithelial ovarian cancer. Front Oncol 3:296, 2013.

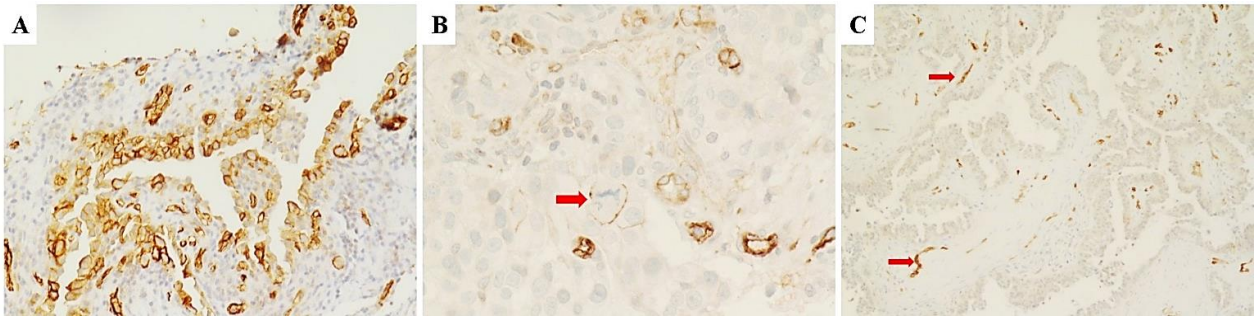

Figure 2. Different Immunohistochemical expression patterns of AQP1 in diagnostic biopsies of high grade serous ovarian carcinomas. A) Diffuse positivity for AQP1 showing linear and circumferential membranous staining is depicted. B) Another serous cacinoma case showing focal staining for AQP1. Arrow indicates two adjacent neoplastic cells (a mitotic figure is shown in the cell on the left) with linear and circumferential membranous staining. C) Negative staining for AQP1 is depicted. Arrow indicates vascular endothelial cells which served as positive internal control.

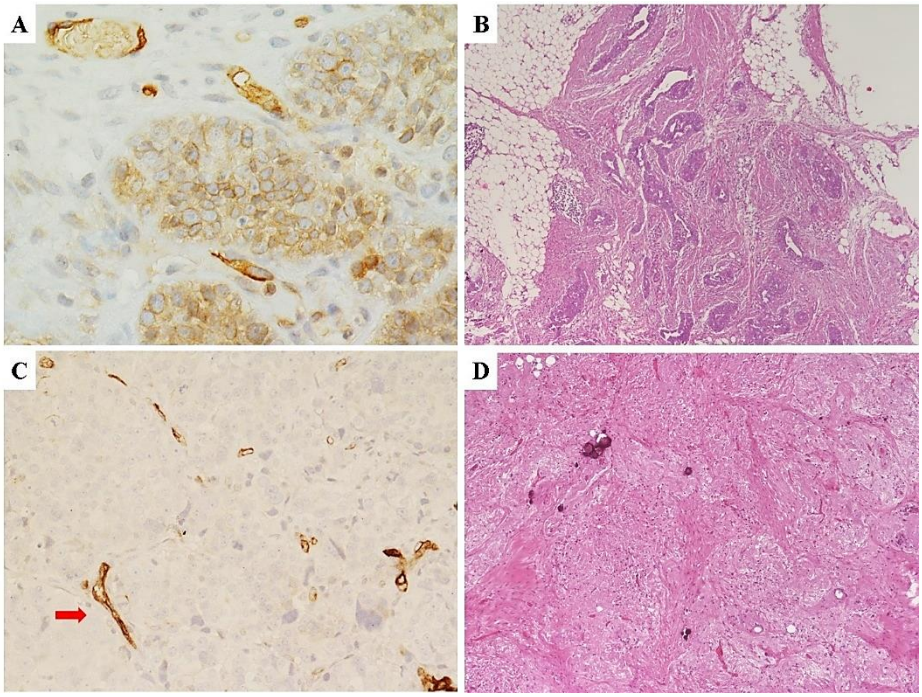

Figure 3. Omental pathological response according to AQP1 IHC.

A) Diagnostic biopsy of a case of high grade serous ovarian carcinomas demonstrating diffuse positivity for AQP1; B) after NACT and IDS this case showed an omental response score of 1: mainly viable tumor with no or minimal regression-associated fibro-inflammatory changes. C) Another serous ovarian carcinoma case showing negative staining for AQP1. Scattered vascular endothelial cells served as positive internal control (arrow). D) After NACT and IDS this case showed an omental response score of 3: extensive fibro-inflammatory changes with no residual tumor identified.

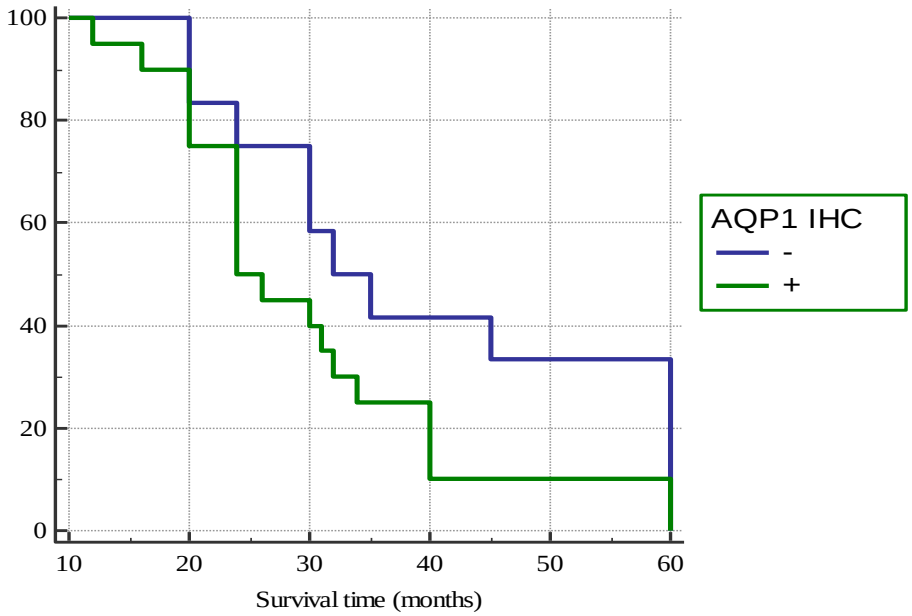

Figure 4. Survival curves of all cases of ovarian high-grade serous carcinomas in relation to immunohistochemical expression of AQP1.

TABLE 1. Patients' Characteristics

Case	Age	Stage	AQP1 IHC	CRS	Follow-up (months)	Outcome
1	49	IIIC	0 (Negative)	3	60	A
2	42	IV	0 (Negative)	3	60	A
3	73	IV	0 (Negative)	3	30	A
4	37	IIIC	0 (Negative)	3	35	A
5	57	IIIC	0 (Negative)	3	45	A
6	55	IIIC	0 (Negative)	2	32	A
7	52	IIIC	0 (Negative)	2	24	A
8	68	IIIC	0 (Negative)	2	20	D
9	45	IIIC	0 (Negative)	2	60	A
10	48	IV	0 (Negative)	2	60	A
11	71	IIIC	0 (Negative)	1	20	D
12	63	IIIC	0 (Negative)	1	30	D
13	58	IIIC	25 (Not uniform)	1	40	A
14	73	IIIC	25 (Not uniform)	1	40	A
15	75	IIIC	25 (Not uniform)	1	12	D
16	68	IIIC	25 (Not uniform)	1	20	D
17	46	IIIC	50 (Diffuse)	1	24	A
18	49	IIIC	50 (Diffuse)	1	24	A
19	55	IIIC	50 (Diffuse)	1	24	A
20	61	IV	50 (Diffuse)	1	32	A
21	75	IIIC	80 (Diffuse)	2	24	A
22	72	IIIC	80 (Diffuse)	2	20	D
23	48	IIIC	5 (Focal)	2	20	D
24	53	IIIC	5 (Focal)	2	16	D
25	57	IIIC	5 (Focal)	2	26	A
26	60	IV	5 (Focal)	2	40	A
27	63	IIIC	1 (Focal)	2	60	A
28	52	IIIC	1 (Focal)	2	60	A
29	59	IIIC	1 (Focal)	2	30	A
30	64	IIIC	1 (Focal)	2	24	D
31	66	IIIC	1 (Focal)	2	34	A
32	50	IIIC	1 (Focal)	2	31	A

Legend: IHC (immunohistochemistry), CRS (complete response score), A (alive), D (dead for the disease)

Table 2. Distribution of CRS scores according to AQP1 staining.

CRS	AQP1-	AQP1+
1-2	7	20
3	5	0
Total patients	12	20