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Characterization of the folate receptor alpha (α) immunoexpression in relapsed/recurrent ovarian cancer

Maria Marta Oliveira Cadima

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INSTITUTO DE **CIÊNCIAS BIOMÉDICAS ABEL SALAZAR**

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Porto, junho de 2025.

Maria Marta Oliveira Cadima

(Maria Marta Oliveira Cadima)

Dedicatória

Dedico esta tese, com todo o meu coração, à minha família e ao meu noivo. Estiveram sempre ao meu lado. Nos dias mais difíceis encontrei em vocês o apoio incondicional, as palavras certas e o abraço de que precisava para continuar.

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Resumo

Introdução: O recetor alfa do folato é frequentemente expresso em tumores serosos de alto grau do ovário. A sua avaliação por imunohistoquímica é utilizada para classificar os tumores como FOLR1 positivos, se a expressão for igual ou superior a 75%. Isto permite a seleção de doentes para terapias dirigidas, como o mirvetuximab soravtansine (MIRV). Um amostra representativa, do tumor primário, metastático ou recorrente, é fiável para determinar a expressão de FOLR1, uma vez que estudos iniciais indicaram que a expressão deste receptor mantém-se ao longo do curso da doença.

Objetivo: O nosso primeiro objetivo foi implementar uma técnica laboratorial baseada em imunohistoquímica para a caracterização de FOLR1 em CEO na nossa instituição. Como subobjetivo, desenvolvemos uma ferramenta digital para avaliar a expressão de FOLR1. O nosso segundo objetivo foi avaliar o FOLR1 no diagnóstico e na doença recorrente de CEO, caracterizando assim a expressão ao longo do curso da doença.

Métodos: Estudo de coorte retrospetivo, consistindo na análise por imunohistoquímica da expressão de FOLR1 em 42 amostras tumorais emparelhadas de cancro epitelial do ovário recorrente entre 2012 e 2023.

Resultados: Entre 1 de janeiro de 2012 e 31 de dezembro de 2023, 42 amostras emparelhadas de 42 doentes (idade mediana de 58,7 anos) com HGSOC recidivante foram avaliadas no diagnóstico e na doença recorrente. A maioria dos doentes apresentava doença avançada no diagnóstico (65,8% estavam no estádio FIGO IIIC). Variantes BRCA foram detetadas em 16 (45,7%) doentes. Como primeira opção de tratamento, 69,1% realizaram cirurgia de citorredução primária e 38,1% quimioterapia neoadjuvante (mediana de 5 ciclos, 3 a 8), seguida de cirurgia de intervalo. Todos os doentes foram expostos a compostos de platina com um PFI mediano de 14 meses (entre 0 e 106 meses). A maioria dos doentes era platino-sensível (n=38, 90,5%), 7,1% (n=3) platino-resistentes e um doente com doença refratária. Espécimes cirúrgicos (61,9%) foram o tipo de amostra mais comum tanto no diagnóstico como na doença recorrente. O local anexial (80,5%) foi o mais frequente no diagnóstico e o peritoneu (76,2%) na recidiva. A grande maioria das amostras (n=80) foi considerada adequada para avaliação de FOLR1. Tanto no diagnóstico como nas recidivas, 46,3% da nossa coorte apresentava estado FOLR1 positivo, com uma expressão mediana de 70,0%. Observou-se heterogeneidade da expressão em 26,8% das amostras no diagnóstico e 17,1% na recidiva. O tratamento neoadjuvante primário associou-se significativamente ao estado FOLR1 positivo no diagnóstico (p=0,047), a sobrevivência livre de progressão mediana foi

significativamente inferior nos doentes FOLR1 positivos no diagnóstico (p=0,035). Observámos 35% (n=14) de alterações no estado de FOLR1 ao comparar as amostras do diagnóstico com as das recidivas, das quais 17,5% (n=7) passaram a positivo (diferença mediana do score de 30) e 17,5% (n=7) passaram a negativo (diferença mediana do score de -35). A heterogeneidade intratumoral da expressão de FOLR1 no diagnóstico associou-se à alteração para positivo (p=0,046) e a predominância do padrão apical (p=0,041) associou-se a qualquer alteração do estado. Não foram encontradas associações significativas entre a alteração do estado de FOLR1 e outras variáveis clinicopatológicas. A comparação entre a classificação visual do patologista e a classificação baseada em script do estado FOLR1 mostrou uma precisão global de 73,6% e concordância moderada (Kappa = 0,45).

Conclusão: A validação e implementação da IHQ automatizada e padronizada para FOLR1 é facilmente exequível em amostras FFPE. O FOLR1 é variavelmente expresso no HGSOC, com presença considerável de heterogeneidade intratumoral e alteração do estado de FOLR1 ao longo do curso da doença. A reavaliação do estado de FOLR1 em diferentes amostras pode ser relevante, contudo, são necessários mais estudos para determinar o impacto da variabilidade da expressão de FOLR1 na resposta a terapias dirigidas como o mirvetuximab. A avaliação digital automatizada necessita de melhorias para aplicação clínica

Abstract

Background: Folate receptor alpha is frequently expressed in high grade ovarian serous tumors. Its assessment using immunohistochemistry is used to classify tumors as FOLR1 positive, if the expression is equal or higher than 75%. This allows selection of patients for targeted therapies, such as mirvetuximab soravtansine (MIRV). A representative sample, from primary, metastatic or recurrent tumor, is thought to be reliable to determine the expression of FOLR1, since initial studies indicated that the expression is maintained throughout the course of disease.

Purpose: Our first goal was to implement a laboratory technique based on immunohistochemistry for FOLR1 characterization in EOC in our institution. As a sub-aim, we developed a digital tool to evaluate FOLR1 expression. Our second goal was to evaluate FOLR1 at diagnosis and recurrent disease of EOC, thus characterizing the expression throughout the course of disease.

Methods: Retrospective cohort study, consisting of immunohistochemistry analysis of FOLR1 expression of 42 paired archival tumor samples of recurrent epithelial ovarian cancer between 2012 and 2023.

Results: Between first of january, 2012 and 31 of december, 2023, 42 paired samples of 42 patients (median age of 58.7 years) with relapsed HGSOC were assessed at diagnosis and recurrent disease. Most patients had advanced disease at diagnosis (65.8% were at FIGO stage IIIC). *BRCA* variants were detected in 16 (45.7%) patients. As first choice of treatment, 69.1% performed primary debulking surgery and 38.1% performed neoadjuvant chemotherapy (median of 5 cycles, 3 to 8) followed by interval debulking surgery. All patients were exposed to platinum compounds with a median PFI of 14 months (between 0 and 106 months). The majority of patients were platino-sensitive (n=38, 90.5%), 7.1% (n=3) platino-resistant and one patient with refractory disease. Surgical specimens (61.9%) were the most common sample both at diagnosis and recurrent disease. Adnexal (80.5%) was the most frequent site of sample at diagnosis and peritoneum (76.2%) at relapse. The vast bulk of samples (n=80) were considered adequate for FOLR1 assessment. Both at diagnosis and recurrences, 46.3% of our cohort had a positive FOLR1 status, with a median expression of 70.0%. Heterogeneity of expression was observed in 26.8% of samples at diagnosis and 17.1% at recurrence. Primary neoadjuvant treatment setting significantly associated with FOLR1 positive status at diagnosis (p=0.047), median progression free survival was significantly lower in FOLR1 positive patients at diagnosis (p=0.035). We observed a 35% (n=14) of changes in status of FOLR1 comparing samples at diagnosis to recurrences, of which 17.5% (n=7) changed to positive (score median difference of 30) and 17.5% (n=7) changed to negative (score median

difference of -35). Intratumoral heterogeneity of FOLR1 expression at diagnosis is associated with change to positive (p=0.046) and predominance of apical pattern (p=0.041) associated with any change of status. No significant associations were found related to the change in status of FOLR1 and other clinicopathological variables. Comparison of pathologist visual classification and script-based classification of FOLR1 status, showed an overall accuracy of 73.6% and moderate agreement (Kappa = 0.45).

Conclusion: Validation and implementation of FOLR1 standardized automated IHC is readily feasible in FFPE samples. FOLR1 is variably expressed in HGSOC, with a considerable presence of intratumoral heterogeneity and change of FOLR1 status over the course of disease. Reassessment of FOLR1 status in different samples may be relevant, however, further studies are needed to determine the impact of FOLR1 expression variability in response to targeted therapies such as mirvetuximab. Automated digital scoring, needs improvement for clinical application.

Lista de abreviaturas

DM4- Maytansinoid microtubule inhibitor

DOR- Duration of Response

EOC- Epithelial ovarian Cancer

FDA-Food and Drug Administration

FFPE- Parraffin-Embedde Tissue

FRα - Folate receptor alpha

HGSOC- High grade serous ovarian carcinoma

IHC- Immunohistochemistry

IPO - Instituto Português de Oncologia do Porto Francisco Gentil

ITH- Intra-tumoral heterogeneity

MIRV- Mirvetuximab Soravtansine-gynx

NACT- Neoadjuvant chemotherapy

NGS- Next Generation Sequencing

OC- Ovarian cancer

ORR- Objective Response Rate

PDS- primary debulking surgery

PFS- Progression Free Survival

PROC- Platinum Resistant Ovarian Cancer

PSOC- Platinum Sensitive Ovarian cancer

VUS-Variant of Uncertain Significance

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1. Introduction

Ovarian cancer (OC) is a complex disease being one of the most lethal gynecological cancers with 324,398 new cases and 206,839 deaths worldwide in 2022.[1,2] OC has several histological subtypes that differ in terms of aggressiveness and treatment response. Epithelial ovarian cancer (EOC) encompasses over 90% of the main histological subtypes.[3]

High grade serous ovarian carcinoma (HGSOC) is one of the most prevalent and lethal forms of EOC, and conceptually includes ovary, fallopian tube and primary peritoneal cancer, given their common origin and biological behavior.[4] Most patients with OC are present in advanced stages, namely FIGO stage III or IV. The current treatment includes debulking surgery and neoadjuvant or adjuvant platinum-based chemotherapy. The initial response to platinum-based chemotherapy in HGSOC is generally high (60-80%). However, eventually 80% of patients will relapse and exhibit platinum resistance. Classic therapies for EOC are guided by the platinum free interval. Platinum free interval is generally defined as the time between stopping a platinum-based regime until recurrence of disease, allowing the following classification: platinum-refractory if ≤3 months, platinum-resistant (PROC or platinum resistant ovarian cancer) if > 3 and ≤ 6 months, and platinum-sensitive (PSOC) if >6 months of EOC. [5,6] Despite treatment, the 5-year survival is approximately 30% in advanced stages and just slightly increased in the last decade, hence remaining a clinical challenge.[4,7]

The molecular characterization of HGSOC has been determinant for prognosis and treatment options, as well as prevention in patient's relatives.[8,9] HGSOC is characterized by *TP53* mutations and recurrent mutations in *BRCA1* and *BRCA2*, where *BRCA1* mutation appears to be more frequent than *BRCA2*.[10,11] Around 13-15% of HGSOC have BRCA 1/2 germ-line mutations and, 5-7% have somatic *BRCA 1/2* mutations. Globally, 50% have homologous recombination deficiency.[12,13] Nowadays genetic testing is mandatory routine clinical practice at diagnosis.

The incorporation of BRCA 1/2 mutations as biomarkers that guide response to therapies such as PARP inhibitors, has radically transformed patient outcomes and is now integrated into both first-line and maintenance treatment settings.

For recurrent platinum resistant ovarian cancer, predictive molecular data is scarce, but very recently, a new biomarker, folate receptor alpha (FRα), was introduced.[14] FRα is overexpressed in approximately 80% of EOC. [15,16] FRα is one of the isoforms of folate receptors present in tissues and normally have a limited expression (0-25% in normal adult tissues), thus its overexpression is frequently related with tumorigenesis.[15-17]

FRα, determined by the FOLR1 gene on chromosome 11, is attached to the plasma membrane via a glycosyl-phosphatidylinositol (GPI) anchor glycoprotein to the apical surface of polarized epithelial cells.[18] FRα has a high affinity for folates and transports them to the cytoplasm via endocytosis, the acidic pH allows further dissociation between the molecules and the return of FRα to the surface.[19] Folic acid or vitamin B9 is a vital nutrient imbedded in one-carbon metabolism for cell division.[20] FRα activation by folates is associated with tumorigenesis, besides instigating cell growth, it acts on multiple intracellular signaling for tumor invasion.[21]

FRα expression is mostly frequent on HGSOC and it is believed to maintain its expression throughout the course of disease and after treatment with chemoterapy.[22-24] Archival tumor samples are considered reliable to identify FRα status in EOC.[25] Immunohistochemistry (IHC) assessments are appropriate to characterize the status of FRα and stratification of patients with recurrent EOC.[25] Microscope observation allows the recognition of a positive FRα status with ≥ 75% of viable tumor cells with moderate (2+) and/or strong (3+) intensity.[26]

The expression pattern of FRα in EOC is abnormal, therefore being possible to recognize the cancer cells with target therapies.[27] The capacity of FRα to internalize molecules allowed the use of target therapies based on antibody-drug conjugate (ADC).[28,29] Mirvetuximab soravtansine-gynx (MIRV) is an ADC with an anti-FRα linked to the maytansinoid microtubule inhibitor (DM4).[30] This new ADC displays its cytotoxic activity because the DM4 molecule induces cell death or cell-stop division by aiming at the microtubules. It delivers maytansinoid payload to FRα-positive cells and some bystander FRα-negative cells. Therefore, the cytotoxic effect is related to the more extensive expression of FRα in the surface of tumor cells.[31]

The adverse reactions (AEs) associated with MIRV were mostly reversible low-grade events, with ophthalmologic and gastrointestinal symptoms being the most common, namely blurred vision, nausea and diarrhea.[32-34] Immunogenicity is another possible outcome to be considered when using ADCs with DM4, despite two Phase I studies reporting rare counts of antibodies formed against DM4.[35,36] A study found that although 7,8% of 734 patients across four different Phase I and Phase III studies develop new antibodies after starting treatment with MIRV, patients showed low immunogenicity with similar levels of the drug and no apparent impact on effectiveness.[37]

Phase II SORAYA trial (NCT04296890) demonstrated significant benefit of MIRV with an ORR of 32,4% (95% CI 23,6-42,2 %), double the ORR of chemotherapy, median duration of response (DOR) of 6,9 months and OS of 15 months (95% CI 11,5-18,7 months).[30,38,39] The Phase III FORWARD I trial (NCT02631876) revealed an objective response rate (ORR) of 24% with MIRV compared to 10% with

chemotherapy.[40] Phase III MIRASOL trial (NCT04209855) demonstrated significant difference in PROC between MIRV over chemotherapy; with ORR of 42,3% (95% CI 35,8-49,0 %), median progression-free survival (mPFS) of 5,6 months (95% CI 4,3-6,0 months), overall survival (OS) and 16,5 months (95% CI 14,4-19,9 months) with MIRV, compared to PFS of 4,0 months (95% CI 2,9-4,5 months) and OS of 13,3 months (95% CI 4,3-6,0 months) with chemotherapy.[41-43] Food and Drug Administration (FDA) in November of 2022 approved the treatment with MIRV for patients with platinum recurrent resistant EOC (PROC) with a positive FRα-status.[44]

Ongoing trials are now focusing on evaluating the use of these therapies in PSOC. Phase II single-arm PICCOLO trial demonstrated that 43,5% of screened population with PSOC were FRα-positive, compared to 32 to 36% of FRα-positive in PROC in previous trials.[38,42,45] The treatment with MIRV elicited the ORR to 51.9 % (95% CI 40.4% to 63.3%), DOR of 8.25 months (95% CI 5.55- 10.78 months) and mPFS of 6,93 months (95% CI 5.85- 9.59 months). A Phase III GLORIOSA trial is going to evaluate the efficacy and safety of bevacizumab solo therapy or associated MIRV with as maintenance therapy for recurrent PSOC for high expression of FOLR1.[46]

MIRASOL, SORAYA and GLORIOSA clinical trials used a IHC validated companion test VENTANA FOLR1 Assay, for patient selection for the treatment with MIRV, thereby establishing a clinical practice for rating the FRα status in recurrent EOC.[26,38,47-49] In Portugal, treatment with MIRV for patients with platinum recurrent resistant EOC is not yet approved. An early access program was launched in january 2025, thus the implementation of FRα testing in laboratories was mandatory.

Our first goal was to implement a laboratory technique based on IHC for the evaluation of FRα in samples from patients with recurrent EOC in our institution. Acknowledging the findings in other studies on status of FRα in tumorigenesis of EOC, validated this technique under our laboratory routine conditions, with appropriate internal controls, using optical microscopy, to be used as a criterion to assort patients for target treatments. As a sub-aim, we also developed a digital tool to evaluate FRα IHC. Our second goal was to evaluate FRα in tumor samples from recurrent EOC and compare it to samples of primary or synchronous metastases at diagnosis of the same patients, thus characterizing the expression throughout the course of disease.

2. Methods

2.1. **Case selection and criteria**

This is a cohort retrospective study examining archival samples of women with recurrent EOC, diagnosed between January 1st of 2012 and December 31st of 2023, under the supervision of Professor Carla Bartosch as the project principal investigator, in Centro de Investigação do Instituto Português de Oncologia do Porto Francisco Gentil, E.P.E. (IPOP). It has been approved by the institutional ethics committee Comissão de Ética para a Saúde do IPO Porto (CES.100_25).

The inclusion criteria for this study were a) 18 years of age or older; b) HGSOC tumor histology; c) date specimen between January 1st2012 and December 31st2023 in IPOP records; d) at least 1 sample of archival tissue of the primary and 1 sample of the recurrent ovarian carcinoma; and e) clinical information available in patients electronic clinical records. The exclusion criteria were a) histological ambiguous diagnosis upon review and b) archival tissue not available or not found. The selection of patients was made using a query based on International Classification of Diseases for Oncology (ICD-O) codification system in IPOP Pathology Department Database.

2.2. **Variables**

Data was collected from patients electronic clinical files, pathology database registries and histological slide review. The following parameters were collected: a) age at diagnosis; b) ECOG stage (ECOG O vs ECOG 1 vs ECOG 2 vs ECOG 3 vs ECOG 4 vs ECOG 5) at diagnosis and recurrence; c) type of sample (surgical specimen vs surgical biopsy vs biopsy); d) date of sample for primary and recurrence; e) sample setting (primary tumor vs synchronous metastasis vs recurrence;); f) treatment at primary and recurrence (chemotherapy vs cytoreductive surgery vs HIPEC); g) extension of cytoreductive surgery (hysterectomy, bilateral salpingo-oophorectomy, omentectomy, lymphadenectomy, other); h) residual disease at cytoreduction (R0 vs R1 vs R2 vs missing); i) location of primary cancer at diagnosis (ovary vs fallopian tube vs peritoneum vs indetermined); j) FIGO stage at diagnosis; k) lymphovascular invasion (present, absent , suspicious, missing); l) gene cancer panel testing (not tested vs tested no mutations vs tested with mutations); m) *BRCA* mutation (Germline *BRCA* 1 vs Somatic *BRCA* 1 vs Germline *BRCA* 2 vs Somatic *BRCA* 2 vs VUS *BRCA* 1 vs VUS *BRCA* 2 vs none); n) number of systemic therapy lines (1 time vs 2 times vs 3 times); o) type of therapy (platinum-based chemotherapy, bevacizumab, PARP inhibitor or taxanes); p) disease response to first, second or last line chemotherapy (complete response vs partial response vs no

response); q) date of last line chemotherapy; r) date of recurrence (clinical recurrence vs imaging recurrence vs histological recurrence); s) platinum free-interval (refractory (≤3 months) vs resistant (> 3 e ≤ 6 months) vs sensitive (>6 months)); t) IHC FRα percentage tumor staining (further categorized as <75% vs ≥75%); u) IHC FRα staining intensity (weak (1+) vs moderate (2+) vs strong (3+)); u) IHC FRα predominant staining patterns (membranar circunferential vs apical vs dot; homogeneous vs heterogeneous); x)progression free survival; y) overall survival [6,22,47-50]

2.3. **Sample preparation**

All available histological slides from each patient were retrieved from the Pathology Department archive and reviewed by a trained pathologist to review diagnosis and select representative samples (one from the primary tumor/metastasis at diagnosis and one matched sample from the patient's tumor recurrence). The corresponding formalin fixed paraffin-embedded (FFPE) tissue blocks were retrieved from the archive and 3µm sections were obtained. IHC was performed using VENTANA FOLR1 (FOLR1-2.1) RxDx assay on an automated BenchMark ULTRA instrument.[26,47]

2.4. **Quality Control**

A control tissue (sample of a normal Fallopian tube) was run simultaneously with the samples, identifying if any failure happened with the reagents in the staining process. Selection of Fallopian tube appropriate control samples was made after a thorough evaluation of 20 samples from young women that underwent prophylactic salpingectomies and elective procedures performed for permanent contraception. The control tissue test was considered acceptable if there wasn't specific staining in the stroma of the epithelium of a normal fallopian tube (negative control) and a moderate circumferential/strong apical membrane staining as positive control. [26]

The evaluation required the use of a light microscope and the specimens must contain a minimum of 100 tumor cells to be considered acceptable.[26] Upon review, samples were selected considering the representativeness of the tumor, avoidance of tumor necrosis and preservation artifacts.

2.5. **Immunohistochemical evaluation**

The VENTANA FOLR1 assay allows both cytoplasmatic and membranous staining, but only membrane staining defines the FRα status.[26,47] The membrane staining pattern can be apical,

circumferential, or dot like, with four staining intensities: negative (0), weak (1+), moderate (2+) or strong (3+).[47] The FRα score was defined as the percentage of viable tumor cells with moderate (2+) and/or strong (3+) intensity. Following the cut-off established in previous MIRV clinical trials, a positive case was defined as having a high FRα score equal or more than 75% and a negative case was defined as having less than 75.[26,38,47,48] IHC FRα score evaluation was performed by a trained pathologist using an optical microscope.

As a complement, we also developed a simple easy-to-use tool using digital image analysis of whole slide images. Slides were digitized at a 40x objective magnification on a Hamamatsu 360 digital scanner, and digital image analysis was performed using QuPath v.0.5.1 software.[51] A custom script was written in Apache Groovy, an object-oriented programming language for the Java platform, making use of its compatibility and integration into QuPath. It encompasses automated cell detection, phenotypic classification and FRα expression evaluation, within previously annotated regions. The script performs cell detection using QuPath's built-in "Watershed Cell Detection" algorithm, instructed with the following parameters: pixel size of 0.5 µm, background radius of 8.0 µm, median filter radius of 1.0 µm, a Gaussian sigma of 1.5 µm, detection threshold of 0.1 and a cell expansion of 6.0 µm. Detected cells were classified based on manually defined parameters applied to morphometric and densitometric features. These parameters were selected by evaluating the most impactful criteria used by the "Artificial neural network (ANN_MLP)" object classifier after manually training tumoral and stromal areas in QuPath. Cells were considered tumoral if they met all of the following criteria: cytoplasmic DAB optical density mean > 0.025, total cell area ≥ 90 µm², and nuclear area ≥ 40 µm². For FRα evaluation, positivity among tumor cells was determined using any of the following thresholds: Cell DAB OD mean ≥ 0.35, Cell DAB OD max ≥ 0.7, or Cytoplasm DAB OD mean ≥ 0.4, whilst the remainder were considered FRα-negative. Finally, the script automatically computes the total number of tumor cells assessed, the counts and percentages of FRα-positive and FRα-negative tumor cells and then prints the results to the QuPath console.

2.6. **Statistical analysis**

Data was tabulated in a datasheet and analysed using excel, STATA 13 and IBM®SPSS Statistics version 30.0. Descriptive statistics were performed using medians, ranges and proportions.

Non-parametric methods were used throughout the analysis due to the small sample size. For paired comparisons of FOLR1 expression between diagnostic and recurrent samples, the McNemar test and the Wilcoxon signed-rank test were applied for binary and continuous variables,

respectively. Kruskal–Wallis test, Wilcoxon rank-sum test (Mann–Whitney U), chi-squared and Fisher's exact tests were used for comparisons of continuous and categorical variables between groups of FOLR1 status change, as adequate. To assess differences in FOLR1 score variability across groups, the Brown–Forsythe test was used.

Survival analysis was performed using Kaplan-Meier curves and log-rank tests. Progression-free survival (PFS) is determined as the time from diagnosis until disease recurrence, death or last of follow-up. Overall survival (OS) was defined at the time from diagnosis to death or last follow-up.

The agreement between visual pathology classification and script-based classification of FOLR1 status, was assessed using confusion matrices (2×2 tables), from which accuracy, sensitivity, and specificity were calculated. Cohen's Kappa statistic was used to measure the level of agreement between the two methods. For continuous FOLR1 scores, Pearson's correlation coefficient was applied to assess the strength of the linear relationship.

All statistical tests were two-sided, and a p-value < 0.05 was considered statistically significant.

The results of this study will be open for publishing in a scientific international journal.

3. Results

3.1. **Patient´s cohort characteristics**

The cohort included 42 patients with HGSOC, whose demographics and baseline characteristics are summarized in Table 1. The median age was 58,5 years (range, 39 to 83 years). The most common primary tumor site was ovarian (n= 23, 57.5 %), followed by fallopian tube (n=16, 40.0 %). Only one patient had confirmed primary peritoneal cancer (n=1, 2.5%). Surgical specimen (n=26, 61.9%) was the most frequent, followed by biopsy (n=13, 31.0%) and surgical biopsy (n=3, 7.1%). Adnexal tissue (n=33, 80.5%) make most of the primary site, samples of metastases were used in 8 patients (19.5%) and 1 patient had missing data. Most patients presented ECOG performance status of 0 (n=25, 71.4 %), followed by ECOG performance status of 1 (n=10, 28.6 %). Most patients had advanced disease at diagnosis, namely 65.8 % of patients were at stage IIIC followed by IIB (n=3, 7.9%), IIIA (n=3, 7.9%) and IIIB (n=3, 7.9 %).

As for the *BRCA* mutation status, 45.7 % (n= 16) had a *BRCA* mutation, including germline BRCA 1 (n=8, 22.9 %), germline *BRCA* 2 (n=3, 8.6 %) and somatic *BRCA* 1 (n=1, 2.3 %). Additionally, 13 (31.0 %) tumors were tested with the TruSight Hereditary Cancer Panel, 61.5 % (n=8) having oncogenic variants in other genes (TP53, CDK2, CHEK2, NFT1 and PIK3CA).

Table 1. Cohort Demographics and Characteristics at diagnosis

Characteristic at diagnosis	All patients (n = 42)
Age, years, median (range)	58,5 (39–83)
FIGO stage, n (%)	(n=38) [a]
IB	1 (2.6 %)
IIA	2 (5.3 %)
IIB	3 (7.9 %)
IIIA	3 (7.9 %)
IIIB	3 (7.9 %)
IIIC	25 (65.8 %)
IV	1 (2.6 %)
ECOG Performance status, n (%)	(n=35) [b]
0	25 (71.4 %)
1	10 (28.6 %)
Primary tumor site, n (%)	(n=40) [c]

Ovarian	23 (57.5 %)
Fallopian tube	16 (40.0 %)
Peritoneal	1 (2.5%)
***BRCA* variant status, n (%)**	(n=35) [d]
Not detected	19 (54.3 %)
Germline *BRCA* 1	8 (22.9%)
Germline *BRCA* 2	3 (8.6 %)
Somatic *BRCA* 1	1 (2.9 %)
VUS *BRCA* 2	4 (11.4%)
Gene Cancer Panel, n (%)	(n=13) [e]
Variants detected [f]	8 (61.5 %)
Not detected	5 (38.1%)

FIGO, International Federation of Gynecology and Obstetrics. ECOG, Eastern Cooperative Oncology Group. PS, performance status. VUS, Variant of Uncertain Significance.

[a]4 had missing data related to FIGO stage at diagnosis.

[b]7 patients had missing data related to ECOG PS at diagnosis.

[c]2 had missing data related to origin of ovarian cancer at diagnosis.

[d]7 had missing data related to BRCA status at diagnosis, usually caused by not testing.

[e]29 had missing data related to results of TruSight Hereditary Cancer Panel.

[f]Detect genetic mutations: in TP53 with c.672G>A p.?; c.537T>G p.(His179GIn); c.198del, p.(Met66 IIefs Ter57); c.743G>A p.(Arg248Gin; NM_000546.5:c.843C>G p. (Asp281Glu); c.574C>T p.(Gln192Ter). CDK2 with c.1931+1G>A p.? and c.1087dup p.(Ser363PhefsTer8). CHEK2 with c.349A>G, p.(Arg117Gly). NFT1 with c.2910_2931del p.(Asn970LysfsTer7). BRIP1 with c.226_227del, p.(Val76LysfsTer2). PIK3CA with c.1634A>C, p.(Glu545Ala).

Regarding treatment, our cohort included patients that underwent primary debulking surgery (n=26, 61.9 %), and patients whose initial treatment was neoadjuvant chemotherapy (n=16, 38.1 %) with a median of 5 cycles (varying between 3 to 8 cycles) followed by interval debulking surgery (table 2). The majority of patients, 83.3% (n=35), received adjuvant chemotherapy with an association of platinum and taxanes, only 3 patients (7.1%) associated bevacizumab to the regime and 4.8% had PARP inhibitor as maintenance therapy. At first debulking surgery (table 2), 46.9 % (n=15) had complete tumor resection (R0), 37.5 % (n=12) had macroscopic residual tumor (R2) and 15.6% (n=5) with R1. For patients submitted to neoadjuvant chemotherapy, 25.0% (n=2) had CRS1,

50.0% (n=4) had CRS2 and 25.0 % (n=2) had CRS3. Patients responded better to the first line of chemotherapy (table 3) compared to the second and last line of chemotherapy, 82.5 % had a CR and 10.0 % a PR with no progression of disease. As the last line of chemotherapy, 30.0 % had SD and 22.5 % had progression of disease.

Table 2. Treatment of choice at diagnosis

Modalities of treatment at diagnosis	All patients (n = 42)
First choice of treatment, n (%)	(n = 42)
Primary debulking surgery	26 (61.9 %)
Neoadjuvant chemotherapy [a]	16 (38.1 %)
Number of cycles of neoadjuvant chemotherapy, n (%)	
Median, (range)	5.0 (3-8)
CRS, n (%)	(n = 8) [b]
CRS 1	2 (25.0 %)
CRS 2	4 (50.0 %)
CRS 3	2 (25.0 %)
Debulking surgery approach, n (%)	(n = 42) [c]
Cytoreductive surgery	42 (100.0 %)
Hysterectomy	41 (97.6 %)
Bilateral Salpingo-Oophorectomy	42 (100.0 %)
Omentectomy	37 (88.1 %)
Lymphadenectomy	8 (19.0 %)
Removal of any visible cancerous tissue in the organs or elsewhere in the abdomen	30 (71.4 %)
HIPEC	1 (2.4 %)

Surgical outcome at first cytoreduction, n (%)	(n = 32) [d]
R0	15 (46.9 %)
R1	5 (15.6 %)
R2	12 (37.5 %)
LVI, no. (%)	(n = 28) [e]
Present	13 (46.4 %)
Suspicious	3 (10.7 %)
Absent	12 (42.9 %)
Adjuvant therapy, n (%)	(n = 42)
No	7 (16.7%)
Yes	35 (83.3 %)
Lines of systemic therapies, n (%)	(n = 42)
Median, (range)	2,5 (1-5)
1	10 (23.8 %)
2	11 (26.2 %)
3	9 (21.4 %)
4	7 (16.7 %)
5	5 (11.9 %)
Exposure systemic therapy, n (%)	(n = 42) [f]
Platinum compounds	42 (100.0 %)
Taxanes	35 (83.3 %)
Bevacizumab	3 (7.1 %)
PARP inibitor	2 (4.8 %)

HIPEC, Hyperthermic Intraperitoneal Chemotherapy. CRS, Chemotherapy Response Score. LVI, Lymphovascular invasion. PARP, poly ADP-ribose polymerase.

[a]15 (93.8%) patients enrolled with carboplatin plus paclitaxel and one patient (6.3%) with cisplatin plus paclitaxel.

[b]CRS1, minimal tumor response. CRS 2, appreciable tumor response. CRS3, complete tumor response. 8 had missing data related to CRS score.

[c]2.4 % (n=1) wasn't submitted to a hysterectomy; 11.9 % (n=5) weren´t submitted to an omentectomy; 81.0 % (n=34) weren´t submitted to a lymphadenectomy; 28.6% (n=12) weren´t submitted to removal of all visible tumor; 1 % (n=2.4%) wasn't submitted to a hysterectomy.

[d] Residual tumor classification: R0, no evidence of macroscopic disease or optimal cytoreduction; R1, residual minimal disease or suboptimal cytoreduction. R2, evidence of macroscopic disease. 10 had missing data related to residual tumor classification.

[e]14 had missing data related to LVI status.

[f]16.7% (n=7) without taxanes, 92.9% (n=39) without bevacizumab and 95.2 % (n=40) without PARP maintenance.

Table 3. Disease response to chemotherapy of diagnosis

	All patients (n = 42)		
Disease response, n (%)	**1st-line chemotherapy (n=40) [a]**	**2nd-line chemotherapy (n=32) [b]**	**Last-line chemotherapy (n=40) [c]**
CR	33 (82.5 %)	13 (40.6 %)	10 (25.0 %)
PR	4 (10.0 %)	14 (43.8 %)	9 (22.5 %)
SD	3 (7.5 %)	2 (6.3 %)	12 (30.0 %)
PD	0 (0.0 %)	3 (9.4 %)	9 (22.5%)

CR, complete response. PR, partial response. SD, stable disease. PD, progressive disease.
[a]2 had missing data related to first line of chemotherapy.

[b]10 had missing data related to the second line of chemotherapy.

[c] 2 had missing data related to the last line of chemotherapy.

3.2. **Patient´s recurrence characteristics and follow-up**

The median progression-free survival (PFS) was 21 months (95% CI, 14.6–27.3 months). The median platinum free interval (PFI) was 14 months (95% CI, 9.5 - 18.5), varying between 0 and 106 months until first recurrence.) Patients classified as PSOC (n=38, 90,5%), 7.1% with PROC and one (2.4%) with refractory disease after 17 days of last chemotherapy. At recurrence (table 4) 59.5% had a second debulking surgery and 76.2% received chemotherapy, 31.0% were submitted to HIPEC and 4.8% with hormonotherapy. According to residual disease (RD), at debulking surgery after recurrence (table 4), 62.5 % (N=10) have complete tumor resection (R0), 12.5 % (n=2) had macroscopic residual tumor (R2) and 25.0 % with R1. Response to treatment, included 6 CR (21.4 %), 19 PR (67.9 %), 2 SD (7.1 %) and 1 (3.6 %) progression of disease at chemotherapy responses at recurrence.

Table 4. Cohort characteristics of recurrence

	All patients (n=42)
ECOG PS, n (%)	(n=40) [a]
0	25 (62.5 %)
1	13 (32.5 %)
2	2 (5.0 %)
Platinum Free Interval, n (%)	(n=42)
Median, (range), months	14.5 (0 – 106.0)
Refractory, recurrence ≤3 months	1 (2.4 %) [b]
Resistant, recurrence > 3 and ≤ 6 months	3 (7.1 %)
Sensitive, recurrence > 6 months	38 (90.5 %)
Approach at recurrence, n (%)	(n=42) [c]
2nd Cytoreductive surgery	25 (59.5 %)
Chemotherapy	32 (76.2 %)
HIPEC	13 (31.0 %)
Hormonotherapy	2 (4.8 %)

Surgical outcome at 2nd cytoreduction, n (%)	(n=16) [d]
R0	10 (62.5 %)
R1	4 (25.0 %)
R2	2 (12.5 %)
Disease response to chemotherapy at recurrence, n (%)	(n=28) [e]
CR	6 (21.4 %)
PR	19 (67.9 %)
SD	2 (7.1 %)
PD	1 (3.6 %)

PS, performance status. PFI, platinum free interval. HIPEC, Hyperthermic Intraperitoneal Chemotherapy. CRS, Chemotherapy Response Score. PR, partial response. SD, stable disease. PD, progressive disease

[a] 2 had missing data related to ECOG PS at recurrence.

[b] One patient without evidence of disease had a recurrence 17 days after finishing its last chemotherapy.

[c] 40.5 % (n=17) had no cytoreductive surgery; 23.8 % (n=10) had no chemotherapy; 69.0 % (n=30) had no HIPEC; 95.2 % (n=40) didn't receive hormonotherapy treatment.

[d] Residual tumor classification: R0, no evidence of macroscopic disease or optimal cytoreduction; R1, residual minimal disease or suboptimal cytoreduction. R2, evidence of macroscopic disease. 9 had missing data related to Residual tumor classification.

[e] 4 had missing data related to status disease response to chemotherapy.

Recurrence samples were obtained in the context of surgical resection of relapsed tumors (67.5 %), confirmation of recurrence (22.5 %) and palliative surgery (10.0 %). Most common samples were surgical specimens (n=26, 61.9%) followed by biopsy (n= 13, 31.0 %). The main site for sample

recurrence was peritoneum (n=32, 76,2%), in serosa of liver, rectum, diaphragm, supra-renal, ileum, spleen, jejunum, psoas muscle, round and falciform ligament parieto-colic, pelvic floor, transverse and sigmoid colon, parametrial and fat tissue adjacent to small bowel. Followed by vagina (n=5, 11.9%), lymphatic ganglia (n=2, 4.8%), colon (n=1, 2.4%), liver (n=1, 2.4%) and colon (n=1, 2.4%).

After the first recurrence 71.4% (n=30) had progression of disease versus 28.6% (n=12) without. Histological confirmation of progression was rarely performed, only 4 patients had a sample of progression, including 3 surgical specimens and 1 biopsy. Median time of follow-up was 119 months. At last follow-up half the patients (57,1%, n=24) were dead from disease, 26,2% (n=11) were alive with disease and 16,7% (n=7) were alive without disease. The median overall survival (OS) was 83 months (95% CI, 70.8–95.2 months).

3.3. **Folate receptor alpha analysis**

The implementation of the FOLR1 immunohistochemistry test, using a standardized and automated system, was rapid and feasible in retrospective FFPE samples, including primary adnexal tumors, metastases, and recurrences; the main challenge was identifying an appropriate control, which required testing a large number of fallopian tube samples. Histological review of the original sample slides, with selection of sections showing the best tumor representativeness while avoiding necrosis and artifacts, ensured sample adequacy in the vast majority of cases.Thus, 97.6%(n=82) fulfilled the adequacy criteria for evaluation, and 2 (1 primary and 1 recurrence sample) had less than 100 tumor cells in the IHC section, thus being inadequate. The primary tumor sample belonged to a patient with CRS3 after neoadjuvant chemotherapy and the recurrence one refers to a small biopsy.

In our cohort (table 5), the median FOLR1 expression was 70.0% and the rate of FOLR1 positive status was 46.3%, both for samples at diagnosis and recurrences. FOLR1 expression was predominantly homogeneous, but heterogeneity (figure 1) of expression was observed in 11 (26.8%) tumors at diagnosis and 7 (17.1%) recurrences. The predominant pattern of FOLR1 (figure 2) was the circumferential membrane expression, followed by the apical and dot-like. At diagnosis, primary tumor samples (median=60%, range 10-100) had significantly lower FOLR1 expression than synchronous peritoneal metastases (median=85%, range 60-100, p=0.010). Accordingly, a positive FOLR1 status was less frequent in adnexal samples compared to peritoneal synchronous metastases (36.4% vs. 87.5%, p=0.016). No significant differences were found regarding FOLR1 expression between recurrence sites. No significant differences were found in FOLR1 expression using paired

analysis of samples at diagnosis and matched recurrences, regarding distribution of FOLR1 score, status, intratumoral heterogeneity and patterns.

We found an association between FOLR1 positive status at diagnosis and neoadjuvancy compared to primary surgery setting (66.7% vs. 34.6% FOLR1 status, respectively, p=0.047). Additionally, median progression free survival (PFS) was significantly lower in patients with positive FOLR1 (20 months, IC95 12.9 - 27.1) compared to patients with negative FOLR1 status diagnosis (24 months, IC95 7.9 - 40.1, p=0.035) (figure 3), i.e., positive FOLR1 tumors relapsed sooner. No association was found between FOLR1 status (both at diagnosis or recurrence) and the remaining clinicopathological variables studied, including age, FIGO stage, lines of treatment, response to treatment and overall survival (data not shown).

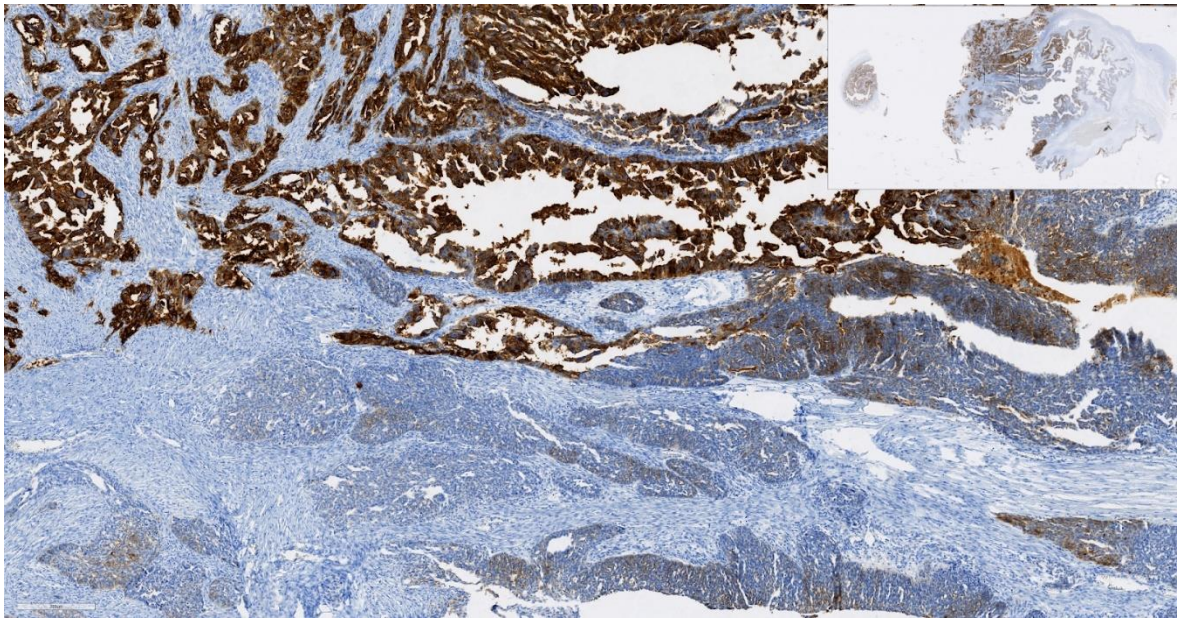

Figure 1. Ovarian tumor showing a heterogeneous expression of FOLR1. Strong membrane expressions are seen in the upper region, in contrast to low expression in the tumor nests below. Inset displays a low-magnification view of the tissue section.

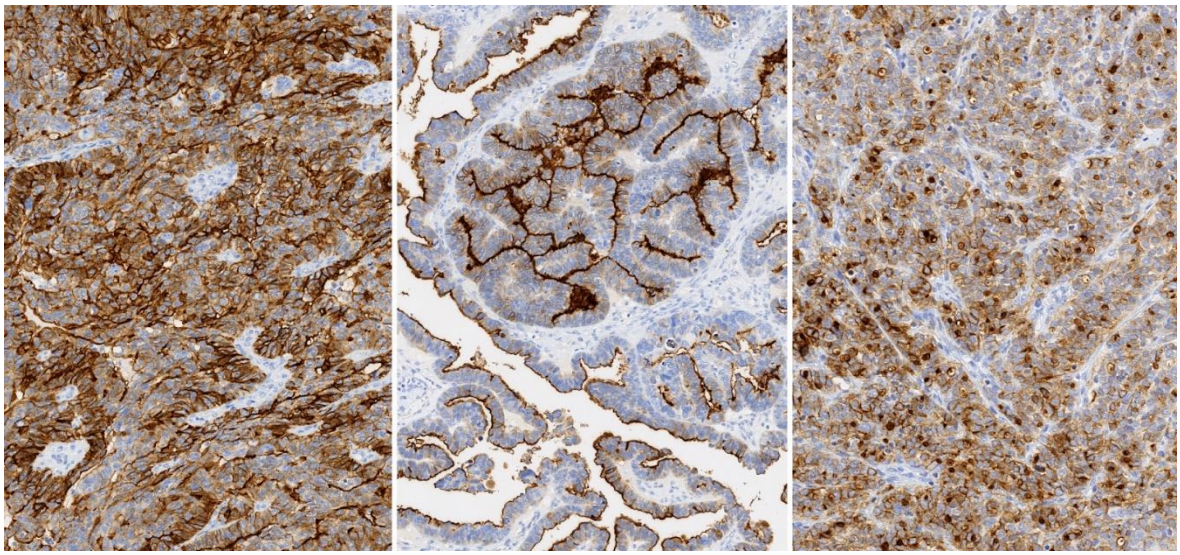

Figure 2. Patterns of FOLR1 expression: a) circumferential, b) apical, c) dot.

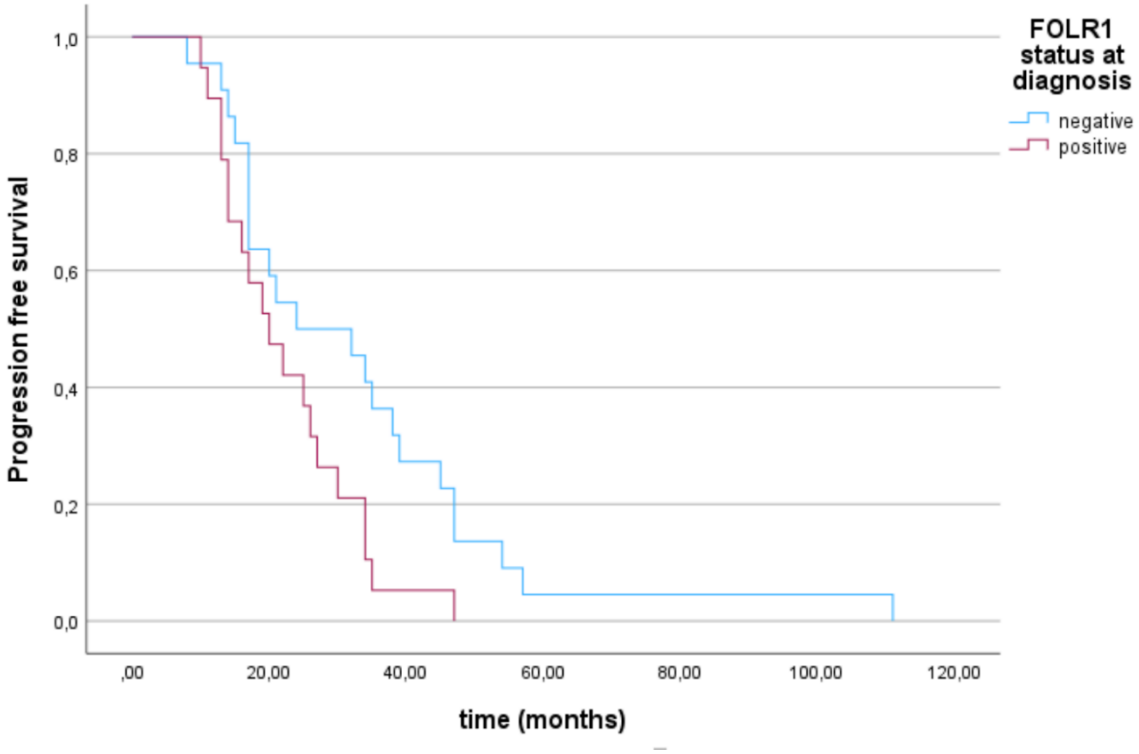

Figure 3. Progression free survival (PFS) in correlation with change of FOLR1 status

Table 5. Folate receptor expression IHC at diagnosis and recurrency

	Samples		***p***
	Diagnosis (n=42)	**Recurrent (n=42)**	
FOLR1 score, n (%)			*p*=0.128
Median, (range)	70.0% (10.0-100%)	70.0% (0.0-100%)	
FOLR1 status, n (%)			*p=1.000*
Positive status	19 (46.3 %)	19 (46.3 %)	
Negative status	22 (53.7 %)	22 (53.7 %)	
Predominant pattern, n (%)			
Circumferential	24 (58.5 %)	24 (64.9 %)	*p*=0.648
Dot-like	15 (36.6 %)	9 (24. 3 %)	*p*=0.549
Apical	16 (39.0 %)	19 (51.4 %)	*p*=0.481
Heterogeneity, n (%)			*p*=0.607
Yes	11 (26.8 %)	7 (17.1 %)	
No	30 (76.2 %)	34 (82.9 %)	

Forty patients had adequate matched samples for both primary and recurrence. The majority, 65% (n=26) maintained FOLR1 status at diagnosis and recurrence, either maintaining it negative (n=14) or positive (n=12). Importantly, 14 (35%) patients change their FOLR1 status in the recurrence, namely, 17.5% (n=7) changed from negative to positive, and 17.5% (n=7) changed from positive to negative. The global score variations and representative examples of changes in FOLR1 expression are shown in figure 4.

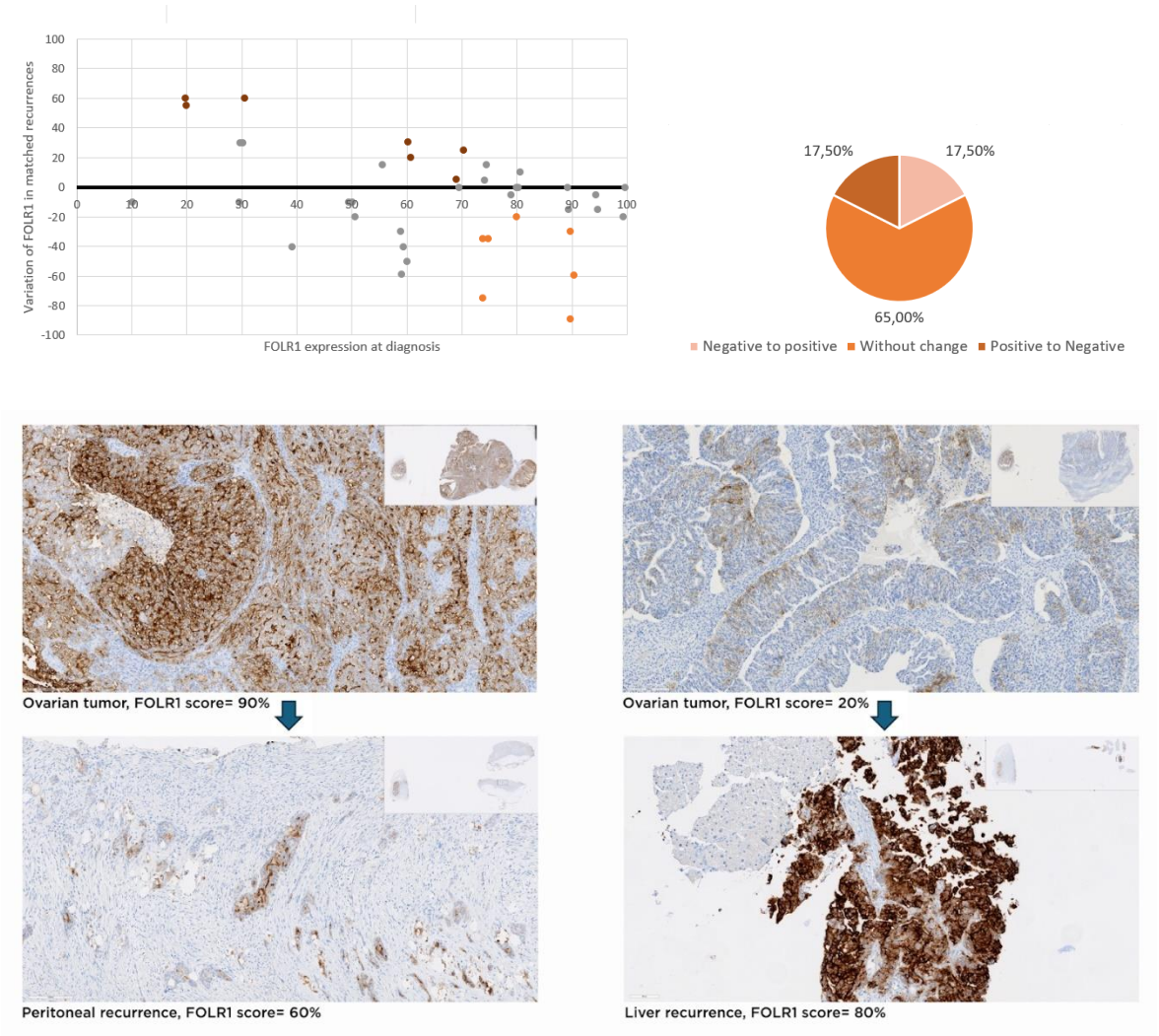

Figure 4. Immunohistochemical tumor expression of FOLR1 at diagnosis and matching recurrences. Scatter plot of the complete cohort showing the quantitative variation of FOLR1 score in recurrences in relation to its expression at diagnosis, in brown are marked the change to positive and as orange the change to negative (a jitter was applied to distinguish similar values in quantitative variation of FOLR1). The pie chart shows the distribution of FOLR1 status changes. Representative images showing a tumor that changed FOLR1 status from positive to negative (on the left) and another that changed from negative to positive (on the right). Insets display low-magnification views of the tissue sections.

Quantitatively, the median difference of FOLR1 score (at diagnosis to recurrence) was -7.5% with a minimum of -89.0% and a maximum of 60.0%. As expected, absolute median score differences were significantly larger in the groups that showed changes (30 and -35 for the groups that change to positive and negative, respectively), compared to the group with no changes (median -7.5).

Nonetheless, the range of variation is large in all groups, with no significant differences in variability (p=0.858). In particular, of the tumors that changed to positive, two had a borderline FOLR1 expression at diagnosis with a score of 70%, two had a score of 60%, and the remaining 3 had equal or lower than 30%. For tumors that changed to negative, their FOLR1 score in the recurrence was less than 40%, except for 2 cases with 60%.

Patients with no changes in FOLR1 status were predominantly homogenous at diagnosis (n= 20, 76.9%). Regarding patients who reclassified as negative in recurrence, all (n=7, 100%) had homogeneous FOLR1 expression in the tumor sample at diagnosis. For patients who reclassified as positive in recurrence, most (n=4, 57.1%) had an heterogeneous intratumoral expression in the tumor sample at diagnosis. A statistically significant association was found between intratumoral heterogeneity at diagnosis and the direction of FOLR1 expression change (p=0.046), indicating a possible relationship with a change to positive. Regarding the type of expression pattern (circumferential, apical, dot-like), there were no significant associations with changes in FOLR1 status, except for the apical pattern that was significantly more frequent at diagnosis in tumors that had any changes in the recurrence compared to those with a maintained FOLR1 status (64.3% vs 26.9%, respectively, p=0.041). Additionally, we also found a possible association of changes with recurrence sample site (p=0.040). A thorough analysis of other clinical-pathological parameters (table 6) that could associate with change in FOLR1, did not demonstrate any significant associations, namely with LVSI, FIGO stage, patients' age, ECOG, BRCA variants, treatment and survival.

Table 6. Clinical-pathological characteristics according to change in FOLR1 status

	FOLR1 status					
Variables	**No Change (n=26)**	**Change to Positive (n=7)**	**Change to Negative (n=7)**	**Any change (n=14)**	**p** (no change vs. any change)	**p** (no change vs. change to positive vs. change to negative)
FOLR1 score difference (diagnosis to recurrence)						
median	-7.5	+30	-35	-7,5	*p<0.001	*p<0.001
minimum, maximum	-59, 30	5, 60	-89, -20	-89, 60	***p*=0.107**	***p*=0.858**

Intratumoral heterogeneity at diagnosis, n (%)						
Heterogeneous	6 (23.1 %)	4 (57.1 %)	0 (0.0 %)	4 (28.6 %)	***p*=0.072**	***p*=0.046**
Homogeneous	20 (76.9%)	3 (42.9 %)	7 (100.0 %)	10 (71.4 %)		
Predominant pattern at diagnosis						
Circumferential, n (%)	17 (65.4 %)	4 (57.1 %)	1 (14.3 %)	5 (35.7 %)	***p*=0.198**	***p*=0.200**
Apical, n (%)	7 (26.9 %)	4 (57.1 %)	5 (71.4 %)	9 (64.3 %)	***p*=0.041**	***p*=0.082**
Dot-like, n (%)	10 (38.5 %)	3 (42.9 %)	1 (14.3 %)	4 (28.6 %)	***p*=0.730**	***p*=0.619**
Sample site at diagnosis, n (%)						
Adnexal	20 (76.9 %)	7 (100.0 %)	5 (71.4 %)	12 (85.7%)	***p*=0.689**	***p*=0.516**
Peritoneum	6 (23.1 %)	0 (0.0 %)	2 (28.6 %)	2 (14.3%)		
Sample site at recurrence, n (%)						
Peritoneum	21 (80.8%)	3 (42.9%)	7 (100%)	10 (71.4%)	***p*=0.694**	***p*=0.040**
Other (vagina, skin, lymph node)	5 (19.2%)	4 (57.1%)	0 (0.0%)	4 (28.6%)		
Type of samples at diagnosis, n (%)						
Surgical specimen	18 (69.2 %)	5 (71.4 %)	2 (28.6 %)	7 (50.0 %)	***p*=0.254**	***p*=0.204**
Surgical biopsy	2 (7.7 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)		
Biopsy	6 (23.1 %)	2 (28.6 %)	5 (71.4 %)	5 (71.4 %)		
LVI, n (%) [a]						
Absent/equivocal	8 (53.3 %)	3 (60.0 %)	3 (50.0 %)	6 (54.5 %)	***p*=1.000**	***p*=1.000**
Present	7 (46.7 %)	2 (40.0 %)	3 (50.0 %)	5 (45.5 %)		
FIGO stage, n (%) [b]						
IB / IIA / IIB	3 (12.5 %)	0 (0.0 %)	2 (33.3 %)	2 (16.7 %)	***p*=1.000**	***p*=0.356**
IIIA / IIIB / IIIC / IV	21 (87.5 %)	6 (100 %)	4 (66.7 %)	10 (83.3 %)		

Age at diagnosis						
Median, years (range)	59.0 (39-83)	50.0 (46-73)	51.0 (39-67)	50.5 (39-67)	***p*=0.394**	***p*=0.621**
ECOG at diagnosis, n (%) [c]						
ECOG 0	17 (77.3 %)	3 (60.0 %)	3 (50.0 %)	6 (54.5 %)	***p*=0.240**	***p*=0.446**
ECOG 1	5 (22.7 %)	2 (40.0 %)	3 (50.0 %)	5 (45.4 %)		
BRCA variants, n (%) [d]						
Not detected	13 (61.9 %)	3 (42.9 %)	3 (60.0 %)	6 (50.0 %)	***p*=0.716**	***p*=0.784**
BRCA variant	8 (38.1 %)	4 (57.1 %)	2 (40.0 %)	6 (50.0 %)		
Primary treatment setting, n (%)						
Primary debulking surgery	18 (69.2 %)	5 (71.43 %)	2 (28.6 %)	7 (50.0 %)	***p*=0.310**	***p*=0.167**
Neoadjuvant chemotherapy	8 (30.8 %)	2 (28.6 %)	5 (71.4 %)	7 (50.0 %)		
Adjuvant therapy, no. (%)						
No	4 (15.4 %)	0 (0.0 %)	2 (28.6 %)	2 (14.3 %)	***p*=1.000**	***p*=0.370**
Yes	22 (84.6 %)	7 (100 %)	5 (71.4 %)	12 (85.7 %)		
Lines of systemic therapies, n (%)						
Median (range)	2 (1-5)	3 (2-4)	2 (1-5)	3 (1-5)	***p*=0.977**	***p*=0.686**
Exposure systemic therapy, n (%)						
Platinum	22(84.6 %)	7 (100.0 %)	5 (71.4 %)	12 (85.7 %)	***p*=1.000**	***p*=0.370**
Taxanes	22 (84.6 %)	7 (100.0 %)	5 (71.4 %)	12 (85.7 %)	***p*=1.000**	***p*=0.370**
Bevacizumab	2 (7.7 %)	0 (0.0 %)	1 (14.3 %)	1 (7.7 %)	***p*=1.000**	***p*=0.737**
PARP inhibitor	0 (0.0 %)	1 (14.3 %)	0 (0.0 %)	1 (7.7 %)	***p*=0.350**	***p*=0.350**
Platinum Free Interval, months						
Median, (95CI)	12 (7.0-17.0)	20 (0.0-43.1)	16 (10.9-21-1)	16 (5.0-27.0)	***p*=0.546**	***p*=0.654**

Refractory, recurrence ≤3 months, n (%) [b]	1 (3.9 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	***p*=0.701**	***p*=1.000**
Resistant, recurrence > 3 and ≤ 6 months, n (%)	3 (11.5 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)		
Sensitive, recurrence > 6 months n (%)	22 (84.6 %)	7 (100.0 %)	7 (100.0 %)	14 (100.0 %)		
Survival, median (95 % CI)						
Progression free survival	20 (15.0-25.0)	32 (11.5-52.5)	22 (14.3-29.7)	24 (18.5-29.5)	***p*=0.732**	***p*=0.543**
Overall survival	84 (65.4-102.6)	92 (54.1-129.9)	59 (8.0-110.0)	80 (43.9-116.1)	***p*=0.507**	***p*=0.750**

PS, performance status. LVI, lymphovascular invasion.

[a]14 patients had missing data for LVI.

[b] 4 patients had missing data for FIGO stage.

[c]5 patients had missing data for ECOG performance status.

[d]7 patients had missing data or BRCA variants status.

* Comparison of variables was made with the absolute values of their difference.

3.4. **Digital Folate receptor alpha evaluation**

Comparison of pathologist visual classification and script-based classification of FOLR1 status (table 7), showed an overall accuracy of 73.6% and moderate agreement (Kappa = 0.45). Specificity was high (100%) correctly identifying all negatives, with no false positives, but there was low sensitivity (44%), missing half of true positives. However, when separately analyzing samples at diagnosis and recurrences, the script showed considerable differences in performance. For diagnostic samples, the script-based method maintained a high specificity (100%) but a very low sensitivity (25%), with an overall accuracy of 62.5% and weak agreement (Cohen's Kappa = 0.25). In contrast, for recurrent tumors, performance improved significantly, with a high specificity (100%) and moderate sensitivity (61%), achieving 82.5% accuracy and a Kappa of 0.63, indicating substantial agreement.

Table 7. Confusion Matrices for FOLR1 Classification

	Script negative	**Script positive**
Primary Tumors/ Metastasis at diagnosis		
Visual Negative	16 (TN)	0 (FP)
Visual Positive	12 (FN)	4 (TP)
Recurrences		
Visual Negative	22 (TN)	0 (FP)
Visual Positive	7 (FN)	11 (TP)
Global (Diagnostic samples + Recurrences)		
Visual Negative	38 (TN)	0 (FP)
Visual Positive	19 (FN)	15 (TP)
FN, False negative. FP, false positive. TN, true negative. TP, true positive.		

Considering FOLR1 absolute scores, there is a strong positive correlation (r=0.80) between visual pathology scores and script-based scores (figure 4). Even though the script generally follows the same scoring trend, it generally reports lower values. The average absolute difference between the two is approximately 15.8 percentual points. Visual inspection of the script classification (figure 5) confirms that misclassification frequently occurs, where several intensely stained regions lacking nuclei are segmented as small nuclei and classified as stromal.

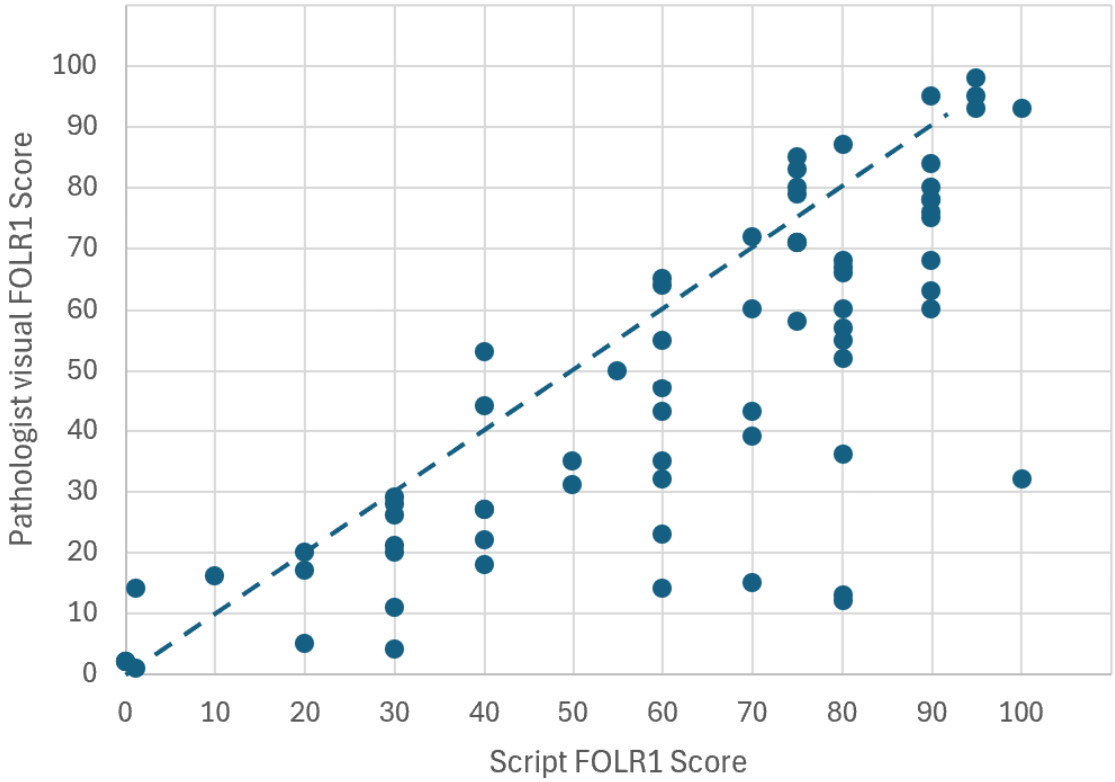

Figure 4. Correlation between script-based and visual pathology FOLR1 scores. Each point represents a tumor sample. The dashed line represents perfect agreement. While there is a strong correlation, most points fall below the line, suggesting the script tends to underestimate FOLR1 expression.

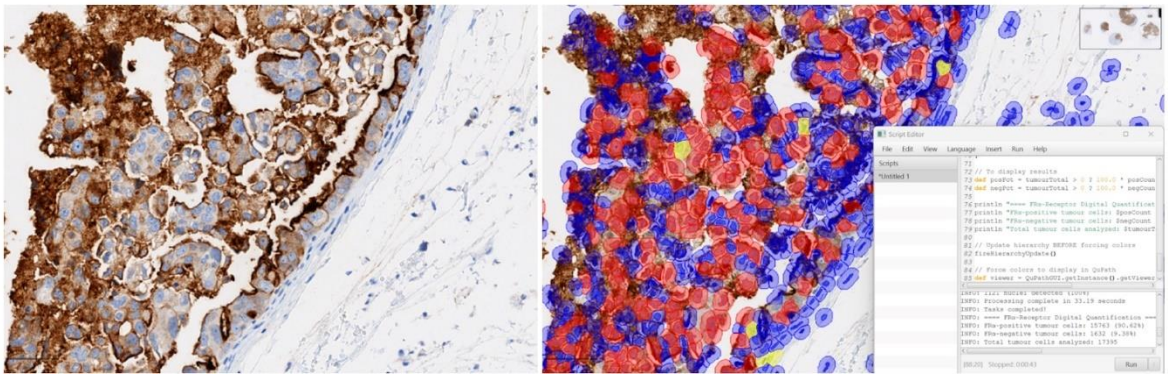

Figure 5. Comparison between visual FOLR1 staining (left) and script-based segmentation and classification (right) in QuPath. Several strongly stained tumor areas are misidentified as small nuclei and incorrectly classified as stromal (blue), denoting a segmentation limitation that may contribute to underestimation of FOLR1-positive (red) tumor cells. Inset shows the script output.

4. Discussion

Epithelial ovarian cancer (EOC) is the main histological subtypes of ovarian cancer. HGSOC is one of its most lethal forms, which treatment relies on debulking surgery and platinum-based chemotherapy. In our cohort, tubo-ovarian was the most common primary site, 68% of patients presented advanced disease (stage III/IV) with a median age of 58.7 years at diagnosis, which is concordant with literature.[52,53] As in previous studies, BRCA variants were present in nearly half of patients, with *BRCA* 1 oncogenic variants being more frequent than *BRCA* 2.[10,11] Most of our cohort (69.1%) were submitted to primary debulking surgery and 38.1% to neoadjuvant chemotherapy followed by interval debulking surgery. Previous literature re-enforces no significant difference of primary treatment setting on survival outcomes for women with advanced EOC.[54-56] As expected, response to initial treatment was high but recurrence of disease shortly ensued. Progression free survival and overall survival of our cohort is also concordant with other series.

FOLR1 is a new biomarker that predicts target treatment response, that is abnormally overexpressed in ovarian cancer. And so, a reliable characterization of this receptor is essential. Archival tissue samples are suitable to determine the tumor immunoexpression frequency and intensity of FOLR1, as well as the distribution pattern, differentiating membrane and cytoplasmic localization.[25] We implemented a standardized fast automated FOLR1 IHC test using FFPE samples, as in previous clinical trials, and so, our main goal was achieved.[38,43,45,47] The main challenge was the selection of appropriate tissue control and histological review of samples was vital for selection of sections with tumor representativeness.

Both resection specimens as biopsy are reliable for the characterization of FRα status in IHC, hence it should be selected the sample with the more amount of tissue, usually being the surgical specimen.[25] Surgical specimens were the most common type of sample (61.9%) both at diagnosis and recurrences. Farther, the attribution of FOLR1 status can be statistically significant difference between biopsy and resection specimens.[53] However, we found no statistically significant difference for type of samples for change of status at diagnosis as well in recurrences. And so, in our study every sample had a previous sample with hematoxylin-eosin stain (H&E stain) to access a representative tumor sample, selecting when possible, the resection specimen, to then be submitted to further characterization of FOLR1 status. The vast bulk of samples (n=82) were adequate for FOLR1 characterization, only two samples were considered inadequate without sufficient tumor cells present to attribute a concordant status.

As a sub aim, we also develop a script base algorithm for an automated characterization of FOLR1. Studies on FOLR1 assessment with a script based score are scarce, and to the best of our best of our

knowledge our studies the only one who compares its accuracy with the pathologist visual scoring.[51] Even thought, a strong positive correlation between visual pathology scores and script-based scores was found, with high specificity, there was a low sensitivity and overall modest accuracy. All in all, it still warrants improvement for use in clinical routine.

As described in previous studies, we also found a high expression of FOLR1 in HGSC, with a median expression of 70%. Importantly, both at diagnosis and recurrences, 46.3% of our patients have a positive FOLR1 status, according to the criteria used to select patients for current target therapies (equal ou higher than 75%). The results are concordant with literature found in recent reviews and in previous clinical PICCOLO trial, who also had a majority of PSOC.[45]

The rate of FOLR1 status (46.3 %) in our cohort is seemingly high compared to previous clinical trials such as MIRASOL and SORAYA, which report rates between 32% to 36%.[38,43] However, we must consider that these trials focused on PROC and thus differ from the clinicopathological characteristics of our cohort, which included approximately 90% platinum-sensitive patients at first recurrence. Our FOLR1 status rates are more similar to the one declared in the PICCOLO trial (42.5%) whose cohort was also platinum-sensitive.[45]

Tumor heterogeneity in a patient, can be defined as variances between different tumor cells, either within the primary tumor itself or their synchronous metastases or recurrences during the course of disease.[57] Tumor microenvironment, epigenetic fluctuations and clonal evolution are some of the theories that can explain the origin of different phylogenies of tumor cells within the same tumor mass.[58] HGSC intratumoral heterogeneity can imply distinct properties of the cells, with differences in invasion and metastatic seeding capacity, and therefore therapeutic resistance.[57,58]

In our study we observed intratumoral heterogeneity of FOLR1 expression of tumor cells within each sample at diagnosis, in 26.8% of patients. Even though the presence of FOLR1 intratumoral heterogeneity is generally described in literature, to the best of our knowledge no study published its frequency within samples. There are a few studies of heterogeneity focused on comparing biopsy samples with resection specimens, and comparison of samples at diagnosis and metastases/recurrences of different sites. In our study we compared samples at diagnosis to recurrences, to characterize FOLR1 expression during the course of disease. Importantly, 35% of our patients changed FOLR1 status, half changed to negative and the other half to positive. This is in contrast a previous study by Kalli et al.[22] (n=24 paired samples), which compared primary and recurrent disease, but described a low frequency of changes (12.5%) of patients. As the expression of FOLR1 can change over time it would imply a different approach to treatment according to results at diagnosis and at its recurrence.

Some studies have tried to identify factors that influenced the change in FOLR1 expression. Previs et al. reported that the primary site, such as ovary and fallopian tube, were more likely to present a higher proportion of FRα-positive status comparative to theirs synchronous metastases.[59] Even though our main goals did not focus on this comparison, in in our study we included primary tumor samples and only when it was not accessible, we included peritoneal metastases at diagnosis. The latter showed a higher FOLR1 expression compared to adnexal samples, but this was a not a paired analysis. Lawson et. al found a statistically significant difference considering the site of sample for positive FOLR1 status.[53] In our study, there also was a significant difference in change to positive or to negative FOLR1 status considering the site of samples at recurrences, but not for diagnosis.

Importantly, in our study, intratumoral heterogeneity at diagnosis and apical pattern significantly associated with changes of status FOLR1 status. Indeed, loss or gain of FOLR1 expression can be due to intratumoral heterogeneity expression by tumor cell clonal selection after exposure to therapies, or tumor evolution. In our study, previous exposure to chemotherapy was not a significant factor for change of status between diagnosis and recurrence. This is in accordance with the study of Crane et al. and Despierre et al., which concluded that chemotherapy did not alter expression rates.[23,60]

Recently, it was demonstrated that target therapies directed to FOLR1 had an apparently significant influence on FOLR1 change of status. Johannet et al. compared pre matched samples (n=12) after treatment with MIRV with the post-treatment samples, demonstrating a significant variation, namely loss of FLOR1 compared to pre-treatmemt.[61] Other studies detect a reduction in the expression of FOLR1 after MIRV, but not statistically significant between (n= 17) paired pre and post treatment samples.[25] Even after considering different sample locations, intratumoral heterogeneity of primary and metastatic foci, effects of prior systemic therapies, the authors consider that the loss of FOLR1 expression represents a tie of resistance with MIRV.[23,25,61]

Higher expression of FOLR1 is correlated with more aggressiveness and worse outcomes.[62] We found a statistically significant association of FOLR1 status and progression-free survival as found in previous studies. In our cohort, patients with positive FOLR1 status had a significantly lower median progression free survival compared to patients with negative FOLR1 status at diagnosis. Furthermore, we found that the primary setting of neoadjuvant chemotherapy was also significantly associated with positive FOLR1 status at diagnosis. Given that choice of neoadjuvant chemotherapy at primary setting is usually offered for disease with a more advanced stage this probably correlates with worse prognosis.[54]

Our study has several limitations, being a retrospective study there is risk for selection bias and limited access to patient's data, and so generalized results should be considered with careful

consideration. Although we have a small cohort of patients, we included a total of 40 paired samples at primary and recurrent disease, which is a larger cohort compared to previous similar studies as Kalli (n=24), Crane (n=12) and Despierre (n=9).[22-24]

5. Conclusion

In summary, we achieved our first goal and successfully validated and implemented a FOLR1 standardized automated IHC based on FFPE samples for EOC. Microscopic optical visual assessment of FOLR1 expression remains the gold standard for evaluation. Digital automated algorithms still warrant improvement and validation for clinical use.

Regarding our second goal, our study supports that FOLR1 is variably expressed in HGSOC, with a considerable frequency of intratumoral heterogeneity and change of FOLR1 status over the course of disease. Changes included both directions equally, ie, from negative to positive or from positive to negative. We did a thorough analysis of parameters that could influence the change in FOLR1 status in recurrences. Only intratumoral heterogeneity at diagnosis and apical pattern, stood out as possible factors. Treatment options, volume and site of sample did influence change in FOLR1 expression.

We concluded that reassessment of FOLR1 status in different samples may be relevant, however, further studies are needed to determine the impact of FOLR1 expression variability in response to targeted therapies such as mirvetuximab.

6. Apendix

Avaliação da expressão de FOLR1 - Protocolo Anátomo-Patológico Sinóptico

Produto: biópsia / peça cirúrgica

Topografia: ovário / peritoneu / outro: __

Origem da amostra: tumor primário / metástase / recidiva

Anticorpo utilizado: VENTANA FOLR1 (FOLR1-2.1) RxDx Assay (CE-IVD)

Avaliação da imunorreatividade:

- Número de células neoplásicas avaliáveis: >= 100 / < 100 (amostragem de células neoplásicas insuficiente para avaliação adequada)

- Resultado: __ %, com intensidade 2+/ 3+

- Descrição (optional): e.g. heterogeneidade da marcação, padrão (circunferencial, dot, apical, misto), presença de artefactos

Conclusão:

Teste imuno-histoquímico negativo / positivo para FOLR1, com expressão moderada ou forte detectável em <75% / ≥ 75% das células tumorais viáveis.

Nota: A categorização em positivo/negativo foi baseada nos critérios atuais aprovados para a terapêutica dirigida ao FOLR1 - mirvetuximab soravtansine.

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