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ORIGINAL ARTICLE

Functional estrogen receptor signal transduction pathway activity and antihormonal therapy response in low-grade ovarian carcinoma

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Abstract

Background: Advanced low-grade ovarian carcinoma (LGOC) is difficult to treat. In several studies, high estrogen receptor (ER) protein expression was observed in patients with LGOC, which suggests that antihormonal therapy (AHT) is a treatment option. However, only a subgroup of patients respond to AHT, and this response cannot be adequately predicted by currently used immunohistochemistry (IHC). A

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possible explanation is that IHC only takes the ligand, but not the activity, of the whole signal transduction pathway (STP) into account. Therefore, in this study, the authors assessed whether functional STP activity can be an alternative tool to predict response to AHT in LGOC.

Methods: Tumor tissue samples were obtained from patients with primary or recurrent LGOC who subsequently received AHT. Histoscores of ER and progesterone receptor (PR) were determined. In addition, STP activity of the ER STP and of six other STPs known to play a role in ovarian cancer was assessed and compared with the STP activity of healthy postmenopausal fallopian tube epithelium.

Results: Patients who had normal ER STP activity had a progression-free survival (PFS) of 16.1 months. This was significantly shorter in patients who had low and very high ER STP activity, with a median PFS of 6.0 and 2.1 months, respectively ($p < .001$). Unlike ER histoscores, PR histoscores were strongly correlated to the ER STP activity and thus to PFS.

Conclusions: Aberrant low and very high functional ER STP activity and low PR histoscores in patients with LGOC indicate decreased response to AHT. ER IHC is not representative of functional ER STP activity and is not related to PFS.

KEYWORDS
antihormonal therapy, immunohistochemistry, ovarian carcinoma, signal transduction pathway, survival, targeted therapy

INTRODUCTION

Ovarian carcinoma is the fifth leading cause of death in women with cancer.[1] Ovarian carcinoma can be classified into high-grade and low-grade tumors. Low-grade ovarian carcinoma (LGOC) can be subclassified into serous, endometrioid, and mucinous LGOC.[2] Despite a favorable overall survival (OS) of 91 months in patients who have advanced LGOC, compared with 41 months in those who have advanced-stage, high-grade serous ovarian carcinoma (HGSOC), the current standard chemotherapy does not improve OS in patients who have LGOC.[3] The chemoresistance of LGOC emphasizes the need for alternative therapy options.[4,5]

LGOC and HGSOC differ clinically;[6] for example, LGOC exhibits higher expression of the estrogen receptor (ER) and the progesterone receptor (PR). Therefore, ER is a potential target for treatment.[7] Antihormonal therapy (AHT) proved to be useful in LGOC because a clinical benefit rate (CBR) of 61%–71% was observed in studies of patients with LGOC.[8,9] However, the commonly used method to predict therapy response—ER and PR immunohistochemistry (IHC)—lacked predictive value in all studies.[8–10] This emphasizes the need for a tool to predict response to AHT.

Several clinical trials have used DNA sequencing or protein expression methods to apply targeted treatment in malignancies.[11–13] However, these might not be accurate methods for all cancers because the functional phenotype of cancer cells is not fully reflected by gene mutations or the expression of proteins.[14] The MOSCATO trial reported that only 33% of patients benefited from matched targeted treatment.[11] This low response rate might be a result of the influence of other factors, such as the tumor microenvironment or changes in cellular oncogenic processes, e.g., crosstalk of signal transduction pathways (STPs).[14]

Verhaegh et al. developed an assay using messenger RNA (mRNA) concentrations from validated target genes of oncogenic STPs. This assay determines the STP activity of cells and might be a more representative measure of the functional phenotype of cells.[15–17] In other hormone-sensitive malignancies, the STP assay has demonstrated the ability to predict prognosis and targeted therapy response.[18–20] Therefore, this assay has the potential to select patients with LGOC who will respond to AHT.

In the current study, our objective was to determine the predictive value of ER STP activity and IHC hormone receptor expression on the response to AHT in patients with LGOC. Because therapy response can be influenced by other activated STPs, we also investigated the relation between the androgen receptor (AR), phosphoinositide-3 kinase (PI3K), hedgehog (HH), transforming growth factor-β (TGF-β) and mitogen-activated protein kinase (MAPK) pathways and the response to AHT in patients with LGOC.

MATERIALS AND METHODS

Design and patient population

In this retrospective study, we performed a search in the Nationwide Network and Registry of Histopathology and Cytopathology in the Netherlands (PALGA) and the Registration System Oncological

Gynecology database to select patients who were diagnosed with either primary borderline serous tumors recurring in LGOC or primary or recurrent LGOC between 1999 and 2019. To be eligible for inclusion, patients must have received AHT, e.g., aromatase inhibitors or antiestrogens, for at least 4 weeks.[21] Combination therapy with other targeted therapy or chemotherapy and maintenance therapy after surgical treatment were not permitted. Tumor tissues obtained from patients during chemotherapy or within a wash-out period of 3 weeks were excluded. Furthermore, patients with other malignancies were excluded. Clinical data were retrieved from the patients' medical files (for extracted variables, see Table S1).

Tumor tissue

Formalin-fixed, paraffin-embedded tumor tissue was obtained from eligible patients (see Table S2). Each formalin-fixed, paraffin-embedded sample was cut into 5-μm-thick sections for mRNA extraction, one 5-μm-thick section for hematoxylin and eosin staining, and two 4-μm-thick sections for ER IHC and PR IHC. A gynecologic pathologist (S.L.B.) determined the tumor cell percentage and annotated tumor area on the hematoxylin and eosin-stained slide. The samples with a tumor cell percentage <40% were excluded to minimize stromal contamination.

Signaling transduction pathway assay

mRNA was extracted from samples, and quantitative reverse transcription-polymerase chain reaction was performed. The tumor area on the unstained slides was macrodissected using the annotated hematoxylin and eosin-stained slides as a reference followed by deparaffinization, mRNA isolation, and quantitative reverse transcription-polymerase chain reaction analysis, as described in the Supporting Methods.

The STP activity of seven STPs was determined using an STP assay (version 1.15.2; Philips).[22–25] The results are reported as quantitative measurements on a scale from 0 to 100, representing the functional activity of each pathway. On this scale, 0 corresponds to the lowest odds, and 100 corresponds to the highest odds in favor of active pathway activity. To identify aberrant STP activity, STP activity scores of healthy postmenopausal fallopian tube epithelium (post-FTE) were used as a reference for normal activity.[26] Growing evidence suggests a tubal origin of serous borderline tumors and serous LGOC, like in HGSOC.[27] Post-FTE scores were used because the hormonal status is similar to that of our study population.[26]

Immunohistochemical analysis and scoring

Two independent pathologists (S.L.B. and M.H.F.M.L.-B.) determined the percentage of IHC staining of ER and PR in the tumor cells (range, 0%–100%). In addition, the slides were scored using histoscores, as previously described.[23]

Statistical analysis

The data were analyzed using the statistical computing software package IBM SPSS Statistics (version 26.0; IBM Corporation). Statistical results with p values $< .05$ were considered statistically significant.

Clinical characteristics were compared between responders and nonresponders. Response was assessed according to Response Evaluation Criteria in Solid Tumors, version 1.1, or biochemically by CA-125 concentrations according to Gynecologic Oncology Group criteria. Responders were defined as patients who experienced clinical benefit (stable disease, partial response, or complete response) for at least 6 months. The CBR was calculated as the proportion of responders. The overall response rate (ORR) was calculated as the proportion of patients with a partial or complete response. Groups were compared using independent-sample t-tests or a Mann–Whitney U test for continuous variables. Fisher exact tests were used for categorical variables. Differences in STP activity between primary and recurrent disease were analyzed in pairs using Wilcoxon rank-sum tests. To explore correlations, the Pearson correlation coefficient was calculated.

Normal pathway activity was defined as STP scores within the range of two standard deviations above or below the mean STP activity score in normal post-FTE. Any scores outside this range were defined as aberrant low or high STP activity.

Progression-free survival (PFS) and OS were analyzed using Kaplan–Meier curves. Univariate Cox hazard analysis was performed, and variables that had p values $< .10$ were subsequently entered into a multivariate Cox hazard model.

Ethics statement

This study was approved by the Dutch Medical Research Ethics Committees United (MEC-U, W19.175, and W18.134). The collection, storage, and use of tissue and patient data were performed in agreement with the *Code for Proper Secondary Use of Human Tissue* in the Netherlands (Committee on Regulation of Health Research; https://www.coreon.org).

RESULTS

Study population

In total, 682 patients were identified from initial screening of 47,951 pathology reports. After subsequent screening of medical files, 577 patients were excluded because they did not receive AHT. In addition, we excluded 78 patients for several reasons, as described in Figure S1, leaving a total of 27 patients for our data analysis. Among the included patients, 15 were identified as responders, and 12 were identified as nonresponders to AHT. Table 1 presents the clinical characteristics stratified by response to AHT. There were no significant differences between groups in age at diagnosis, hormonal status, histology of

TABLE 1 Baseline clinical characteristics stratified by response to antihormonal therapy after 6 months.

	Responders (n = 15), No. (%)	Nonresponders (n = 12), No. (%)	Total (n = 27), No. (%)	p
Age at diagnosis: Mean ± SD, years	51.7 ± 16.5	55.1 ± 17.1	53.2 ± 16.5	.60
Hormonal status at AHT treatment				.36
Premenopausal	1 (6.67)	0 (0.0)	1 (3.7)	
Postmenopausal	14 (93.3)	12 (100)	26 (96.3)	
Parity: Mean ± SD	1.21 ± 1.25	1.70 ± 1.41	1.42 ± 1.32	.39
Histology of primary tumor				.90
Borderline	4 (26.7)	3 (25.0)	7 (25.9)	
Low-grade serous	10 (66.7)	7 (58.3)	17 (63.0)	
Endometrioid	1 (6.7)	1 (8.3)	2 (7.4)	
Mucinous	0 (0.0)	1 (8.3)	1 (3.7)	
FIGO stage				.25
I	0 (0.0)	3 (30.0)	3 (12.5)	
II	3 (21.4)	2 (20.0)	5 (20.8)	
III	9 (64.3)	4 (40.0)	14 (54.2)	
IV	2 (14.3)	1 (10.0)	3 (12.5)	
AHT				.59
Tamoxifen	8 (53.3)	8 (66.7)	16 (59.3)	
Letrozole	5 (33.3)	4 (33.3)	9 (33.3)	
Anastrozole	2 (13.3)	0 (0.0)	2 (7.4)	
PFS, median (CI 95%), months	19.7 (13.9–39.0)	4.0 (3.0–5.5)	11.5 (8.6–24.6)	.00*
OS, median (CI 95%), months	66.0 (63.1–149.5)	62.1 (36.3–88.6)	66.0 (60.3–113.3)	.12

Note: Response was assessed according to Response Evaluation Criteria in Solid Tumors, version 1.1, or as the biochemical response according to Gynecologic Oncology Group criteria.

Abbreviations: AHT, antihormonal therapy; CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; OS, overall survival; PFS, progression-free survival; SD, standard deviation.

*Statistically significant.

primary and recurrent disease, International Federation of Gynecology and Obstetrics stage, or treatment type. Responders to AHT had significantly longer PFS compared with nonresponders, with a PFS of 19.7 and 4.0 months, respectively ($p < .001$). There was no significant difference in OS between the groups ($p = .12$). The calculated ORR was 18.5%, and the CBR was 55.6% (Table 2).

TABLE 2 Response rates at 3, 6, 9, and 12 months after the start of antihormonal therapy.

	No. of patients (%)			
	3 months	6 months	9 months	12 months
Progression	6 (22.2)	12 (44.4)	13 (48.1)	16 (59.3)
Stable disease	14 (52.9)	9 (33.3)	9 (33.3)	4 (14.8)
Partial response	2 (7.4)	1 (3.7)	0 (0.0)	2 (7.4)
Complete response	0 (0.0)	0 (0.0)	1 (3.7)	3 (11.1)
Unknown	5 (18.5)	5 (18.5)	4 (14.8)	2 (7.4)

Note: Response was assessed according to Response Evaluation Criteria in Solid Tumors, version 1.1, or as the biochemical response according to Gynecologic Oncology Group criteria.

STP activity analysis

In total, 36 tumor tissue samples from 27 patients were available for analysis, including 24 primary and 12 recurrent tumor tissues. When comparing the STP activity in LGOC with the STP activity in post-FTE, we observed significantly lower ER STP activity in LGOC ($p = .006$). Moreover, significantly higher MAPK ($p = .002$), HH ($p < .001$), and TGF-β ($p = .003$) STP activity was observed in LGOC. For the AR, Notch, and PI3K pathways, no significant differences in STP activity were observed (see Figure S2). Aberrantly high or low STP activity was identified in 92% of samples compared with post-FTE pathway scores (see Table S3). In LGOC, the ER STP activity was most often aberrant (44.4%): of these, 87.5% had (very) low ER pathway activity, followed by (overall high) TGF-β pathway activity (41.7%), (high) MAPK pathway activity (33.3%), (high) HH pathway

activity (30.6%), (overall high) PI3K pathway activity (25%), and (overall low) Notch pathway activity (11.1%). Figure S3 provides an overview of individual clinical characteristics and STP activity scores.

In the MAPK, Notch, TGF-β, and PI3K pathways, the median STP scores were lower in responders compared with nonresponders. In contrast, the median STP activity of the AR, ER, and HH pathways was higher in responders compared with nonresponders. However, no significant differences were observed (Figure 1).

Aberrant STP activity in at least one of the seven assessed STPs was identified in 80% of responders and in all nonresponders. In responders, ER STP activity was aberrant in 33.3% of responders versus 66.7% of nonresponders. Although most nonresponders had low aberrant ER STP activity, one patient had a remarkably high ER STP score.

Survival analysis

PFS and OS were analyzed for all assessed STPs and stratified were for normal, low, and high STP activity. For the ER pathway, low and high STP activity was correlated with a significantly shorter PFS, with a median PFS of 6.0 and 2.1 months, respectively, compared with a median of 16.1 months in patients with normal ER STP activity (log-rank $p < .001$). Other assessed STP activity was not associated with PFS (Figure 2). For OS, the analysis did not reveal noteworthy differences (see Figure S4).

From the univariate Cox hazard regression analysis, we identified aberrant ER and Notch activity as possible predictors of PFS, with respective hazard ratios of 2.17 (95% CI, 0.93–5.07; $p = .07$) and 2.17 (95% CI, 0.93–5.07; $p = .08$; see Table S4). Unadjusted multivariate analysis with aberrant ER and Notch activity showed significant hazard ratios of 2.72 ($p = .03$) for aberrant ER STP activity and 4.98 ($p = .02$) for aberrant Notch STP activity.

Estrogen and progesterone receptor IHC and STP activity

We assessed ER and PR IHC in 37 LGOC tissue samples. ER IHC was not correlated with ER STP activity, PFS, or OS. PR histoscores and functional ER STP activity were found to have a strong positive correlation ($r = 0.85$; $p < .001$; see Figure S5).

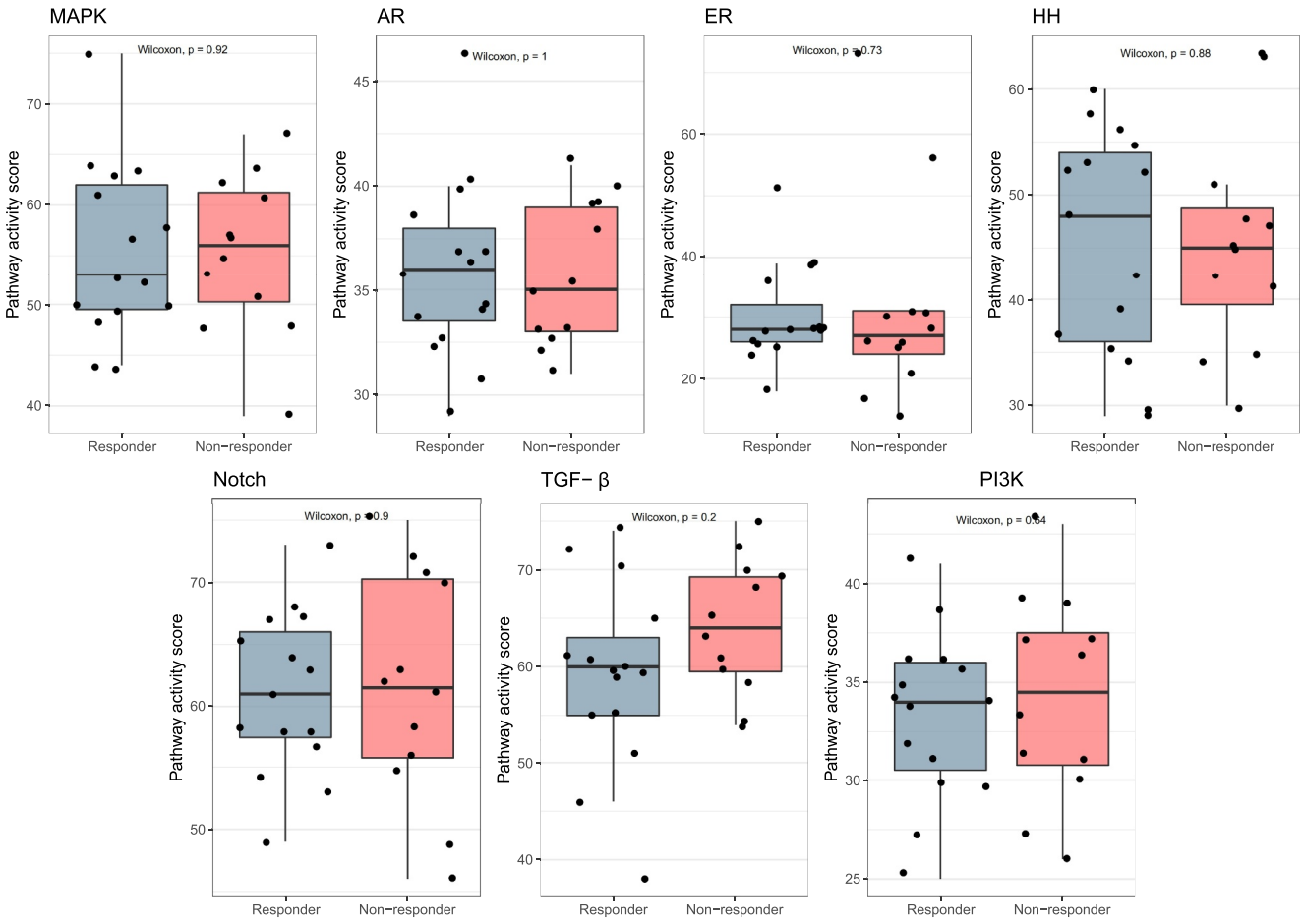

FIGURE 1 Pathway activity score of responders compared with nonresponders of the seven assessed signal transduction pathways. AR indicates androgen receptor; ER, estrogen receptor; HH, hedgehog; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3 kinase; TGF-β, transforming growth factor beta.

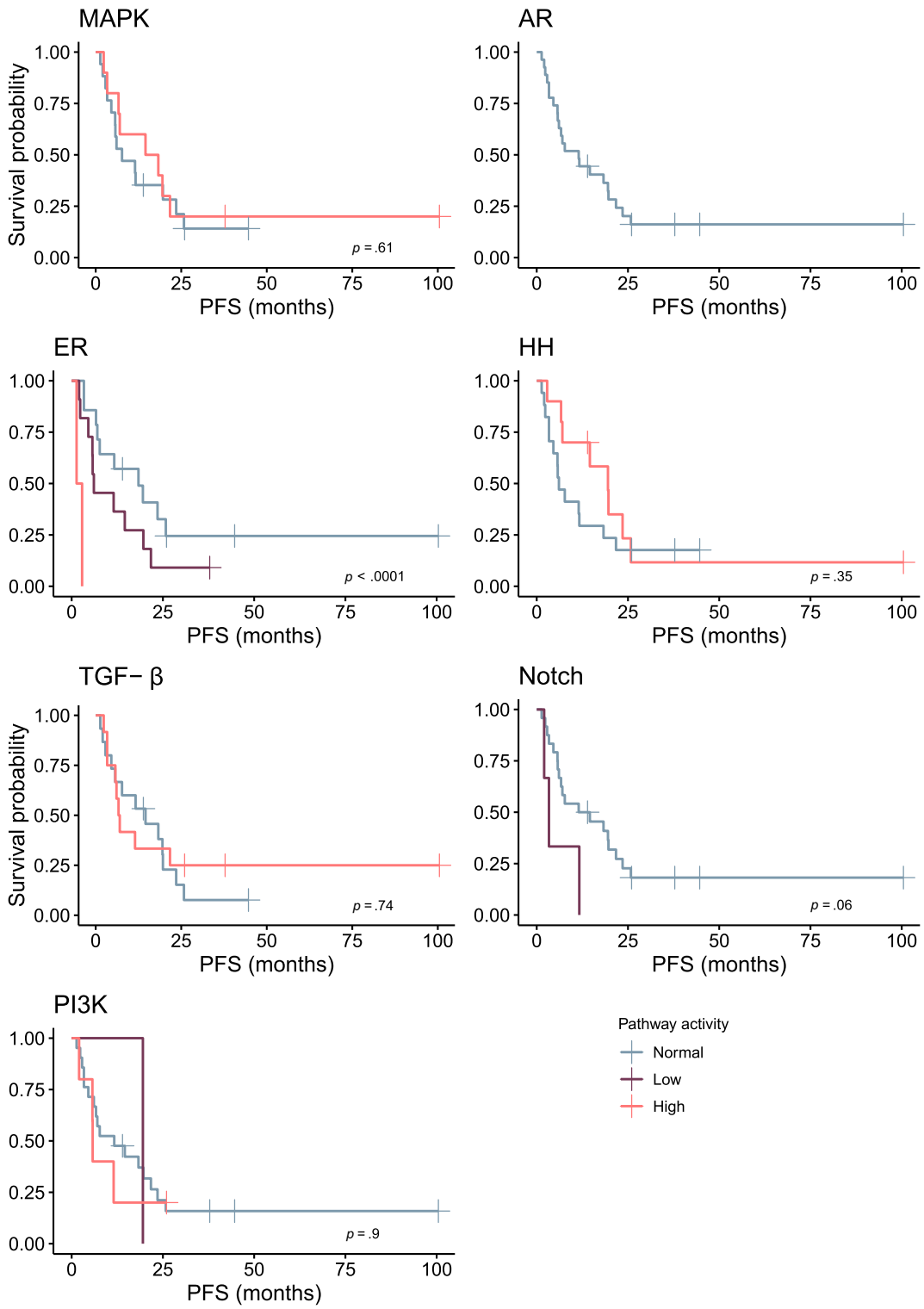

FIGURE 2 Kaplan–Meier curves for PFS of the seven assessed signal transduction pathways stratified by normal, low, and high signal transduction pathway activity. AR indicates androgen receptor; ER, estrogen receptor; HH, hedgehog; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3 kinase; PFS, progression-free survival; TGF-β, transforming growth factor beta.

From eight patients, tissue samples of both the primary and recurrent tumors were available for analysis. The percentage of ER-stained cells and ER histoscores increased in recurrent disease, although no significant differences were observed. However, both the percentage of positive PR-stained cells and the PR histoscore were significantly lower in recurrent disease compared with primary disease ($p = .02$; Figure 3).

In contrast to the higher observed ER IHC percentage and histoscores, significantly lower ER STP activity was observed ($p = .03$) in recurrences. No other significant differences were identified in other

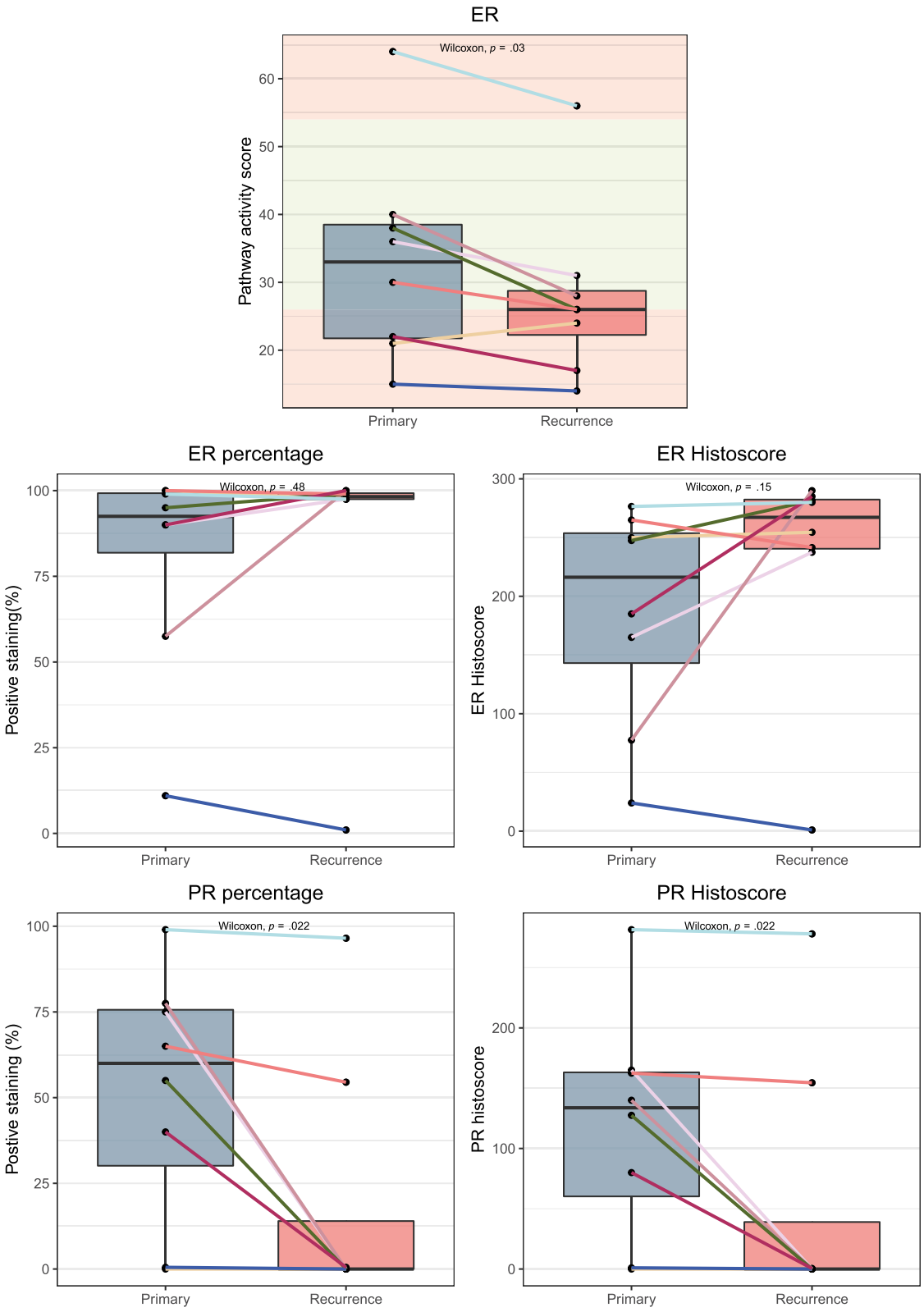

FIGURE 3 Paired analysis of pathway activity scores for primary and recurrent disease in the ER STP ($n = 8$) and paired analysis of ER/PR percentages and ER/PR histoscores. Individual colored lines represent one patient. Red and green background areas represent cutoff values for aberrant and normal STP activity, respectively. ER indicates estrogen receptor; PR, progesterone receptor; STP, signal transduction pathway.

STPs (Figure 4). In all paired samples, an inverse correlation was observed in the difference between the primary and recurrent samples in MAPK and ER STP activity ($r = 0.77$; $p = .03$).

DISCUSSION

In the current study, we observed that STP activity did not predict response to AHT in patients with LGOC. However, patients who had aberrant high or low ER STP activity generally did not respond to AHT and had a significantly shorter PFS compared with patients who had normal ER STP activity. PR histoscores were strongly correlated with functional ER STP activity and thus PFS; whereas ER IHC was not correlated with survival. Our results are in contrast to findings in endometrial and breast cancer, in which a subgroup of patients who responded to (neo)adjuvant AHT could be identified by (high) ER STP activity.[18,28] Contrary to our expectations, the patient who had an aberrantly high ER STP activity (without concurrent aberrant STPs) did not benefit from AHT. This suggests other underlying AHT-resistance mechanisms in which possibly both intracellular factors, such as crosstalk, and extracellular factors, such as local estrogen production causing an immune response, play a key role in the development of resistance.[29]

Based on high ER protein expression, previous studies suggested that the ER STP is generally highly active in (LG)OC. However, in LGOC, the ER STP might not be as active as is believed because only one patient exhibited aberrantly high ER STP activity in recurrent LGOC.[8,9,30] High STP activity can be possibly explained by ER signaling mechanisms, such as autocrine production of estradiol (direct-genomic signaling).[29] Also, local estrogens call for an immune response, changing the function of subpopulations of cells from tumor-suppressing to tumor-promoting. The change to a tumor-promoting environment possibly results from the STP activity of immune cells themselves, particularly the PI3K STP, which plays an important positive and negative role in cellular response.[31]

Conversely, the self-regulation of ER expression can also be established through indirect-genomic signaling through crosstalk by the PI3K and MAPK pathways.[32] These STPs have the ability to phosphorylate downstream components of the ER STP through crosstalk, leading to a decrease in ER expression.[33–35] We observed

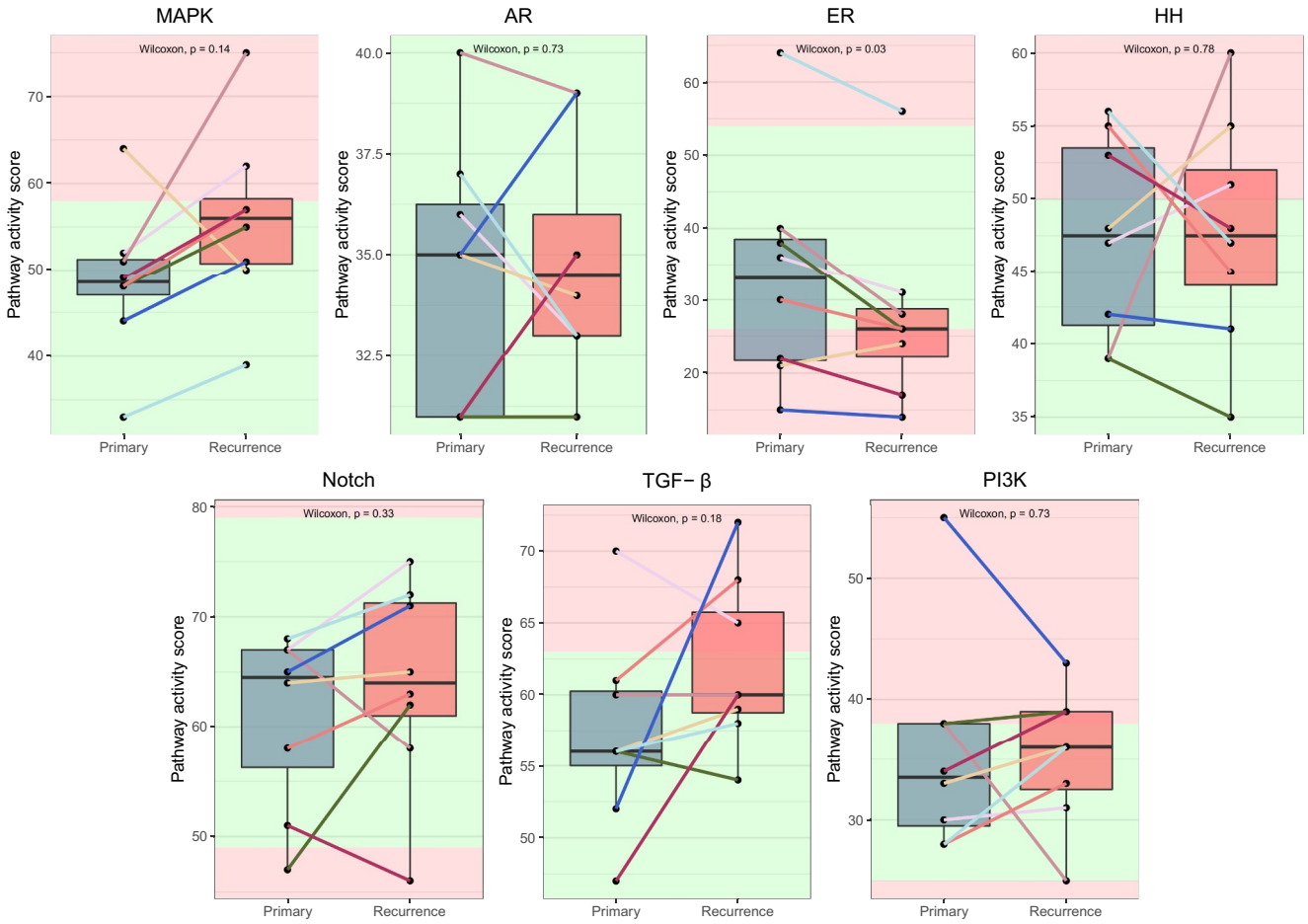

FIGURE 4 Paired analysis of pathway activity scores for primary and recurrent disease in each STP ($n = 8$). Individual colored lines represent one patient. Red and green background areas represent cutoff values for aberrant and normal STP activity, respectively. AR indicates androgen receptor; ER, estrogen receptor; HH, hedgehog; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3 kinase; STP, signal transduction pathway; TGF-β, transforming growth factor beta.

an inverse correlation in the difference in MAPK and ER STP activity between primary and recurrent disease. A similar inverse correlation has been observed in several preclinical studies through the observation of improved AHT sensitivity after targeted MAPK inhibition.[33,34,36] LGOC frequently harbor *KRAS* or *BRAF* genomic mutations, which are known MAPK gain-of-function mutations.[37] Hyperactive MAPK pathway activity is a cause for AHT resistance in breast cancer; we observed an increase in MAPK activity in recurrent LGOC: a possible similar resistance mechanism.[32,38]

In our study, we observed a CBR of 55.6% and an ORR of 18.5% after 6–12 months of AHT. These results are in line with studies that had a similar population, reporting a CBR of 61% and an ORR of 9%–14%.[8,9] Concurrent tumor driving aberrant STPs can be an explanation why not all patients responded to AHT. In the analyzed tissue samples of recurrent disease obtained before AHT from non-responding patients, we observed more aberrant STPs and a much higher proportion of patients with (very) low ER STP activity compared with those in primary disease. These observations demonstrate that multiple, dominant STPs and loss of ER functional STP activity indicate a possible cause for AHT resistance. Therefore, other aberrant pathways might be considered in the choice of therapy.

In all analyzed samples, ER IHC suggested that ER STP activity is higher than it functionally is. Therefore, ER IHC is not reliable to represent functional ER STP activity. These results are supported by a previous study in HGSOC in which no association between ER IHC and ER STP activity was identified.[23] However, we did observe an association between PR histoscores and ER STP activity in LGOC ($R = 0.85$; $p < .001$), resembling the findings from previous research in endometrial cancer.[28] Low PR expression or complete loss thereof have previously been related to a worse survival and are thought to be caused by inactivity of the ER STP.[7,39] Our findings contribute to this theory that low PR expression is caused by ER STP inactivity and thus is a possible better predictor than ER IHC.

Of clinical importance, we observed significantly lower functional ER STP activity in recurrent disease, indicating that performing an STP assay on primary disease is not accurate for predicting the response to AHT in recurrent disease. Furthermore, differences in the STP activity profile (and in PR expression) between primary and recurrent disease suggest the development of therapy resistance after primary treatment. Based on our observations, tissue samples should be obtained close to the start of in the intended (targeted) treatment.

The strength of this study is the clearly defined cohort of patients who had LGOC with tissue samples obtained before the initiation of AHT. Also, the availability of paired samples of primary disease and recurrent disease allowed us to make (careful) conclusions for different phases of the clinical course of LGOC. However, this study has several shortcomings. First, the retrospective design led to missing data and differences in follow-up between patients. In addition, only patients with IHC-confirmed, positive ER/PR LGOC are treated with AHT, resulting in possible selection bias. Second, only a small set of patients were eligible for inclusion because of the low incidence of LGOC and the low numbers who received AHT treatment. Finally, the time between tissue collection and the initiation of AHT differed substantially in several patients. The median time was 0.8 months for tissues collected shortly before AHT initiation and 17.1 months for tissues collected long before AHT initiation, respectively. The finding that tissue of recurrence usually is not obtained before the start of second-line or third-line therapy explains why only 50% of the available tissue samples were obtained shortly before the initiation of AHT.

To our knowledge, this study is the first study to determine functional STP activity in LGOC. We observed a relation between the response to AHT and aberrant ER STP activity in a small group of patients. Our findings should be confirmed in a larger cohort, preferably in a prospective study. Based on the possible interaction of the MAPK and ER STPs, a combination treatment with a MAPK STP inhibitor and AHT could be considered in patients with low ER pathway activity to increase sensitivity to AHT. In addition, differences in STP activity between primary disease and recurrent disease provide motivation for further research to gain knowledge about therapy resistance mechanisms and to improve patient stratification for (targeted) therapy.

To conclude, in patients with LGOC, ER STP activity is not an accurate predictor of response to AHT; however, low PR histoscores and aberrant low or very high functional ER STP activity are associated with shorter PFS. The commonly used ER IHC has no correlation with functional ER STP activity and has no predictive value for response to AHT or PFS. Furthermore, we observed that activation of the MAPK STP might play a key role in the development of AHT resistance in LGOC.

AUTHOR CONTRIBUTIONS

Cynthia S. E. Hendrikse: Accrued clinical data, contributed to the formal analysis, and wrote the original draft. **Phyllis van der Ploeg:** Involved in conceptualization, accrued clinical data, contributed to the formal analysis, involved in data acquisition, contributed to data interpretation, and reviewed and edited the article. **Nienke M. A. van de Kruis:** Accrued clinical data and contributed to formal analysis. **Jody H. C. Wilting:** Accrued clinical data and contributed to formal analysis. **Floor Oosterkamp:** Accrued clinical data and contributed to formal analysis. **Pauline M. M. Theelen:** Accrued clinical data and contributed to formal analysis. **Christianne A. R. Lok:** Involved in data acquisition and reviewed and edited the article. **Joanne A. de Hullu:** Involved in data acquisition and reviewed and edited the article. **Huberdina P. M. Smedts:** Involved in data acquisition and reviewed and edited the article. **M. Caroline Vos:** Involved in data acquisition and reviewed and edited the article. **Brenda M. Pijlman:** Involved in data acquisition and reviewed and edited the article. **Loes F. S. Kooreman:** Involved in data acquisition and reviewed and edited the article. **Johan Bulten:** Involved in data acquisition and reviewed and edited the article. **Marjolein H. F. M. Lentjes-Beer:** Performed pathologic assessments. **Steven L. Bosch:** Performed pathologic assessments and reviewed and edited the article. **Anja van de Stolpe:** Contributed to data interpretation and reviewed and

edited the article. **Sandrina Lambrechts**: Involved in data acquisition and reviewed and edited the article. **Ruud L. M. Bekkers:** Involved in data acquisition and reviewed and edited the article. **Jurgen M. J. Piek:** Involved in conceptualization, involved in data acquisition, contributed to data interpretation, reviewed and edited the article, and supervised the project.

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CONFLICTS OF INTEREST

Anja van de Stolpe was employed by Philips Molecular Pathway Dx (currently InnoSIGN) during this work. Jurgen M. J. Piek reports personal fees from Philips outside the submitted work. The other authors declare no conflicts of interest.

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