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RESEARCH ARTICLE

Tumor Markers and Signatures

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Gene expression markers in peripheral blood and outcome in patients with platinum-resistant ovarian cancer: A study of the European GANNET53 consortium

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Abstract

Disease progression is a major problem in ovarian cancer. There are very few treatment options for patients with platinum-resistant ovarian cancer (PROC), and therefore, these patients have a particularly poor prognosis. The aim of the present study was to identify markers for monitoring the response of 123 PROC patients enrolled in the Phase I/II GANNET53 clinical trial, which evaluated the efficacy of Ganetespib in combination with standard chemotherapy versus standard chemotherapy alone. In total, 474 blood samples were collected, comprising baseline samples taken before the first administration of the study drugs and serial samples taken during treatment until further disease progression (PD). After microfluidic enrichment, 27 gene transcripts were analyzed using quantitative polymerase chain reaction and their utility

for disease monitoring was evaluated. At baseline, *ERCC1* was associated with an increased risk of PD (hazard ratio [HR] 1.75, 95% confidence interval [CI]: 1.20–2.55; $p = 0.005$), while baseline *CDH1* and *ESR1* may have a risk-reducing effect (*CDH1* HR 0.66, 95% CI: 0.46–0.96; $p = 0.024$; *ESR1* HR 0.58, 95% CI: 0.39–0.86; $p = 0.002$). *ERCC1* was observed significantly more often (72.7% vs. 53.9%; $p = 0.032$) and *ESR1* significantly less frequently (59.1% vs. 78.3%; $p = 0.018$) in blood samples taken at radiologically confirmed PD than at controlled disease. At any time during treatment, *ERCC1*-presence and *ESR1*-absence were associated with short PFS and with higher odds of PD within 6 months (odds ratio 12.77, 95% CI: 4.08–39.97; $p < 0.001$). Our study demonstrates the clinical relevance of *ESR1* and *ERCC1* and may encourage the analysis of liquid biopsy samples for the management of PROC patients.

KEYWORDS

circulating tumor cells, *ESR1* (estrogen receptor 1), *ERCC1* (ERCC excision repair 1 endonuclease non-catalytic subunit), Hsp90 inhibitor

What's new?

Treatment options for patients with platinum-resistant ovarian cancer (PROC) are limited, leaving these patients highly vulnerable to disease progression. With the possibility of serial testing to monitor therapeutic response, however, it may now be possible to predict treatment outcomes in PROC patients using liquid biopsy (LB). Here, the authors applied a molecular LB approach in PROC patients with recurring disease and found that *ESR1* is a predictive marker for lengthened progression-free survival. Meanwhile, *ERCC1* was associated with disease progression and short survival. The discovery of a prognostic role for *ESR1* offers new insight into the biology and treatment of PROC.

1 | INTRODUCTION

Ovarian cancer is the most fatal cancer of the female reproductive tract, causing more than 200,000 deaths worldwide annually.[1] In many cases, the disease is diagnosed late and although the patients often respond well to initial treatment with cytotoxic drugs such as carboplatin and paclitaxel, which cause DNA damage, most relapse within a short period of time, develop progressive resistance over time, and ultimately die of this disease.[2]

High-grade serous ovarian carcinoma (HGSOC) is the major ovarian cancer subtype and accounts for about 70%–80% of ovarian cancer-related deaths.[3] HGSOC is characterized by a high frequency of *TP53* gene mutations mainly located in the DNA-binding domain of the gene. Missense mutations make up more than 85% of all p53 alterations and lead to the formation of missense mutp53 proteins. The higher proliferation rate, invasive and metastatic potential, and chemoresistance of p53 mutated cancer cells are not only due to the loss of the wild-type (wt) tumor suppressor function but also to new oncogenic functions of the mutp53 proteins.[4] Furthermore, mutp53 proteins are very stable, while wtp53 proteins do not accumulate in the cell due to tight regulation by the MDM2 E3 ubiquitin ligase.[5] Degradation of mutp53 is prevented by interaction with the heat shock protein HSP90, a chaperone that promotes protein folding, thereby leading to the accumulation of aberrant mutp53.[6]

In the past, there have been numerous efforts to use HSP90 inhibitors, among them the small molecule inhibitor Ganetespib (G), in the treatment of cancer patients.[7] While almost 40 clinical trials have evaluated the efficacy of G in patients with solid tumors, the Phase I/II GANNET53 trial was the first which employed this treatment concept to combat metastatic p53-mutant platinum-resistant ovarian cancer (PROC). As part of the Phase II GANNET53 trial, we investigated the use of liquid biopsy specimens that may contain circulating tumor cells (CTCs) to monitor response to therapy. The idea is based on earlier observations suggesting the clinical value of CTCs in patients with ovarian cancer.[8–15] In the present translational study we used the microfluidic Parsortix® technology to isolate cells by size and deformability from blood samples taken at baseline and at serial time points during treatment. In the isolated cells, we measured the expression of a targeted panel of genes and evaluated the possible applicability of these CTC-related transcripts in therapy monitoring.

2 | MATERIALS AND METHODS

2.1 | Patients and blood samples

Between May 2015 and September 2016, 133 patients were enrolled in the GANNET53 Phase II clinical trial (EudraCT 2013-003868-31;

NCT02012192; DRKS00005501). The main inclusion criteria were metastatic p53-mutant PROC, high-grade serous, endometrioid or undifferentiated ovarian carcinoma, and recurrent disease measurable according to RECIST 1.1 criteria[16] or assessable according to Gynecologic Cancer Intergroup (GCIG) CA-125 criteria,[17] and a maximum of four chemotherapy lines as previous treatment. Eligible patients were randomized in a 1:2 ratio to receive either Paclitaxel (P) or the combination of G and Paclitaxel (G + P; Figure S1A). Each 20 mL peripheral blood was collected by venepuncture in CellFree DNA BCT tubes (Streck) before administration of the study drug of Cycle 1 (C1D1, i.e., baseline sample), Cycle 2, and from Cycle 3 on every other cycle (C3, C5, C7, C9, etc.) until disease progression. Exclusively at Cycle 1, a second blood sample was taken 24 h after the first administration of the study drug (C1D2; Figure S1B). The primary endpoint of the GANNET53 clinical trial was progression-free survival (PFS) and PFS rates at 6 months.

2.2 | Tumor response assessment

Post-baseline assessments were performed every 8 weeks (±1 week) from the date of randomization and/or cohort assignment (e.g., on Day 1 of Cycle 3, 5, 7, 9, 11, etc.), independent of any treatment interruptions, using the same imaging technique as used during screening until disease progression. Response or progression was evaluated according to RECIST 1.1, CA-125 (GCIG criteria[17]) and by the investigators based on physical and/or gynecological examinations. Evidence of disease progression was based on radiological, clinical, or symptomatic evidence. CA-125 elevation alone was not scored as disease progression.

2.3 | Blood sample processing

Immediately after the blood draw, the samples were sent by overnight courier at ambient temperature to the Department of Obstetrics and Gynecology, Medical University of Vienna. There the samples were processed using a two-step protocol employing density gradient centrifugation and final microfluidic enrichment using the validated Parsortix® technology (ANGLE plc.) as described.[18] In short, the density gradient centrifugation was performed using 15 mL Percoll (GE Healthcare RRID:SCR_000004; $d = 1.065$ g/mL, 305 mOsm/kg) in Sepmate® tubes (STEMCELL Technologies) at 1350 × *g* for 20 min at 4°C with disabled brake. After centrifugation, the top layer above the separation barrier containing the enriched cells at the interphase to the plasma fraction was harvested. Further depletion of unwanted white blood cells was achieved by pressurizing the enriched cells through the Parsortix® GEN3D10 microfluidic separation cassette at 23 mbar (ANGLE plc.). The separation cassette was flushed with 350 µL RLT lysis buffer (Qiagen) to retrieve and lyse the captured cells. The lysates were stored at −20°C until RNA extraction.

2.4 | Gene selection

We previously performed a differential gene expression (DEG) analysis of peripheral blood mononuclear cells from healthy donors and established cancer cell lines[19] and tumor tissue from patients with primary ovarian cancer,[9] respectively. A final set of genes was defined after validating the resulting DEGs with quantitative polymerase chain reaction (qPCR); in addition, we included markers of epithelial cells (*EpCAM*, *CK19*, and *CDH1*), epithelial-to-mesenchymal transition (EMT; *CDH2*, *CDH3*, *CDH5*, *FN*, *VIM*, *ERBB2*, *ERBB3*) and drug resistance (*ERCC1*). *B2M* and *CDKN1B* were included to estimate RNA quality and quantity.

2.5 | Molecular analysis

Total RNA was extracted from cell lysates using the RNeasy Micro Kit (Qiagen) and reverse transcribed using the SuperScript VILO Mastermix (Invitrogen). After a target-specific pre-amplification, the transcript levels of the selected genes were analyzed in duplicates using the TaqMan® Universal Mastermix II and exon-spanning TaqMan® assays on the ViiA7 Real-Time PCR System (all from Applied Biosystems) employing standard thermal cycling parameters. A qPCR specific for *CK19* was performed at 65°C annealing/extension using published primer/probe sequences (Table S1).[20] Raw data were analyzed using the ViiA7 Software v1.1 with automatic threshold setting and baseline correction. From each duplicate, the mean cycle threshold (C_t) value was calculated. If the fluorescent signal did not reach the threshold in both duplicate reactions, the sample was regarded as negative, and so were samples with mean C_t values ≥35.0. A sample was regarded as positive for a specific gene transcript if the mean C_t value was <35.0. Samples with undetectable transcript levels of the reference genes *B2M* or *CDKN1B* in conjunction with high *VIM*-specific C_t values indicating low RNA quality or quantity were excluded from further analyses.

2.6 | Statistics

The association of the CTC-related gene markers with progressive disease (PD) and with the PFS rate at 6 months was assessed using a two-tailed Fisher's exact test. *p* Values were adjusted for multiple testing using the Bonferroni method. The sensitivity of each gene marker to detect PD was calculated by dividing the number of positive samples by the total number of samples taken at radiologically confirmed PD. Similarly, specificity was calculated as the proportion of negative samples among the sum of samples with controlled disease, namely partial remission (PR), stable disease (SD), or complete response (CR). The accuracy was calculated by dividing the sum of positive samples at PD and negative samples at controlled disease by the total number of samples.

To explore the association between gene expression and survival the landmark analysis approach was used, with the time point when

blood was sampled as landmarks. PFS and overall survival (OS) were defined as the days between blood draw and the date of documented PD or death of any cause. When survival or progression status could not be determined, survival data were censored at the last documented date for which the patient was alive or had no progression. The association of marker positivity and survival was assessed at every time point of blood draw using Kaplan–Meier curves and log rank (Mantel–Cox) tests. Survival analyses were performed using GraphPad Prism (RRID:SCR_002798) version 9.4.1. The level of significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Patients and blood samples

Of the 133 PROC patients, 90 were randomized into the G + P arm and 43 to the P arm. The median follow-up was 10.0 months, with confirmed progression in 116 (87.2%; G + P $n = 76$; P $n = 40$) cases.[21] However, the median PFS was not significantly different between the treatment arms (G + P 3.5 months, P 5.3 months; log rank $p = 0.16$). The PFS rate at 6 months was 22% (95% confidence interval [CI]: 14%–31%) in the G + P and 33% (95% CI: 20%–48%) in the P arm.[21] By the time of data lock and closure of the trial in December 2017, 95 (71.4%) patients had died.

After excluding samples from six patients who had withdrawn their consent and five samples with low RNA quality or quantity, a total number of 474 samples from 123 patients remained in the translational study (breakdown of samples see Table S2), with four samples (range 1–9) per patient on average. For each transcript, a sample was considered positive with a mean C_t value of <35.0. For *VIM* and *ERCC1*, the threshold for positivity was set at the median C_t value. For *TFF1* no further analysis was undertaken because most of the blood samples were *TFF1*-negative. At baseline, the prevalence of the remaining 26 gene transcripts ranged from 9.6% (*SCGB2A2*) to 71.9% (*ERCC1*), with a median prevalence of 32.5% (Table S1).

3.2 | Potential drug-related changes in gene expression

To determine whether a change in gene expression was related to the administration of the study drugs or, rather, to PD, we stratified the patients by the presence or absence of each marker at C1D1 and C1D2 into three groups; this was done for each treatment arm separately. The ratio of samples with stable (C1D1+/D2+), decreasing (C1D1+/D2−), or increasing (C1D1−/D2+) gene expression was equally distributed between the treatment arms. An exception was *PPIC*, but here the association did not reach statistical significance ($p = 0.051$; Table S3). Considering the lack of survival benefit in the G + P versus the P arm, in all subsequent analyses, the patients randomized in the G + P or P arms were pooled.

3.3 | Gene transcripts associated with disease progression

In an attempt to evaluate the potential role of CTC-related gene transcripts in monitoring the response to treatment, we compared the presence of each gene transcript in samples taken at radiologically confirmed PD or controlled disease. Tumor assessment was carried out before or within 4 weeks of blood sampling. A total of 159 samples from 90 patients met these criteria, with 22 being taken at C2, 67 at C3, 38 at C5, and 16 at C7. Nine samples were taken at even later time points or by unscheduled sampling at even-numbered treatment cycles. Patients were excluded when no blood sample close to the radiological tumor assessment was available, or when treatment was terminated after the first cycle (e.g., due to early progression) and in these cases, no radiological tumor assessment was performed. PD was detected shortly before or after the blood draw in 44 cases, and controlled disease (SD $n = 68$, PR $n = 45$, and CR $n = 2$) in 115 cases.

The sensitivity of all gene transcripts to detect PD is shown in Table S4. After adjusting for multiple testing only the presence of *ERCC1* and *ESR1* transcripts was significantly different between the samples taken at PD and those taken at controlled disease. *ERCC1* transcript levels were observed in 32 (72.7%) of the PD samples, but in only 62 (53.9%) of the non-PD samples (Fisher's exact test, $p = 0.032$). In contrast, elevated *ESR1* was found in 90 (78.3%) of the non-PD samples, but only in 26 (59.1%) of the PD samples (Fisher's exact test, $p = 0.018$), indicating that *ESR1* may be a marker of favorable prognosis.

3.4 | Predictive value of CTC-associated transcripts at baseline

Next, we compared the presence of each CTC-associated transcript at baseline (C1D1) in patients with PFS <6 months ($n = 89$) to patients with PFS ≥6 months ($n = 25$). Significant associations were found for *CDH1*, *ERCC1*, and *ESR1*. The presence of *ERCC1* transcripts at baseline was more frequently observed in patients who developed early progression (79.8% vs. 44.0%, chi-square $p < 0.001$). In contrast, the presence of *CDH1* and *ESR1* was less likely in this group of patients (41.6% vs. 68.0%, chi-square $p < 0.001$ and 50.6% vs. 80.0%; chi-square $p = 0.009$, respectively).

Baseline *ERCC1* was significantly associated with an increased risk of PD, confirming the previously identified association with early progression (Table 1). In contrast, *CDH1* and *ESR1* were related to a reduced risk of PD, and *SCGB2A2* and *LAMB1* with increased risk of death (Table 1).

3.5 | Prognostic value of CTC-associated gene transcripts during treatment

Next, we explored the potential prognostic value of the 26 gene transcripts at each treatment cycle, starting at C2. Blood

TABLE 1 Progression-free and overall survival stratified by the presence of *ERCC1*, *ESR1*, *CDH1*, *LAMB1*, and *SCGB2A2* at baseline.

Symbol	Patients at risk	Progression-free survival		Overall survival	
		HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
ESR1	65 (56.5%)	0.58 (0.39–0.86)	**0.002**	0.69 (0.45–1.07)	0.085
ERCC1	82 (71.9%)	1.75 (1.20–2.55)	**0.005**	1.55 (0.98–2.45)	0.081
CDH1	54 (47.4%)	0.66 (0.46–0.96)	**0.024**	0.79 (0.52–1.22)	0.287
SCGB2A2	11 (9.6%)	1.45 (0.70–2.99)	0.236	2.03 (0.84–4.92)	**0.031**
LAMB1	24 (21.1%)	1.22 (0.75–1.97)	0.384	1.63 (0.92–2.88)	**0.049**

Note: The hazard ratios (HRs) and 95% confidence intervals (CIs) are given as an odd of the patient with marker positivity at C1D1 for death or PD. *p* Values were calculated by the log rank (Mantel–Cox) test, $p < 0.05$ in bold.

TABLE 2 Hazard ratios for progression-free and overall survival of *ERCC1* or *ESR1* positive versus negative patients.

	Patients at risk	Progression-free survival		Overall survival	
		HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
ERCC1 at cycle					
C2	72 (72.7%)	1.83 (1.22–2.74)	**0.005**	2.32 (1.42–3.74)	**0.004**
C3	56 (71.8%)	2.22 (1.42–3.47)	**<0.001**	1.98 (1.13–3.48)	**0.032**
C5	29 (65.9%)	1.96 (1.08–3.56)	**0.028**	1.21 (0.56–2.62)	0.627
C7	5 (26.3%)	2.29 (0.61–8.55)	0.092	2.30 (0.61–8.72)	0.133
ESR1 at cycle					
C2	62 (62.6%)	0.63 (0.41–0.98)	**0.023**	0.76 (0.46–1.25)	0.255
C3	50 (64.1%)	0.61 (0.36–1.01)	**0.028**	0.75 (0.42–1.34)	0.297
C5	33 (76.7%)	0.37 (0.14–1.00)	**0.003**	0.82 (0.34–1.94)	0.626
C7	17 (89.5%)	0.93 (0.21–4.20)	0.922	3.91 (1.08–14.14)	0.077

Note: Gene expression levels were assessed at each treatment cycle, and the absolute numbers of positive samples at each cycle (patients at risk) are indicated. *p* Values were calculated with the log rank (Mantel–Cox) test, $p < 0.05$ in bold.
Abbreviations: CI, confidence interval; HR, hazard ratio.

samples taken at C9 or later were excluded due to small sample sizes. Kaplan–Meier analyses were performed for each CTC-related transcript using the time points when blood had been collected as landmarks. From C2 onwards, patients with *ERCC1* presence had approximately twice the risk of progression compared to *ERCC1*-negative patients. In contrast, the presence of *ESR1* was associated with about half the risk of progression (Table 2).

Of note, a significantly increased hazard for OS in *ERCC1*-positive patients was only observed at C2 and C3; at no point in time was a significant association of *ESR1* and OS observed. The hazards of reaching the endpoint at a given cycle of treatment are shown in Figures S2 and S3 for all 26 CTC-related transcripts.

3.6 | Combined *ERCC1* and *ESR1* gene transcripts to indicate disease progression

As *ERCC1* and *ESR1* were the only gene transcripts with significant association with PD (Table S4), we next examined the prognostic value of a combination of these two markers. We stratified the samples by the presence or absence of *ERCC1* and *ESR1* into four groups (i.e., *ERCC1*+/*ESR1*+, *ERCC1*+/*ESR1*−, *ERCC1*−/*ESR1*+, and *ERCC1*−/*ESR1*−). An *ESR1*-specific C_t value of 35 was chosen as the threshold for *ESR1* positivity, and the median *ERCC1*-specific C_t value of all samples was analyzed as the threshold for *ERCC1* positivity. Then, we evaluated the PFS of the respective patients by designating the time point of each blood draw as a landmark and analyzing only those patients who had not progressed up to that point. As shown by the Kaplan–Meier plots in Figure 1, PFS is significantly different between the four groups of patients at all landmark time points, with the *ERCC1*−/*ESR1*+ group surviving the longest without disease progression.

At baseline (Figure 1A), the patients are clearly separated into an *ERCC1*−/*ESR1*+ favorable ($n = 18$) and an unfavorable prognostic group comprising patients with either *ESR1* absence (*ERCC1*−/*ESR1*−, $n = 14$; *ERCC1*+/*ESR1*−, $n = 34$) or the simultaneous presence of both markers (*ERCC1*+/*ESR1*+, $n = 48$). In the 159 samples collected later in treatment, the unfavorable transcript combination was four times more likely in samples taken close in time to radiologically

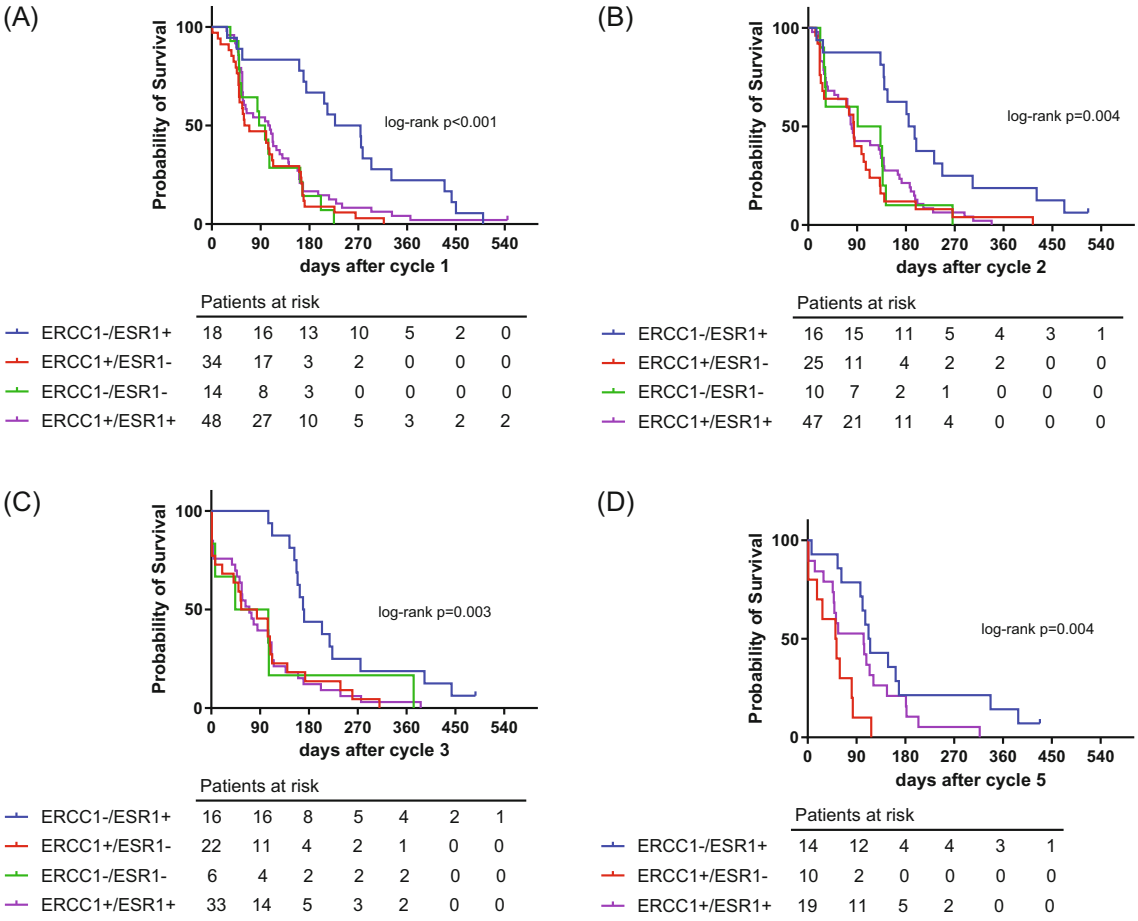

FIGURE 1 Kaplan–Meier plots showing the progression-free survival of patients stratified by *ERCC1* and *ESR1* gene expression at baseline (A), and at administration of cycle C2 (B), cycle C3 (C), and cycle C5 (D) of treatment. *p* Values were calculated by the log rank (Mantel–Cox) test.

TABLE 3 Prevalence of baseline samples with favorable and unfavorable combinations of *ESR1* and *ERCC1* transcripts before treatment starts.

	Favorable	Unfavorable			
	ERCC1−/ESR1+	***ERCC1+/ESR1−***	***ERCC1−/ESR1−***	***ERCC1+/ESR1+***	***p* Value**
PFS ≥ 6 months ($n = 25$)	12 (48.0%)	3 (12.0%)	2 (8.0%)	8 (32.0%)	**<0.001**
PFS < 6 months ($n = 89$)	6 (6.7%)	31 (34.8%)	12 (13.5%)	40 (44.9%)	

Note: The association with the PFS rate at 6 months was assessed using the chi-square test. $p < 0.05$ in bold.
Abbreviation: PFS, progression-free survival.

confirmed PD than to controlled disease (odds ratio [OR] 3.7, 95% CI: 1.5–8.9).

Finally, we investigated the combined predictive power of *ERCC1* and *ESR1* transcripts for early progression of the disease by re-evaluating the association of the unfavorable prognostic combination at baseline (*ESR1* absence or simultaneous presence of *ESR1* and *ERCC1*) with the PFS rate at 6 months. A chi-square test of independence showed that there was a highly significant association between these variables ($\chi^2(1,\ N = 114) = 27.0;\ p < 0.001$). At baseline, the favorable prognostic combination (*ERCC1−/ESR1+*) was observed in half of the 25 patients (48%) with PD later than 6 months after enrolment, but in just 6 (6.7%) patients with earlier progression of the disease (Table 3). Patients with an unfavorable transcript combination (*ESR1−* and/or *ERCC1+*) at baseline had significantly higher odds of early progression compared to those with a favorable transcript combination (OR 12.77, 95% CI: 4.08–39.97; $p < 0.001$).

Furthermore, we observed an increase of *ERCC1−/ESR1+* samples over the course of treatment, suggesting that patients who survived longer without progression (and thus received more cycles of treatment) were more likely to be *ERCC1−/ESR1+* (Figure 2A). In addition, these patients were very likely consistently *ERCC1−/ESR1+* from baseline throughout all treatment cycles (Figure 2B).

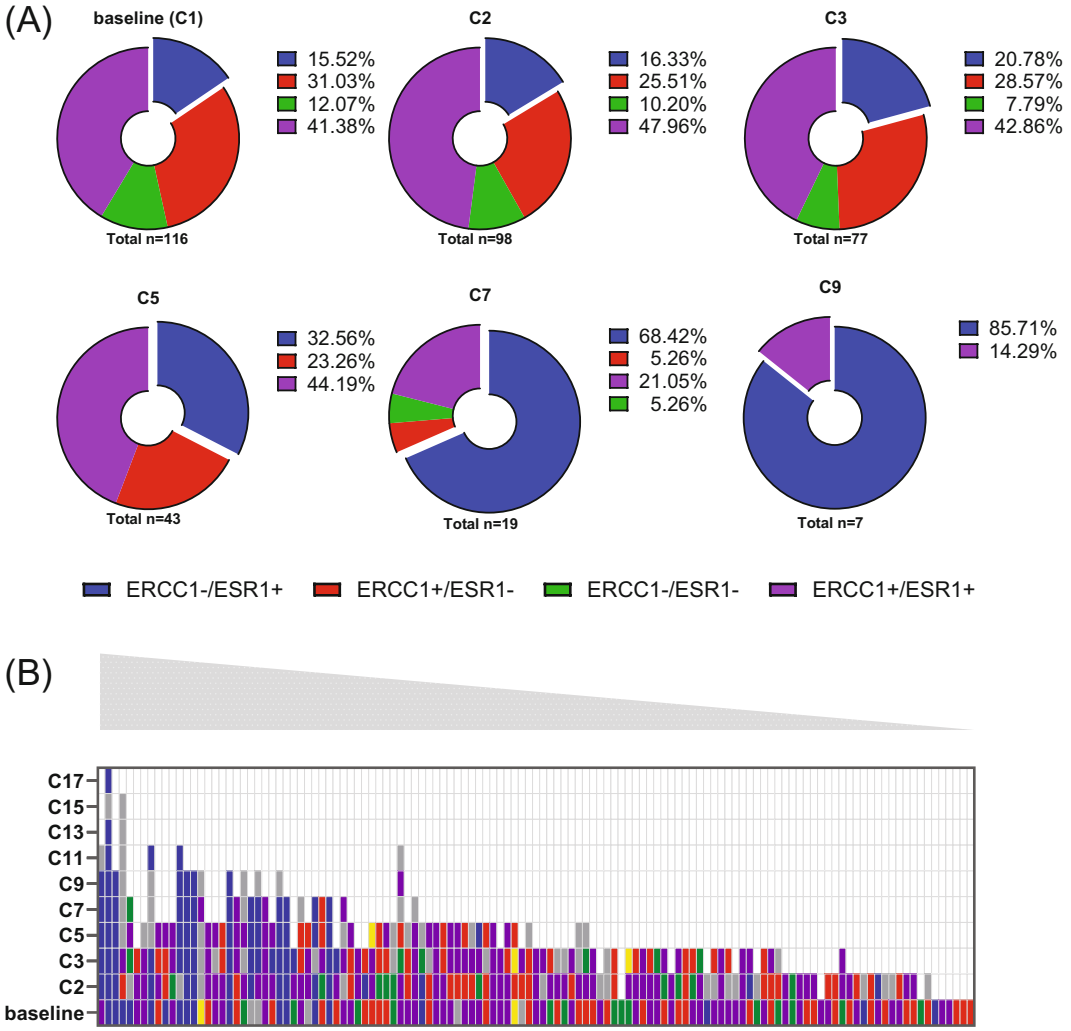

FIGURE 2 (A) The doughnut plots show the percentages of samples stratified by *ERCC1* and *ESR1* presence or absence at baseline and during treatment from cycle C2 to C9. (B) The heat map depicts the distribution of the four combinations of *ERCC1* and *ESR1* absence or presence for the 123 study patients, arranged from left to right with descending PFS (illustrated by the gray bar), and the treatment cycles in rows. Empty cells (white) represent a cycle treatment not administered, and gray cells represent treatment cycles without a corresponding blood sample available. Yellow cells represent samples excluded due to insufficient RNA quantity or quality.

4 | DISCUSSION

Although several studies have demonstrated the presence of CTCs in the peripheral blood of OC patients, the clinical importance of CTCs in this cancer type has long been underestimated.[8–15] A major difficulty not only with OC but also with other types of cancer is the extremely low number of CTCs. A second problem is the heterogeneity of these cells. In recent years, more and more evidence has accrued revealing the predictive value of CTC signatures under different treatment regimens.[22–26] The aim of the present study was to examine 27 CTC-associated gene transcripts for monitoring the response and prognosis of PROC patients enrolled in the GANNET53 clinical trial. To reach this aim, we compared the presence of the CTC-associated gene transcripts in blood samples taken at confirmed progression of the disease and in samples taken at CR, SD, or PR. Furthermore, we evaluated the association of each transcript with PFS. To the best of our knowledge, this is the largest study of CTCs in ovarian cancer in terms of the number of patient samples analyzed, assessing several defined longitudinal time points during treatment with G + P or with P alone. Our results underscore the value of CTC characterization at the molecular level, strongly suggesting that analysis of *ERCC1* and *ESR1* transcripts before treatment may identify patients with poor prognosis and can be useful in monitoring ovarian cancer patients.

In line with previous studies on CTCs in OC patients, the presence of *ERCC1* transcripts in a blood cell fraction enriched for CTCs was associated with poor patient outcome.[27,28] In the present study, *ERCC1* was significantly more often observed in samples taken at radiologically confirmed PD than at controlled disease. Furthermore, high *ERCC1* transcript levels were associated with an approximately twofold risk of developing PD. That association was statistically significant for *ERCC1* levels at baseline (before treatment) and at every time

point throughout treatment, except at the start of Cycle C7, when the observed difference was no longer statistically different at the 0.05 level, likely due to the small sample size ($n = 19$) at C7. The sensitivity of *ERCC1* to detect PD confirmed by imaging diagnostics was 72.7%, while the specificity of 46.1% was only moderate due to the presence of *ERCC1* transcripts in almost half the samples with confirmed controlled disease. There are several possible explanations for the moderate specificity. A possible explanation relates to the *ERCC1* gene functions and the selection criteria of the GANNET53 trial. *ERCC1* is a key gene of the nucleotide excision repair pathway and is essential for the removal of platinum-induced DNA damage. As the GANNET53 patient cohort comprises platinum-resistant (PROC) patients after receiving multiple lines of treatment, it seems plausible that only about one third of the baseline blood sample were *ERCC1*-negative (Figure 2A,B). The majority of the PROC patients were found to be *ERCC1*-positive at baseline, being twice as likely to develop PD on study treatment compared to *ERCC1*-negative patients. Alternatively, the moderate specificity of *ERCC1* gene expression to detect PD may be explained by the fact that a complete response assessed at the same time point as the blood sample was taken occurred in only two samples, and thus an optimum "negative control" group for assessing specificity was not available. Finally, background *ERCC1* gene expression in leukocytes could have caused a certain number of false positive samples with controlled disease.

Clinical trials suggested that ovarian cancer patients with low *ERCC1* levels benefit preferentially from cisplatin-based chemotherapy.[29–32] In addition to ovarian cancer, the role of *ERCC1* in the mechanism of platinum resistance has been evaluated in other types of cancer, including head and neck cancer,[33] non-small cell lung cancer,[34,35] and gastrointestinal cancer.[36] In liquid biopsy samples, the clinical relevance of *ERCC1* has been investigated in ovarian cancer CTCs[27] and furthermore, in extracellular vesicles isolated from blood plasma in metastatic breast cancer patients.[37] Kuhlmann et al. observed that primary OC patients with *ERCC1*-positive CTCs at baseline had a significantly worse OS and a more than fivefold risk of platinum resistance.[27] It should be noted that the association of *ERCC1* positivity and outcome was not confirmed in the tumor tissue in that study, which again points to the importance of liquid biomarkers in monitoring and therapy decision-making of OC patients at primary diagnosis.

A second major finding of our study is the association between *ESR1* transcripts and improved patient outcomes. In general, the expression of ERα, which is encoded by the *ESR1* gene, is linked to increased proliferation and metastasis and, thus poor patient outcomes. Furthermore, ERα mediates the downregulation of epithelial traits such as E-cadherin,[38] thereby fostering EMT of cancer cells, which in turn facilitates migration and invasion to distant sites. In CTCs, ERα protein expression was found to be very heterogeneous and only loosely consistent with the corresponding protein expression in the primary or metastatic tumor.[39] In breast cancer, ERα has been reported to be associated with the capacity of DNA repair,[40] but the crosstalk between ER signaling and the nucleotide excision repair pathway has not been fully elucidated. *ESR1* is involved in the regulation of many of the CTC-associated genes investigated in the present study, and, therefore, may indeed play an important role in OC.

In line with other studies, we found a positive association of *CDH1* (E-cadherin, a marker of epithelial integrity) at baseline and survival. *CDH1* is upregulated in epithelial cells and downregulated by EMT and was found to be positively associated with good prognosis in colorectal cancer patients.[41] In lung cancer cell lines, Nakamichi et al. observed that G could induce E-cadherin protein expression, revert EMT, and thereby overcome acquired resistance to treatment.[42] One of the few studies examining the immediate phenotypic change in CTCs caused by chemotherapy found that CTCs with EMT-like features disappeared 8 days after treatment with eribulin, a non-taxane inhibitor of microtubule dynamics, but re-appeared at disease progression, while the number of epithelial-like CTCs was not altered.[43] In that study, immunofluorescent staining of the various cell populations allowed not only an enumeration, but also an allocation of concomitantly existing subpopulations. A limitation of our study and of many molecular studies in general is that it is not possible to discern between subpopulations of cells, because all cells are lysed and all analytes are pooled. Future work should undertake a close analysis of EMT and its reversion due to drug administration in liquid biopsy samples using single-cell RNAseq techniques.

Despite of the two-step protocol employing density gradient centrifugation and final microfluidic enrichment using the validated Parsortix® technology,[44] we observed almost half of the baseline samples being positive for the epithelial markers *EpCAM* and/or *CK19*. In our previous study evaluating several types of pre-enrichment procedures, the recovery rate of several ovarian cancer cell lines spiked into blood samples was about 20%.[18] Since the GANNET53 study used blood collection tubes with a cell preservative, a higher yield is plausible because the preservative leads to a higher stiffness of cells and, thus, to a higher capture rate by the microfluidic system. This assumption is also based on our recent study comparing blood collection tubes with and without preservatives, showing an about 1.5-fold increased recovery in CellFree DNA BCT tubes.[45]

An important issue in molecular analyses is the use of a reference gene to normalize the data, and the presence of non-detects, here defined as mean C_t values ≥35.0. In the present study, the transcript levels were not normalized to reference genes because contaminating leukocytes would have introduced a bias by restraining positive findings in samples containing just a few CTCs and a relatively large number of leukocytes. In previous studies, we observed that It is widely accepted that CTCs interact with blood cells and that they "never travel alone."[46] As the 27 CTC-related gene transcripts were selected due to their absence in blood cells, it was not surprising that most transcripts (except *ERCC1* and *VIM*) were indeed absent in many samples. These reactions failing to produce a C_t value, so-called non-detects, usually pose a substantial issue in data analysis in general. Because the presence of non-detects in our study may indicate the absence of CTCs and thus is an important finding, we refrained from imputing missing values by computational approaches as suggested by Sherina et al.[47]

A final note on the GANNET53 clinical trial. Due to an unsecured drug supply and concerns about the efficacy of the tested drug combination, recruitment was prematurely closed with 133 patients

enrolled. Finally, the addition of G to P did not improve patient survival.[21] For this reason, the total number of blood samples was smaller than expected at baseline and at disease progression. For 28 patients in the study, the treatment ceased before disease progression occurred, and thus, in these cases, no blood samples at radiologically confirmed PD were available. The unexpectedly small sample size was also the reason why the landmark analysis evaluating the impact of gene transcripts on PFS was not done separately for each treatment arm. A further limitation of our study, is that we did not validate the results in an independent set of samples, and the lack of multivariate statistical analysis.

Within the limitations noted above, our findings indicate that in CTC-enriched liquid biopsies of PROC patients, the presence of *ESR1* transcripts together with the concurrent absence of *ERCC1* transcripts at baseline and before administration of each treatment cycle is predictive for improved PFS. Conversely, the presence of *ERCC1* is associated with disease progression and worse outcomes. Our findings support the analysis of liquid biopsy samples for the management of ovarian cancer patients. In particular, *ESR1* as a predictor of better prognosis is the most novel finding and may lead to translational insights into the biology of this deadly cancer as well as to new treatment strategies.

AUTHOR CONTRIBUTIONS

Eva Obermayr: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing – original draft. **Thomas Mohr:** Data curation; software; visualization; writing – review and editing. **Eva Schuster:** Investigation; methodology. **Elena Ioana Braicu:** Project administration; resources. **Eliane Taube:** Resources. **Jalid Sehouli:** Resources. **Ignace Vergote:** Resources; writing – review and editing. **Eric Pujade-Lauraine:** Resources. **Isabelle Ray-Coquard:** Resources; writing – review and editing. **Philipp Harter:** Resources. **Pauline Wimberger:** Resources; writing – review and editing. **Florence Joly-Lobbedez:** Resources. **Sven Mahner:** Resources; writing – review and editing. **Ute Martha Moll:** Investigation; writing – review and editing. **Nicole Concin:** Conceptualization; funding acquisition; project administration; resources; supervision. **Robert Zeillinger:** Conceptualization; funding acquisition; project administration; supervision; writing – review and editing. The work reported in the paper has been performed by the authors unless clearly specified in the text.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

ETHICS STATEMENT

This study was conducted in compliance with the GANNET53 clinical trial with approval from on-site Ethics Committees of the participating clinical sites. The analysis of CTC-related gene transcripts was approved by the ethics committee of the Medical University of Vienna (EK2266_2018). All patients gave written informed consent to participate in the translational study before blood collection.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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