ERCC1-Positive Circulating Tumor Cells in the Blood of Ovarian Cancer Patients as a Predictive Biomarker for Platinum Resistance

Jan Dominik Kuhlmann,1,2* Pauline Wimberger,1,2 Agnes Bankfalvi,3 Thomas Keller,4 Sarah Schöler,3 Bahriye Aktas,1 Paul Buderath,1 Siegfried Hauch,3 Friedrich Otterbach,3 Rainer Kimmig,1 and Sabine Kasimir-Bauer1

BACKGROUND: Platinum resistance constitutes one of the most recognized clinical challenges for ovarian cancer. Notably, primary tumor-based excision repair cross-complementation group 1 (ERCC1) detection by immunohistochemistry was recently shown to be inaccurate for the prediction of platinum resistance. On the basis of the previous finding that circulating tumor cells (CTC) in the blood of ovarian cancer patients are prognostically significant, and given our hypothesis that the negative prognostic impact of CTC may arise from a cellular phenotype associated with platinum resistance, we asked whether excision repair cross-complementation group 1 (ERCC1) expression in CTC may be a suitable blood-based biomarker for platinum resistance.

METHODS: The presence of CTC was analyzed by immunomagnetic CTC enrichment (n = 143 patients) targeting the epithelial epitopes epithelial cell adhesion molecule (EPCAM) (also known as GA733-2) and mucin 1, cell surface associated (MUC1), followed by multiplex reverse-transcription PCR to detect the transcripts EPCAM, MUC1, and mucin 16, cell surface associated (MUC16) (also known as CA125), including ERCC1 transcripts in a separate approach. ERCC1 expression in primary tumors was comparatively assessed by immunohistochemistry, using the antibody 8F1.

RESULTS: At primary diagnosis, the presence of CTC was observed in 14% of patients and constituted an independent predictor of overall survival (OS) (P = 0.041). ERCC1-positive CTC (ERCC1+ CTC) were observed in 8% of patients and constituted an independent predictor, not only for OS but also for progression-free survival (PFS) (P = 0.026 and P = 0.009, respectively). More interestingly, we discovered the presence of ERCC1+ CTC at primary diagnosis to be likewise an independent predictor of platinum resistance (P = 0.010), whereas ERCC1 expression in corresponding primary tumor tissue predicted neither platinum resistance nor prognosis.

CONCLUSIONS: The presence of ERCC1+ CTC can serve as a blood-based diagnostic biomarker for predicting platinum resistance at primary diagnosis of ovarian cancer.

* Address correspondence to this author at: Department of Gynecology and Obstetrics, Technische Universität Dresden, Fetscherstraße 74, D-01307 Dresden, Germany. Fax: +49-351-458-5844; e-mail jan.kuhlmann@uniklinikum-dresden.de.

Epithelial ovarian cancer accounts for the majority of tumor-related deaths among female malignancies and at primary diagnosis. Approximately 70% of all ovarian cancer patients already present with tumors at advanced stages (1). Standard treatment of advanced ovarian cancer constitutes primary surgery aiming at macroscopic complete tumor resection and subsequent platinum- and paclitaxel-based chemotherapy (2). So far, residual postoperative tumor load is one of the most important prognostic factors for the outcome of ovarian cancer. However, despite recent advances in treatment, more than 50% of all patients suffer from recurrent disease, resulting in worse overall prognosis (1). Importantly, resistance to platinum-based chemotherapy constitutes one of the most recognized clinical challenges for ovarian cancer. Resistance occurs in about 20% of patients and can be assessed only retrospectively within the follow-up period after adjuvant chemotherapy (3).

Functionally, platinum resistance can be caused by either increased tolerance toward DNA–platinum adducts or enhanced DNA repair capacity of tumor cells (4, 5). In this regard, the nucleotide excision repair
NER pathway is believed to be an essential mechanism for the repair of DNA-platinum adducts (5). In terms of the cellular NER, excision repair cross-complementing complementation group 1 (ERCC1) nuclease forms a heterodimer with XPR (xeroderma pigmentosum group A) and accomplishes repair of bulky DNA-platinum adducts (6–8). So far, the expression of excision repair cross-complementation group 1 [ERCC1; also replaces xeroderma pigmentosum, complementation group F (XPF)]\(^7\) has been extensively studied in primary tumor tissue of several cancer entities and has been proposed as a potential predictor for response to platinum-based chemotherapy. However, this concept has been controversial, particularly in the context of immunohistochemical ERCC1 detection, and has not yet been translated into clinical practice (9–19). A recent key publication in the New England Journal of Medicine reported on a comprehensive reevaluation study on 494 lung cancer patients and concluded that immunohistochemical ERCC1 detection in the primary tumor with all currently available antibodies is principally inappropriate for clinicians in terms of predicting platinum sensitivity and guiding therapy decisions (20). Given this discouraging finding, and considering that primary tumor tissue is uniquely available only by resection, it would be of clinical interest to establish a noninvasive blood-based biomarker for stratifying response to platinum-based chemotherapy at primary diagnosis and for guiding individualized therapy decisions.

Following our previous observation that circulating tumor cells (CTC) in the blood of ovarian cancer patients are predictive for a poor overall survival (OS) (21), and given our hypothesis that the negative prognostic impact of CTC may arise from a cellular phenotype, being associated with platinum resistance, we asked whether ERCC1-expressing CTC may be superior to commonly studied primary tumor-based ERCC1 detection in predicting response to platinum-based chemotherapy.

\(^{6}\) Nonstandard abbreviations: NER, nucleotide-excision repair; ERCC1, excision-repair cross-complementation group 1; CTC, circulating tumor cells; OS, overall survival; RT, reverse transcription; TMA, tissue microarray; FFPE, formalin-fixed and paraffin-embedded; PS, proportion score; IS, intensity score; TS, total Allred score; PFS, progression-free survival; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; OR, odds ratio.

\(^{7}\) Human genes: ERCC1, excision repair cross-complementation group 1 (also replaces xeroderma pigmentosum, complementation group F (XPF)); EPCAM, epithelial cell adhesion molecule (also known as GA733–2); MUC1, mucin 1, cell surface associated; MUC16, mucin 16, cell surface associated (also known as CA125); ACTB, actin, beta; PPIC, peptidylprolyl isomerase C (cyclophilin C).

**Materials and Methods**

**PATIENT CHARACTERISTICS**

The present study was conducted at the Department of Gynecology and Obstetrics at the University Hospital of Essen, Germany. In this study, a total of 143 patients with histologically confirmed epithelial ovarian cancer were enrolled. Informed written consent was obtained from all patients and the study was approved by the Local Research Ethics Committee (05–2870). The patients’ clinical data are summarized in Table 1. Tumors were classified according to the WHO classification of tumors of the female genital tract, grading was conducted using the grading system proposed by Silverberg (22), and tumor staging was classified according to the Fédération Internationale de Gynécologie et d’Obstétrique (23). The whole study population received primary radical surgery aiming at macroscopic complete tumor resection. Total abdominal hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy, peritoneal stripping, and pelvic as well as paraaortic lymphadenectomy were performed, where feasible. All patients received platinum-based chemotherapy. Tumors were clinically defined as platinum resistant if they recurred within 6 months after the completion of platinum-based chemotherapy.

**ENRICHMENT AND MOLECULAR CHARACTERIZATION OF CTC**

Peripheral blood was collected for CTC isolation with an S-Monovette\(^6\) (Sarstedt AG & Co.) and was processed within 4 hours after withdrawal. Blood samples were subjected to immunomagnetic tumor cell enrichment using the AdnaTest OvarianCancerSelect (AdnaGen AG). All experimental steps were performed, according to the manufacturer’s instructions. Briefly, epithelial cell adhesion molecule (EPCAM; also known as GA733–2)-positive and mucin 1, cell surface associated (MUC1)-positive cells were targeted, followed by RNA isolation and subsequent gene expression analysis by reverse-transcription (RT) and multiplex PCR, detecting the tumor-associated transcripts EPCAM, MUC1, and mucin 16, cell surface associated (MUC1; also known as CA125) (AdnaTest OvarianCancerDetect; AdnaGen AG). ERCC1 transcripts were assessed in a separate singleplex RT-PCR. PCR reactions were performed with the HotStarTaq Master Mix (Qiagen), using actin, beta (ACTB) as an internal positive control. PCR composition and cycling conditions of ERCC1 transcript detection exactly corresponded to the detection of the already established AdnaTest OvarianCancerDetect marker panel (15 min at 95 °C for initial activation, followed by 35 cycles of 30 s at 94 °C for denaturation, 30 s at 60 °C for annealing, 60 s at 72 °C for extension, and 10 min at 72 °C for termination). The primers generated amplicons with the fol-
lowing sizes: EPCAM, 395 bp; MUC1, 293 bp; MUC16, 432 bp; ERCC1, 366 bp; and ACTB, 114 bp.

**EVALUATION OF CTC POSITIVITY ACCORDING TO ADNATEST OVARIAN CANCER**

A given blood sample, processed with the AdnaTest OvarianCancer, was considered CTC positive if at least one of the tumor-associated transcripts EPCAM, MUC1, or MUC16 was detectable with an amplicon concentration above the indicated diagnostic threshold (>0.15 ng/µL). Analysis of the PCR fragments was carried out with the 2100 Bioanalyzer using the DNA 1000 LabChip kit and the Expert Software Package (version B.02.03.Si307; Agilent Technologies).

**IMMUNOHISTOCHEMICAL STAINING FOR ERCC1**

*ERCC1* expression in primary tumor tissues was analyzed by immunohistochemistry, using tissue microarrays (TMA). Routinely formalin-fixed and paraffin-embedded (FFPE) tissue blocks were retrieved from the Institute of Pathology and Neuropathology of the University Hospital of Essen, Germany. Hematoxylin- and-eosin–stained sections were prepared and reviewed by a pathologist. Tissue cores of 3 mm in size with the greatest possible intratissue tumor content (in most cases at least 60%) were punched from a pre-defined region of a given tumor block and assembled to a TMA block, each comprising 24 tumor samples. Subsequently, TMA-sections of 4-µm thickness were processed for *ERCC1* immunohistochemistry.

Immunohistochemical staining was performed with an automated staining device (Dako Autostainer; Dako), using a monoclonal mouse antihuman ERCC1–IgG2b antibody (Clone 8F1; Laboratory Vision). After deparaffinization of TMA sections, antigen retrieval was performed in 0.01 mol/L sodium citrate buffer at pH 6.0 for 20 min in a hot water bath (95 °C). Incubation with the primary antibody was carried out for 30 min at room temperature, using a dilution of 1:200. Secondary and tertiary immunoreactions were performed using a commercially available anti-mouse IgG detection kit (En-Vision, DakoCytomation). Normal tonsil tissue was used as positive control; replacement of the primary antibody with mouse immunoglobulin and omission of the primary antibody served as negative controls. ERCC1 positivity was graded by the Allred score, which is based on the percentage of stained tumor cell nuclei [proportion score (PS) ranging from 0 to 5] and staining intensity [intensity score (IS) ranging from 0 to 3]. The total Allred score (TS) was calculated by the sum of both scores (TS = IS + PS), ranging from 0 to 8. Samples with a TS of >2 were considered ERCC1 positive, whereas a TS of >6 indicated ERCC1 high positivity.

**STATISTICAL ANALYSIS**

Statistical analysis was performed using SPSS Statistics software 20.0 (IBM). To evaluate the clinical significance of CTC or ERCC1-positive CTC (ERCC1*+* CTC), univariable as well as multivariable regression analyses were performed. For univariable regression analysis, 3 independent analyses were performed in which progression-free survival (PFS), OS, or platinum resis-
tance were individually regressed by International Federation of Gynecology and Obstetrics (FIGO) stage, grading, residual tumor, CTC, or ERCC1 expression, respectively. In the case of multivariable regression analysis, 3 independent analyses for PFS, OS, or platinum resistance as the selected outcome variable in dependence on ERCC1 expression were performed and adjusted for the clinical parameters FIGO stage, tumor grading, and residual tumor. “Negativity” for ERCC1 expression was defined as ERCC1 expression, ERCC1 expression, or ERCC1 expression. Complete models were used to report hazard ratios (HR), odds ratios (OR), and related (2-tailed) P values. In case of significant findings, Kaplan–Meier analyses were performed to create survival curves.

Results

CTC Positivity in Patient Blood According to Established Adnatest OvarianCancer

From a cohort of 143 patients with epithelial ovarian cancer, preoperative blood samples obtained at primary diagnosis were subjected to immunomagnetic CTC enrichment and subsequent molecular CTC characterization, analyzing the transcripts of EPCAM, MUC1, and MUC16 (Adnatest OvarianCancer). CTC were detected in 20/143 patients (14%) and the presence of CTC was significantly associated with reduced OS (HR, 2.16; 95% CI, 1.22–3.84; P = 0.009) (Fig. 1), but not with PFS (HR, 1.50; 95% CI, 0.81–2.79; P = 0.199). Moreover, according to multivariable analysis, the presence of CTC was an independent predictor of OS (HR, 1.85; 95% CI, 1.03–3.32; P = 0.041).

Establishing ERCC1 Transcript Detection as a Novel CTC Marker

To establish an appropriate threshold for CTC-based ERCC1 detection, ROC curve plots were created to compare the CTC-derived ERCC1 signal in our patient samples to the corresponding ERCC1 signal in the blood of 21 healthy controls previously subjected to the AdnaTest OvarianCancerSelect (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue10). An amplicon threshold concentration of >0.2 ng/μL was defined as ERCC1 positive (ERCC1 expression), whereas a value <0.2 ng/μL was considered ERCC1 negative (ERCC1 expression). Notably, the selection of this threshold ensured the highly specific performance of our CTC-based ERCC1 detection by stratifying 100% of the evaluated healthy controls as ERCC1 negative.

ERCC1 expression CTC Predict Poor Prognosis

For our patient cohort, univariable analysis revealed that FIGO stage and residual tumor after surgery significantly correlated with PFS, OS, and platinum resistance (Table 2). Moreover, following multivariable analysis, FIGO stage and residual tumor burden after surgery were independent predictors of PFS, OS, and platinum resistance, whereas tumor grading constituted an independent predictor of PFS and platinum resistance (Table 3).

Considering ERCC1 transcripts as an additional CTC-associated biomarker, we intended to compile the presence of the established Adnatest OvarianCancer CTC marker with the presence of ERCC1 transcripts. In particular, we were interested in the incidence and clinical relevance of CTC being positive for at least one of the established Adnatest marker-transcripts (EPCAM, MUC1, or MUC16) and addi-
tionally for ERCC1 transcripts. Those CTC were herein defined as ERCC1⁺ CTC).

From the 143 preoperative blood samples processed by Adnatest OvarianCancer, cDNA of 120 patients was available for additional ERCC1 singleplex RT-PCR. ERCC1⁺ CTC were detected in 10/120 patients (8%). Following univariable analysis, the presence of ERCC1⁺ CTC correlated with decreased OS (HR, 2.18; 95% CI, 1.03–4.62; P = 0.042) (Table 2 and Fig. 2), but not with PFS (HR, 2.14; 95% CI, 0.92–4.98; P = 0.079). Moreover, multivariable analysis revealed the presence of ERCC1⁺ CTC at primary diagnosis to be an independent predictor of a poor PFS (HR, 3.4; 95% CI, 1.1–8.3; P = 0.009) and OS (HR, 2.5; 95% CI, 1.1–5.5; P = 0.026) (Table 3), whereas the presence of ERCC1 CTC was noninformative for PFS according to univariable and multivariable analysis.

ERCC1⁺ CTC PREDICT PLATINUM RESISTANCE

The next step was to inquire whether the detection of ERCC1⁺ CTC could serve as a blood-based biomarker for stratifying response to platinum-based chemotherapy. Using the Adnatest OvarianCancer, we revealed that the presence of CTC at primary diagnosis significantly correlated with platinum resistance in univariable analysis (OR, 3.00; 95% CI, 1.13–7.94; P = 0.027).

Notably, the presence of ERCC1⁺ CTC at primary diagnosis was likewise associated with platinum resistance, as determined by univariable analysis (OR, 5.79; 95% CI, 1.40–23.96; P = 0.015) (Table 2) and additionally constituted an independent predictor of platinum resistance, as revealed by multivariable analysis (OR, 8.5; 95% CI, 1.7–43.6; P = 0.010) (Table 3). Accuracy of ERCC1⁺ CTC detection for identifying platinum-resistant ovarian cancer patients was documented with a positive predictive value of 70% and a negative predictive value of 71%. Moreover, the presence of ERCC1⁺ CTC was still an independent predictor of platinum resistance when patients with serous histology were considered exclusively (OR, 11.3; 95% CI, 1.3–95.7; P = 0.026).

ERCC1⁺ CTC DETECTION IS SUPERIOR TO PRIMARY-TUMOR-BASED ERCC1 DETECTION IN PREDICTING PLATINUM RESISTANCE

We intended to relate clinical relevance of our CTC-based ERCC1 assay to the commonly studied primary-tumor-based ERCC1 detection (20). Out of the 120 patients studied for ERCC1⁺ CTC, corresponding primary tumor tissue (FFPE) was available in 77 cases and subjected to comparative immunohistochemical ERCC1 detection. Interestingly, in 2/77 patients (3%), concordant ERCC1 positivity in CTC and corresponding primary tumor was observed, whereas 44/57 patients

### Table 3. Multivariable analysis to evaluate clinical relevance of ERCC1⁺ CTC with regard to the patient’s survival and platinum resistance.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cox regression PFS</th>
<th>Cox regression OS</th>
<th>Log regression platinum resistance*</th>
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<tbody>
<tr>
<td></td>
<td>Independent HR 95% CI</td>
<td>Independent HR 95% CI</td>
<td>Independent OR 95% CI</td>
</tr>
<tr>
<td>FIGO stage</td>
<td>P &lt; 0.0005 16.6 3.7–74</td>
<td>P = 0.011 14.1 1.9–108</td>
<td>P = 0.021 14.0 1.5–131</td>
</tr>
<tr>
<td>Grading</td>
<td>P = 0.043 0.57 0.33–0.98</td>
<td>P = 0.561 0.85 0.49–1.48</td>
<td>P = 0.009 0.27 0.10–0.72</td>
</tr>
<tr>
<td>Residual tumor</td>
<td>P = 0.030 1.8 1.1–3.0</td>
<td>P &lt; 0.0005 3.2 1.9–5.7</td>
<td>P = 0.021 3.1 1.2–8.0</td>
</tr>
<tr>
<td>ERCC1⁺ CTC</td>
<td>P = 0.009 3.4 1.4–8.3</td>
<td>P = 0.026 2.5 1.1–5.5</td>
<td>P = 0.010 8.5 1.7–43.6</td>
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* Platinum resistance status for 9 patients was unknown.
(57%) were negative for ERCC1 expression in both compartments. Moreover, in 25/77 patients (33%), ERCC1 positivity was exclusively recorded in the tumor tissue but not in CTC, whereas in 6/77 patients (8%), ERCC1 positivity was exclusively observed in CTC but not in corresponding tumor tissue. The overall concordance rate of ERCC1 expression in these 2 compartments was 60% [resulting from concordant ERCC1 expression in 46/77 patients (see online Supplemental Table 1)], and the Cohen’s kappa coefficient was -0.055 (95% CI, -0.214 to 0.104).

Next, we investigated whether ERCC1 expression in primary ovarian cancer tissue is clinically significant and may predict prognosis or platinum resistance. However, considering that a statistically substantiated absence in only 77 patients was not feasible, we extended the ERCC1 analysis with primary tumor tissues. Moreover, in the present study we successfully established a CTC-based RT-PCR assay for ERCC1 expression analysis, intending to complement the previous marker panel and to extend clinical utility of molecular CTC characterization to the most recognized clinical challenge for ovarian cancer, the detection of platinum-resistant disease. Notably, ERCC1+CTC at primary diagnosis were superior in predicting the patient’s prognosis than ERCC1−CTC, by additionally constituting an independent predictor of a poor PFS. The prognostic relevance of ERCC1+CTC is principally in accordance with a recent very small pilot study analyzing 17 patients with metastatic non–small-cell lung cancer who were receiving platinum-based chemotherapy (36). Increased ERCC1 protein expression in the patient’s CTC was associated with reduced PFS. However, this pilot study did not resolve any association between CTC-derived ERCC1 expression and the patient’s response to platinum-based chemotherapy (36). In this context, our findings support the idea that the additional evaluation of CTC-based ERCC1 transcripts in ovarian cancer patients significantly improves the prognostic impact and clinical utility of CTC as a blood-based biomarker for stratifying prognosis.

Most interestingly, we revealed the presence of ERCC1+CTC at primary diagnosis as an independent predictor of platinum resistance, with a reasonable predictive value. In this context, a recent comprehensive study of the OV CAD (Ovarian Cancer Diagnosis) consortium, analyzing a cohort of 216 patients, found that peptidylprolyl isomerase C (cyclophilin C) (PPIC)-positive CTC were significantly more abundant in platinum-resistant than in platinum-sensitive patients (32). Importantly, however, this observation exclusively referred to the follow-up situation after accomplished chemotherapy and not to primary diagnosis. To the best of our knowledge, we provide the first report suggesting a prospective CTC-based biomarker.
for the detection of platinum resistance at primary diagnosis of ovarian cancer. Moreover, given that ERCC1 + CTC retain this predictive capacity for platinum resistance, when only patients with serous tumors were considered, our findings may likewise be representative for the major histologic subtype of ovarian cancer. These findings are of high clinical significance because they show that the additional characterization of ERCC1 transcripts renders CTC a liquid biomarker for the stratification of platinum-resistant patients before the initiation of chemotherapy. In this regard, the detection of ERCC1 + CTC could aid clinicians in guiding individual therapy decisions by minimally invasive blood collection. Once platinum resistance is predicted, an alternative therapeutic strategy could be scheduled, preventing the patient from unnecessary systemic toxicity and side effects of platinum-based chemotherapy.

We confirmed the superiority of CTC-based ERCC1 evaluation in predicting response to cisplatinum therapy compared to commonly studied primary tumor-based ERCC1 detection. Notably, we observed that corresponding ERCC1 expression in primary tumor tissue predicted neither prognosis nor platinum resistance. These findings were not surprising because they agree with the results from a publication reporting on the discouraging finding that immunohistochemical ERCC1 detection in the primary tumor, with all currently available antibodies, constitutes an inappropriate diagnostic tool for predicting platinum sensitivity and for guiding therapy decisions (20). Moreover, we observed virtually no concordance between ERCC1 positivity in blood vs primary tumor, which was also reported in a recent study on advanced breast cancer, in which poor correlation between ERCC1 expression between the primary tumor vs CTC and in the primary tumor vs biopsied metastatic sites was reported (37). These findings indicate that ERCC1 detection in these 2 compartments may capture “orthogonal” snapshots due to the different biological behavior of primary tumor vs blood-derived tumor cells and may provide complementary and independent clinical information on prognosis and platinum sensitivity. However, this interpretation must be used with care, because ERCC1 expression in CTC and primary tumor tissue was analyzed with different methods in our study, and therefore the lack of concordant ERCC1 expression could also be due to differing analytical sensitivities of the applied methodologies.

Given our present experimental framework, we cannot distinguish whether an ERCC1 + CTC event is derived from a coexisting population of “AdnaTest-positive” CTC and separately present ERCC1-positive CTC or whether it is derived from CTC with combined marker positivity in the same cell. However, we can hypothesize that ERCC1-(over)expressing CTC in the blood may be characterized by an enhanced capacity to resolve DNA-platinum adducts, consequently bypassing cisplatinum-mediated cytotoxicity and possibly converting to the well-known molecular phenotype of “on-target” platinum resistance (5). Consecutively, these cells may survive multiple cycles of chemotherapy and, in line with the fact that metastasis-initiating cells are present among CTC in the blood (38), ERCC1 + CTC may be responsible for platinum-resistant recurrence, metastasis, and poor prognosis. Importantly, our data also indicate the presence of a clinically informative subpopulation of CTC at primary diagnosis, possibly providing innate platinum resistance and expressing a certain ERCC1 level without any previous contact with cisplatinum.

This is the first report proposing a noninvasive biomarker for stratifying response of ovarian cancer patients to platinum-based chemotherapy. This CTC-based biomarker constitutes a promising blood-based alternative to the commonly suggested primary tumor-based ERCC1 detection for guiding therapeutic decisions at primary diagnosis of ovarian cancer. Due to the limited number of patients, the present study should be considered explorative and our results need to be validated in larger patient cohorts.

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References


