Serum calretinin as an independent predictor for platinum resistance and prognosis in ovarian cancer

Theresa Link\textsuperscript{1,2,3}, Simon Passek\textsuperscript{1,2,3}, Pauline Wimberger\textsuperscript{1,2,3}, Kerstin Frank\textsuperscript{4}, Yana Damyanova Vassileva\textsuperscript{1,2,3}, Michael Kramer\textsuperscript{5} and Jan Dominik Kuhlmann\textsuperscript{1,2,3}

\textsuperscript{1}Department of Gynecology and Obstetrics, Medical Faculty and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany
\textsuperscript{2}National Center for Tumor Diseases (NCT), Dresden, Germany; German Cancer Research Center (DKFZ), Heidelberg, Germany; Faculty of Medicine and University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany, Helmholtz-Zentrum Dresden-Rossendorf (HZDR), Dresden, Germany
\textsuperscript{3}German Cancer Consortium (DKTK), Dresden and German Cancer Research Center (DKFZ), Heidelberg, Germany
\textsuperscript{4}DKK-Blood Donor Service, ITM Plauen, Plauen, Germany
\textsuperscript{5}Medizinische Klinik und Poliklinik I, Medical Faculty and University Hospital, Technische Universität Dresden, Dresden, Germany

Calretinin (CRT) is a calcium-binding protein that controls intracellular calcium signaling. Besides its prominent expression in neurons, serum CRT (sCRT) has recently been suggested as blood-based biomarker for prediagnostic mesothelioma detection. CRT is expressed in ovarian cancer tissues in up to 40\% of cases; however, its clinical relevance as blood-based biomarker for ovarian cancer is unknown. sCRT was determined by calretinin enzyme-linked immunosorbent assay (Calretinin-ELISA, DLD Diagnostika GmbH, Hamburg, Germany) in a total of 515 serum samples from 116 healthy controls and 134 ovarian cancer patients (thereof 86\% with Fédération Internationale de Gynécologie et d’Obstétrique [FIGO] III/IV), including samples at primary diagnosis and at four longitudinal follow-up time points in the course of treatment and at recurrence. sCRT level was significantly increased in ovarian cancer patients compared to healthy controls (estimated difference = 0.3 ng/ml, \( p < 0.001 \)), was mostly independent from CA125 (\( r = 0.388 \)) and enabled accurate discrimination between cases and controls (area under the curve = 0.85). Higher sCRT level at primary diagnosis predicted suboptimal debulking (\( p < 0.001 \)) and was associated with advanced FIGO-stage (\( p < 0.001 \)) and increased amount of ascites (\( p < 0.001 \)). sCRT levels at primary diagnosis and its dynamics in the course of chemotherapy were independent predictors for poor progression-free survival (hazard ratio [HR] = 1.99, confidence interval [CI] = [1.13–3.52], \( p = 0.0181 \)) and overall survival (HR = 15.4, CI = [1.92–124], \( p = 0.0099 \)). Furthermore, sCRT at primary diagnosis or a relative sCRT increase in the time interval between surgery and the onset of chemotherapy were both independent predictors of platinum resistance (OR = 4.99, CI = [3.50–16.001], \( p = 0.0016 \); OR = 2.41, CI = [1.37–6.026], \( p = 0.0271 \), respectively). This is the first study that suggests sCRT as liquid biopsy marker for independent prediction of platinum resistance and prognosis.

Introduction

Epithelial ovarian cancer is the leading cause of death among patients with gynecologic malignancies. At primary diagnosis, more than 70\% of ovarian cancer patients present with advanced disease. Standard treatment of advanced ovarian cancer consists of primary radical debulking surgery aiming at macroscopic complete tumor resection followed by platinum- and paclitaxel-based chemotherapy, which extends progression-free...
What’s new?

Calretinin is a calcium-binding protein with diagnostic implications in cancer, particularly in mesothelioma. This study shows that serum calretinin (sCRT) also has strong potential as a liquid biopsy marker in ovarian cancer. In ovarian cancer patients, sCRT level was found to be elevated at primary diagnosis. While sCRT levels declined following surgery and the initiation of platinum-based chemotherapy, baseline levels were reestablished upon disease recurrence. Moreover, sCRT level at primary diagnosis predicted both poor survival and platinum resistance. The findings support the incorporation of sCRT as an auxiliary marker in diagnostic and prognostic assessments of ovarian cancer.

Calretinin (CRT) was originally discovered in 1987 and was named based on its structural similarity with calbindin-1 and its initial site of characterization, the chicken retina.10 CRT belongs, together with calbindin and secretagogin, to the “EF-hand family” of calcium binding proteins, which contain a helix–loop–helix motif that can act as calcium-binding site.11 Human CRT has a molecular weight of approximately 31 kDa and is predominantly located in the cytosol. It contains six helix–loop–helix domains (also referred to as EF-hand motifs), five of which are able to bind Ca2+ ions.12 Cellular functions of CRT are closely related to calcium signaling. CRT can act as a calcium buffer, a calcium sensor or a modulator of neuronal excitability.13,14 It has a broad tissue distribution and is expressed in a subset of neurons of the central and peripheral nervous system.12,15 However, physiological CRT expression has been observed in nonneural cells, particularly mesothelial cells, but also in adipocytes or ovarian stroma and during embryonic development.15–17

CRT is expressed in several epithelial and nonepithelial tumor entities, especially in mesotheliomas, in which it is used as an immunohistochemical marker to differentiate mesotheliomas from adenocarcinomas.18 The diagnostic impact of CRT for mesothelioma has recently been transferred to a liquid biopsy approach, in which plasma CRT was suggested as blood-based marker for mesothelioma detection, particularly in a prediagnostic setting.19,20 Among malignancies of the female genital tract, CRT expression has commonly been reported in sex cord stromal tumors with an expression rate ranging from 40% up to 100%.19 There is evidence that CRT is variably expressed in ovarian cancer, with highest prevalence among serous histotypes (0–40%) followed by endometrioid subtypes (0–33%) and clear cell carcinomas (0–14%).15

In contrast to mesothelioma, clinical relevance of CRT as potential blood-based biomarker for ovarian cancer is completely unknown. Therefore, we interrogated (i) whether we can detect serum CRT (sCRT) in ovarian cancer patients at a level that significantly differs from healthy controls and (ii) whether sCRT level at primary diagnosis and in the course of surgery and adjuvant chemotherapy could potentially serve as a predictive (and/or prognostic) liquid biopsy marker for ovarian cancer.

Patients and Methods

Patient characteristics

The present study was conducted at the Department of Gynecology and Obstetrics at the Carl Gustav Carus University of Dresden, Technische Universität Dresden, Dresden, Germany. In total, 134 patients with histologically confirmed primary epithelial ovarian cancer and adjuvant treatment were included. Informed written consent was obtained from all patients, and the study was approved by the Local Research Ethics Committee in Dresden (EK74032013). The patients’ clinical data are reported in Supplementary Table S1. Tumors were classified in line with the WHO-classification of tumors derived from female genital tract. Grading was conducted using the grading system established by Silverberg21 and tumor staging was classified according to the Fédération Internationale de Gynécologie et d’Obstétrique (FIGO),22 which was revised in 2014.23 The revised version of the FIGO classification was used for all patients, who underwent primary surgery in 2014 or later. The whole study population received primary radical surgery aiming at macroscopic complete tumor resection followed by platinum- and paclitaxel-based adjuvant chemotherapy. Bevacizumab, which is approved for patients with a tumor stage of at least FIGO IIIb, was additionally administered to 91/134 patients (68%).

Healthy donors

In total, 116 female healthy individuals without any history of benign or malignant disease were recruited for obtaining control serum samples. Informed written consent was obtained from all donors, and the study was approved by the Local
Research Ethics Committee in Dresden (EK74032013). There was no correlation between sCRT and age as confirmed by our own data set ($p = 0.15$) and by previous studies. Nevertheless, individuals for blood donation were selected in an age range matching the included ovarian cancer patients, with the majority of donors ranging between 50 and 75 years. Furthermore, serum preparation of control samples was performed with exactly the same protocol as for the patient samples to ensure comparability.

**Serum preparation**

After blood withdrawal with a 7.5 ml S-Monovette® (Sarstedt AG & Co., Nümbrecht, Germany), blood samples were incubated at room temperature for at least 30 min to allow complete blood coagulation. Within 1 hr after blood drawing, serum was prepared by centrifugation for 8 min at 1,800g at room temperature. The obtained cell free serum fraction was immediately frozen at $-80^\circ\text{C}$ until further processing. Unnecessary freeze-thaw cycles were avoided. Samples were blinded so that neither time of blood drawing nor any other information was disclosed during the investigation. Samples were thawed on ice and were immediately processed after complete thawing.

**Detection of sCRT**

Concentrations of sCRT were determined as described by Johnnen et al., using the commercially available Calretinin enzyme-linked immunoabsorbent assay kit by DLD Diagnostika GmbH. The assay is based on polyclonal antibodies developed by Raiko et al. All reagents and samples were equilibrated to room temperature ($20\text{–}25^\circ\text{C}$) and the incubations were performed at room temperature ($20\text{–}25^\circ\text{C}$) using a plate shaker. Serum samples ($2 \times 15 \mu\text{l}$) were diluted 1:5 in the provided dilution buffer. The diluted samples were determined in duplicate. Details of the procedure are outlined in the manufacturer’s instructions. Optical densities at 450 nm were measured by a photometer (Molecular Devices, Sunnyvale, CA). A standard curve was obtained by four-parameter curve fitting using SoftMax Pro 4.0 from Molecular Devices.

**Statistical analysis**

The statistical analysis was conducted with R, Version 3.5.1 and GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla, CA). $p$-Values $<0.05$ were considered statistically significant. All confidence intervals (CIs) were specified as 95% CI. Nonparametric two-sided Mann–Whitney $U$-test was used to compare sCRT levels of ovarian cancer patients at primary diagnosis with those of healthy controls. Using receiver operating characteristic (ROC) curve analysis, we assessed the capacity of sCRT concentrations to discriminate between ovarian cancer patients and healthy controls. By Spearman’s rank-order test, the correlation between sCRT and CA125 was evaluated. For analyzing sCRT level change in the course of treatment, two-sided Wilcoxon rank-sum tests for paired data were applied. Nonparametric two-sided Mann–Whitney $U$-tests were used to analyze the associations of sCRT and clinicopathological parameters. Uni- and multivariable Cox regression analyses were performed to study the prognostic relevance of sCRT in ovarian cancer. For all Cox regression analyses, respective hazard ratios (HRs) are indicated. In order to assess prognostic characteristics of sCRT for early recurrences, multivariable logistic regression analysis was applied and odds ratios (ORs) are provided. Firth’s bias-reduced logistic regression analysis was used to determine whether sCRT can predict platinum resistance. Additionally, Kaplan–Meier analyses and log-rank tests were performed. All models were tested for OS and PFS as separate outcome variables. The correlation between sCRT levels at primary diagnosis and at recurrence was assessed by Spearman’s rank-order test. For all regression and Kaplan–Meier analyses, the cutoff determination for stratifying the patients either into a sCRT high or a sCRT low group (or sCRT-area under the curve [AUC]-high vs. sCRT-AUC-low) was conducted by Maximally Selected Rank Statistics. Cutoff selection was supported by a test for independence between the outcome variables OS/PFS and sCRT/sCRT-AUC.

**Data availability**

The article contains all relevant data, including the patient’s clinicopathological parameters. The original set of raw data will be made available upon reasonable request.

**Results**

**sCRT level is elevated in ovarian cancer and declines in the course of treatment**

We analyzed levels of sCRT in a comprehensive set of clinically documented ovarian cancer patients at primary diagnosis ($n = 133$) and compared it to healthy controls ($n = 116$). Moreover, we tracked sCRT in the course of primary surgery and adjuvant chemotherapy, represented by four additional longitudinal follow-up samples, obtained (i) one week after primary surgery ($n = 68$), (ii) before the onset of platinum-based chemotherapy ($n = 76$), (iii) after the third cycle of chemotherapy ($n = 55$) and (iv) after the completion of chemotherapy ($n = 48$).

sCRT was highly upregulated at primary diagnosis of ovarian cancer (estimated difference [ED] = 0.3 ng/ml, CI = [0.243–0.359], $p < 0.0001$ compared to healthy controls (Fig. 1). In patients with exclusively low-stage disease (FIGO I or II; $n = 19$), ROC curve analysis showed that sCRT at primary diagnosis enabled discrimination between ovarian cancer patients and healthy controls with an AUC of 0.67 (CI = [0.524–0.823]; Supplementary Fig. S1). In the total patient cohort, irrespective of FIGO stage, discrimination was possible with an AUC of 0.85 (CI = [0.803–0.900]; Fig. 2). Choosing 0.35 ng/ml sCRT as a diagnostic cut-off from ROC analysis, ovarian cancer was detected by sCRT in the total cohort with a sensitivity of 90% and a specificity of 74% (Supplementary Table S2).

After surgery, there was a strong and highly significant decline of sCRT (ED = 0.22 ng/ml, CI = [0.124–0.315], $p < 0.0001$) to a level, which remained grossly stable in the
course of platinum-based chemotherapy, however, which remained significantly higher than the sCRT levels of healthy controls. After the completion of chemotherapy, sCRT was still slightly increased compared to healthy controls (ED = 0.098, CI = [0.054–0.142], p < 0.0001; Fig. 1).

Conclusively, sCRT level is significantly elevated in serum of ovarian cancer patients at primary diagnosis, sharply discriminates ovarian cancer from controls and strongly declines in the course of surgery and adjuvant chemotherapy.

Higher sCRT level parallels advanced ovarian cancer and predicts suboptimal primary debulking

We correlated sCRT levels with the patient’s clinicopathological data. Higher levels of sCRT at primary diagnosis correlated with advanced disease, indicated by a higher FIGO stage (ED = 0.243 ng/ml, CI = [0.142–0.351], p < 0.0001) and an increased amount of malignant ascites (ED = 0.251 ng/ml, CI = [0.135–0.375], p < 0.0001, Figs. 3a and 3b). Furthermore, higher levels of sCRT at primary diagnosis were associated with the presence of a residual tumor burden left after primary debulking (ED = 0.215 ng/ml, CI = [0.109–0.321], p < 0.0001; Fig. 3c).

To sum up, higher sCRT levels at primary diagnosis of ovarian cancer correlate with advanced disease and predict suboptimal primary debulking surgery without achieving macroscopically complete tumor resection.

High sCRT level at baseline and in the course of treatment is an independent predictor of poor survival in ovarian cancer

In order to study the prognostic relevance of sCRT in ovarian cancer, we performed survival analysis at primary diagnosis and at all four follow-up time points in the course of surgery.
and adjuvant chemotherapy (Supplementary Tables S3–S8). According to univariable Cox and logistic regression analysis, we observed that high sCRT levels at primary diagnosis and before the onset of platinum-based chemotherapy indicated poor PFS (HR = 2.47, CI = [1.55–3.93], p = 0.0001; HR = 2.23, CI = [1.07–4.63], p = 0.032, respectively) and were associated with increased risk of recurrence <12 months after the completion of chemotherapy (OR = 4.69, CI = [1.94–12.2], p = 0.0099; OR = 5.33, CI = [1.52–25.2], p = 0.016, respectively). Moreover, high sCRT levels at primary diagnosis, after surgery, before the onset of chemotherapy and after completion of chemotherapy correlated with poor OS (HR = 2.49, CI = [1.27–4.87], p = 0.0078; HR = 5.35, CI = [1.25–22.9], p = 0.024; HR = 11.19, CI = [1.51–82.95], p = 0.0181; HR = 3.47, CI = [1.16–10.3], p = 0.0256, respectively; Supplementary Tables S3–S8). Prognostic relevance of sCRT, concluded from the analysis above, was additionally demonstrated by Kaplan–Meier analysis and the log-rank test (Fig. 4).

We subsequently performed multivariable Cox regression analysis with PFS or OS as separate outcome variables, including sCRT levels and established risk factors of ovarian cancer, that is, age at primary diagnosis, amount of malignant ascites, FIGO stage, tumor grading and residual tumor burden left after primary debulking. High sCRT levels at primary diagnosis were an independent predictor for a poor PFS (HR = 1.99, CI = [1.13–3.52], p = 0.0181). In addition, multivariable logistic regression analysis revealed that sCRT levels at primary diagnosis and before the onset of chemotherapy were independent predictors for increased risk of recurrence <12 months (OR = 3.33 CI = [1.18–10.0], p = 0.0263; OR = 8.00, CI = [1.25–74.0], p = 0.0409, respectively). Furthermore, high sCRT levels before the onset of platinum-based chemotherapy and after completion of chemotherapy were independent predictors of poor OS (HR = 15.4, CI = [1.92–124], p = 0.00995; HR = 5.59, CI = [1.23–25.4], p = 0.0260, respectively; Supplementary Tables S3–S8).

In conclusion, high sCRT levels in the course of surgery and adjuvant platinum-based chemotherapy is an accurate and independent blood-based predictor for poor survival in ovarian cancer. Its strongest prognostic significance was observed immediately before the onset of chemotherapy.

Individual sCRT dynamic in the course of treatment is an independent predictor for poor survival

From 48/134 patients, a complete set of longitudinal serum samples was available. In this context, we interrogated, whether the individual dynamics of sCRT across these samples is of prognostic relevance. Therefore, assuming a linear and continuous change of sCRT values between the investigated time points, we plotted a dynamic curve for each patient by interconnecting sCRT values from all longitudinal blood samples. Considering the different time points of blood...
drawing as categorical variables, we calculated an individual AUC for each patient, reflecting the individual sCRT curve in the course of surgery and adjuvant chemotherapy. Using the AUC value with the best discriminative power as cutoff (according to the R package “Maximally Selected Rank Statistics”; Supplementary Fig. S2 and Table S9), all patients were

Figure 4. Prognostic relevance of sCRT at primary diagnosis and in the course of adjuvant chemotherapy. Kaplan–Meier analysis comparing progression-free survival (PFS) and overall survival (OS) of patients with high sCRT levels versus patients with low sCRT levels (a) at primary diagnosis, (b) 1 week after primary surgery, (c) before platinum-based chemotherapy and (d) after completion of chemotherapy. p-Values and hazard ratio (HR) were calculated according to log-rank test. The sCRT cutoff for stratifying the patients into a sCRT high and low group was selected by Maximally Selected Rank Statistics as cutoff with the best discriminative power. [Color figure can be viewed at wileyonlinelibrary.com]
stratified into an "sCRT-AUC-high" group or into a "sCRT-AUC-low" group (Fig. 5). According to univariable Cox and logistic regression analysis, the sCRT-AUC-high condition indicated poor PFS (HR = 2.44, CI = [1.18–5.05], p = 0.016), increased risk of recurrence <12 months (OR = 3.78, CI = [1.10–14.3], p = 0.0403) and poor OS (HR = 8.76, CI = [1.93–39.8], p = 0.005; Supplementary Tables S10 and S11). Moreover, prognostic relevance of sCRT-AUC, as reported above, was additionally demonstrated by Kaplan–Meier analysis and the log-rank test (Fig. 5). According to multivariable Cox regression, the sCRT-AUC-high condition was an independent predictor for poor PFS (HR = 2.90, CI = [1.08–7.82], p = 0.035) and OS (HR = 7.38, CI = [1.17–46.6], p = 0.0336; Supplementary Tables S10 and S11).

Diagnostic capacity of the patient’s individual sCRT dynamics, as reported above, could similarly be reproduced when only the first three time points of serum collection (primary diagnosis, one week after primary surgery and before the onset of platinum-based chemotherapy; available from 66/134 patients) were considered for sCRT-AUC calculation (Supplementary Fig. S3, Tables S12 and S13).

We conclude that longitudinal sCRT values, considered separately at the given time points or in terms of individual sCRT dynamics, have a strong and independent prognostic relevance in ovarian cancer.

**sCRT level in recurrent ovarian cancer**

From 16 patients, corresponding serum samples at primary diagnosis and at first recurrence of ovarian cancer were available. Interestingly, patients with a relatively higher baseline sCRT level experienced sCRT decline due to surgery and adjuvant chemotherapy and later recovered similarly higher sCRT levels in the presence of a recurrent tumor (Figures 6a and 6b). In 3/16 patients, a further sample at second recurrence was available. In two of those patients, sCRT at second recurrence again matched the respective baseline level at primary diagnosis (Fig. 6a). There was as strong correlation between sCRT level at primary diagnosis and at first recurrence (r = 0.834, CI = [0.567–0.943], p < 0.0001; Fig. 6c).

To sum up, we demonstrate that sCRT levels significantly drop in the course of primary surgery and platinum-based chemotherapy but individual baseline levels reestablish at recurrence.

**sCRT level is an independent predictor for primary platinum resistance**

We interrogated, whether sCRT level (i) at separate individual follow-up time points or (ii) its dynamics in the course of surgery and chemotherapy may predict response to platinum-based chemotherapy (platinum resistance defined as PFS < 6 months). According to Firth’s bias-reduced logistic regression analysis, we revealed that sCRT level at primary diagnosis was highly predictive for platinum resistance (univariate: OR = 32.3, CI = [3.57–4.282], p = 0.0005), independently from established risk factors of ovarian cancer (multivariable: OR = 4.99, CI = [3.50–16.001], p = 0.0016; Supplementary Table S14). Additionally, a relative sCRT rise

---

**Figure 5.** Prognostic relevance of sCRT AUCs in the course of surgery and adjuvant chemotherapy. (a) Representative patient’s dynamic curve showing sCRT concentrations between primary diagnosis and the completion of adjuvant chemotherapy. The sCRT AUC cutoff for stratifying the patients into a sCRT-AUC-high and -low group was selected by maximally selected rank statistics as the sCRT AUC value with the best discriminative power. Kaplan–Meier analysis was carried out, comparing (b) progression-free survival (PFS) and (c) overall survival (OS) of ovarian cancer patients with sCRT-AUC-high versus sCRT-AUC-low. Hazard ratios (HR) and p-values, according to log-rank test, are indicated. [Color figure can be viewed at wileyonlinelibrary.com]
in the time interval between surgery and the onset of chemotherapy (sCRT before the onset of chemotherapy > 90%) predicted primary platinum resistant disease. This was independent from established risk factors of ovarian cancer and also independent from resistance prediction at primary diagnosis (OR = 2.41, CI = [1.37–6.026], p = 0.0271; Supplementary Table S14).
For comparison, there was no statistically significant association between CA125 values at primary diagnosis and platinum resistance.

Conclusively, we show that sCRT level at primary diagnosis and a postsurgery sCRT rise independently predict primary platinum resistance in ovarian cancer.

Comparison between sCRT and CA125

There was a weak correlation between sCRT and CA125 at primary diagnosis ($r = 0.367$, CI = [0.202–0.512], $p < 0.0001$), after the third cycle of chemotherapy ($r = 0.317$, CI = [0.0397–0.549], $p = 0.02$) and after the completion of chemotherapy ($r = 0.388$, CI = [0.0404–0.651], $p = 0.03$; Supplementary Fig. S4). After surgery and at the onset of chemotherapy, no correlation was observed ($r = 0.214$, CI = [−0.03–0.44], $p = 0.08$; $r = 0.134$, CI = [−0.1–0.35], $p = 0.26$). Therefore, assuming that both markers provide independent and possibly complementary diagnostic information, we were particularly interested in sCRT level in patients with low CA125. In total, matched sCRT and CA125 values at primary diagnosis were available from 128/133 patients (Supplementary Fig. S5). Of those, 20 patients had a relatively low CA125 value (<100 U/ml, 15th percentile of the CA125 range) and were selected for comparison. Interestingly, 50% of these patients (10/20) had a higher sCRT value than the majority of healthy controls (75%) and tended to have a higher FIGO stage.

Taken together, we report a low correlation between sCRT and CA125 at primary diagnosis and in the course of chemotherapy in ovarian cancer and identify a subgroup of patients with low CA125 but conspicuous sCRT level.

Discussion

In the present study, we systematically analyzed clinical relevance of sCRT in ovarian cancer and report as our key finding that sCRT level at primary diagnosis as well as its dynamics in the course of surgery and platinum-based chemotherapy are independent predictors for poor prognosis and platinum resistance.

sCRT was highly elevated at primary diagnosis of ovarian cancer and accurately discriminated cases from healthy controls (AUC = 0.85), which is comparable to a study on German and Australian mesothelioma patients, reporting on sCRT elevation in patient blood and a discrimination of cases and controls with an AUC of 0.83 and 0.91, respectively. We additionally reported that sCRT allows, albeit with less discriminative power, detection of particularly early disease (FIGO I-II; AUC = 0.67). However, this analysis was based on only 19 patients and does not allow a statistically substantiated conclusion. Considering sCRT as potential screening marker for ovarian cancer, our results encourage future investigation with independent patient cohorts enriched by FIGO I-II ovarian cancer patients (in the present study 86% had FIGO III or IV). Such an early detection approach has already been published for mesothelioma, reporting that sCRT (in combination with serum mesothelin) enabled prediagnostic detection of mesothelioma with an AUC of 0.74.

The biology and origin of CRT release into the bloodstream remains an open question. Since immunohistochemical studies on serous ovarian cancer reported variable CRT positivity in 0–40%, it is possible that CRT promotes growth and survival of ovarian cancer cells, as it has been shown in vitro for mesothelioma. In this scenario, CRT could possibly be released by tumor cells themselves, either by active secretion or by passive release upon tumor cell apoptosis, which is consistent with the generally high apoptotic index of malignant tumors. However, this is not necessarily the most likely way of interpretation, since CRT expression in epithelial ovarian cancer is by far lower than in sex cord stromal tumors (0–40% vs. up to 100%) and staining has often been described as rather focal than homogeneous. Moreover, global gene expression analysis in ovarian/peritoneal serous carcinoma (O/P-SC) versus diffuse peritoneal malignant mesothelioma (DMPM) revealed that CRT was overexpressed in DMPM but not in O/P-SC, suggesting that CRT could be (along a potential gene-expression signature) a marker for differentiating DMPM from O/P-SC. In breast cancer, for comparison, strong CRT expression was predominantly observed in high-grade tumors with basal-like phenotypes and paralleled poor prognosis. Moreover, an alternatively spliced form of CRT (CRT-22k) was detected in serum of breast cancer patients.

Considering that CRT is also highly expressed in mesothelial cells of the peritoneum, it is alternatively possible that elevated sCRT in advanced ovarian cancer patients is related to the tumor microenvironment within peritoneal metastases. In this context, increased CRT-release from mesothelial peritoneal cells could be due to either an “educational niche response,” in which ovarian cancer cells reprogram mesothelial cells toward an active CRT secretion or it could be due to an unspecific release of CRT from apoptotic/necrotic mesothelial cells, which are superseded by ovarian cancer cells.

High sCRT level at primary diagnosis predicted advanced (platinum resistant) disease and poor overall prognosis. After primary debulking, we observed a strong sCRT decline, approaching the level of controls. We therefore conclude that sCRT could be a sensitive blood-based biomarker for assessing individual tumor load and the patient’s initial risk profile at primary diagnosis, including platinum sensitivity status. Considering that higher sCRT level was predictive for inefficient primary debulking, the detection of this marker may additionally help to identify tumors with aggressive and complex growth patterns, which are more difficult to debulk. sCRT levels at primary diagnosis were mostly independent from CA125 and were elevated in a subset of patients with low CA125 and high FIGO stage, suggesting that sCRT is partly complementary to CA125 and could be useful as auxiliary tumor marker in ovarian cancer, particularly for those patients with CA125 failure.
The strength of our study is that we analyzed sCRT not only at primary diagnosis but also in a comprehensive serum set of four longitudinal follow-up samples per patient in the course of surgery, chemotherapy and at recurrence. We report that sCRT level provides independent prognostic information not only at primary diagnosis but also among longitudinal sampling in the course of surgery and chemotherapy. Therefore, sCRT could additionally be useful as blood-based biomarker for therapy monitoring, which identifies aggressive disease with high risk of recurrence and poor prognosis (particularly in patients with macroscopically complete resection). This hypothesis is further supported by the fact that sCRT predicted platinum resistance not only at primary diagnosis but also in patients, who showed a relative sCRT rise between surgery and the onset of chemotherapy. Therefore, post-surgery sCRT may indicate active disease, possibly associated with a platinum resistant phenotype, which is subsequently triggered by the directional selection pressure of chemotherapy, leading to the outgrowth of possibly resistant tumor cells.31 However, all cutoff values for survival analysis, reported herein, are exploratory and need to be validated in independent patient cohorts. Furthermore, we reported, that individual baselines of sCRT at primary diagnosis dropped after surgery and chemotherapy. In most cases, sCRT levels recovered to this individual baseline at recurrence. This points to unknown and patient intrinsic characteristics of CRT release, which recovers after chemotherapy and obviously remains stable across tumor evolution. Overall, our finding suggests that sCRT could be useful as blood-based biomarker for monitoring (early) recurrence.

Conclusion
This is the first study reporting that serum CRT (sCRT) has strong potential as liquid biopsy marker for ovarian cancer with a wide field of potential clinical application, particularly independent prediction of primary platinum resistance and prognosis. Along our previous liquid biopsy approaches in ovarian cancer, suggesting innovative biomarkers concepts on the level of circulating tumor cells or microvesicle-associated microRNAs,32,33 our results show clinical relevance of CRT as a single and easily detectable serum parameter, which could straightforwardly be implemented into routine diagnostics as a CA125 auxiliary tumor marker for improving personalized medicine in ovarian cancer. High-risk patients, as identified by sCRT, could potentially benefit from an intensified therapy regime, including PARP inhibitors or immunotherapy.

References

1. Goodman MT, Howe HL, Tung KH, et al. Incidence of ovarian cancer by race and ethnicity in the United States, 1992-1997. Cancer 2003;97:2676–85.
2. du Bois A, Quinn M, Thigpen T, et al. 2004 consensus statements on the management of ovarian cancer: final document of the 3rd International Gynecologic Cancer Intergroup Ovarian Cancer Consensus Conference (GGIG OCCC 2004). Ann Oncol 2005;16:vii7–vii12.
3. Karam A, Ledermann JA, Kim JW, et al. Fifth Ovarian Cancer Consensus Conference of the Gynecologic Cancer Intergroup: first-line interventions. Ann Oncol 2017;28:711–7.
4. Wimberger P, Wehling M, Lehmann N, et al. Influence of residual tumor on outcome in ovarian cancer patients with FIGO stage IV disease: an exploratory analysis of the AGO-OVAR (Arbeitsgemeinschaft Gynaekologische Onkologie Ovarian Cancer Study Group). Ann Surg Oncol 2010;17:1642–8.
5. Burger RA, Brady MF, Bookman MA, et al. Incorporation of bevacizumab in the primary treatment of ovarian cancer. N Engl J Med 2011;365:2479–83.
6. Ledermann J, Harter P, Gourley C, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. Lancet Oncol 2014;15:852–61.
7. Galluzzi L, Senovilla L, Vitale I, et al. Molecular mechanisms of cisplatin resistance. Oncogene 2012;31:1869–83.
8. Mantia-Smaldone GM, Edwards RP, Vlad AM. Targeted treatment of recurrent platinum-resistant ovarian cancer: current and emerging therapies. Cancer Manag Res 2011;3:25–38.
9. Cannistra SA. Cancer of the ovary. N Engl J Med 2004;351:2519–29.
10. Rogers JH. Calretinin: a gene for a novel calcium-binding protein expressed principally in neurons. J Cell Biol 1987;105:1343–53.
11. Heizmann CW, Hunziker W. Intracellular calcium-binding proteins: more sites than insights. Trends Biochem Sci 1991;16:98–103.
12. Rogers J, Khan M, Ellis J. Calretinin and other Ca2+Ps in the nervous system. Adv Exp Med Biol 2009;669:195–203.
13. Camp AJ, Wijesinghe R. Calretinin: modulator of neuronal excitability. Int J Biochem Cell Biol 2009;41:2118–21.
14. Schurmans S, Schiffmann SN, Gurden H, et al. Impaired long-term potentiation induction in dentate gyrus of calretinin-deficient mice. Proc Natl Acad Sci U S A 1997;94:10415–20.
15. Ordonez NG. Value of calretinin immunostaining in diagnostic pathology: a review and update. Appl Immunohistochem Mol Morphol 2014;22:401–15.
16. Dogliotti C, Dei Tos AP, Laurino L, et al. Calretinin: a novel immunohistochemical marker for mesothelioma. Am J Surg Pathol 1996;20:1037–46.
17. Schwaller B. Calretinin: from a ‘simple’ Ca2+(+) buffer to a multifunctional protein implicated in many biological processes. Front Neuroanat 2014;8:3.
18. Gottron V, Vogt P, Cello MR. The calcium binding protein calretinin is a selective marker for malignant pleural mesotheliomas of the epithelial type. Pathol Res Pract 1996;192:137–47.
19. Jochen G, Burck K, Raiko I, et al. Prediagnostic detection of mesothelioma by circulating calretinin and mesothelin - a case-control comparison nested into a prospective cohort of asbestos-exposed workers. Sci Rep 2018;8:14321.
20. Jochen G, Gawrych K, Raiko I, et al. Calretinin as a blood-based biomarker for mesothelioma. BMC Cancer 2017;17:386.
21. Silverberg SG. Histopathologic grading of ovarian carcinoma: a review and proposal. Int J Gynecol Pathol 2000;19:7–15.
22. FIGO Committee on Gynecologic Oncology. Current FIGO staging for cancer of the vagina, fallopian tube, ovary, and gestational trophoblastic neoplasia. Int J Gynecol Obstet 2009;105:3–4.
23. Prat J, FIGO Committee on Gynecologic Oncol. Staging classification for cancer of the ovary, fallopian tube, and peritoneum. Int J Gynecol Obstet 2014;124:1–5.
24. Raiko I, Sander L, Weber DG, et al. Development of an enzyme-linked immunosorbent assay for the detection of human calretinin in plasma and serum of mesothelioma patients. BMC Cancer 2010;10:242.
25. Blum W, Schwallier B. Calretinin is essential for mesothelioma cell growth/survival in vitro: a potential new target for malignant mesothelioma therapy? Int J Cancer 2013;133:2077–88.
26. Wang RA, Li QL, Li ZS, et al. Apoptosis drives cancer cells proliferate and metastasize. J Cell Mol Med 2013;17:205–11.
27. Davidson B, Zhang Z, Kleinberg L, et al. Gene expression signatures differentiate ovarian/peritoneal serous carcinoma from diffuse malignant peritoneal mesothelioma. Clin Cancer Res 2006;12:5944–50.
28. Talianu RJ, Lu S, Singh K, et al. Calretinin expression in high-grade invasive ductal carcinoma of the breast is associated with basal-like subtype and unfavorable prognosis. Hum Pathol 2013;44:2743–50.
29. Micello D, Bossi A, Marando A, et al. Expression of calretinin in high-grade hormone receptor-negative invasive breast carcinomas: correlation with histological and molecular subtypes. Virchows Arch 2017;471:13–21.

30. Schwallier B, Meyer-Monard S, Gander JC, et al. The calcium-binding protein calretinin-22k is detectable in the serum and specific cells of cancer patients. Anticancer Res 1998;18:3661–7.

31. Burrell RA, Swanton C. Tumour heterogeneity and the evolution of polyclonal drug resistance. Mol Oncol 2014;8:1095–111.

32. Kuhlmann JD, Wimberger P, Bankfalvi A, et al. ERCC1-positive circulating tumor cells in the blood of ovarian cancer patients as a predictive biomarker for platinum resistance. Clin Chem 2014;60:1282–9.

33. Kuhlmann JD, Chebouti I, Kimmig R, et al. Extracellular vesicle-associated miRNAs in ovarian cancer - design of an integrated NGS-based workflow for the identification of blood-based biomarkers for platinum resistance. Clin Chem Lab Med 2018;57:1053–62.