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Review: Current and future role of circulating tumor cells in patients with epithelial ovarian cancer

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Abstract

Circulating tumor cells (CTCs) are viable tumor cells that are released into the circulatory system. CTCs have shown a prognostic value in numerous solid tumors. CTC research in epithelial ovarian carcinoma (EOC) has attracted only little attention. Since the primary route of metastasis in EOC is considered to be direct peritoneal spread in the abdominal cavity and distant metastases only occur in one third of the patients, it was thought that there is not enough shedding of tumor cells in the circulation. Nevertheless recent studies revealed an important role of hematogenous spread in EOC and showed that CTC status is associated with advanced tumor stage, CA-125 levels and residual disease after surgery. Furthermore the presence of CTCs correlates with shorter overall and disease free survival. However this prognostic value of CTCs in EOC seems to depend on the used isolation and detection methods. In EOC function- or density based enrichment methods seem to offer more promising results than epithelial cellular adhesion molecule (EpCAM)-based approaches. This can be explained by a low number of EpCAM positive CTCs in EOC and the downregulation of EpCAM during epithelial-to-mesenchymal transition (EMT). The presence of CTCs might also have predictive value as CTC status was associated with treatment response in two studies and CTCs showed to be a better monitoring tool than CA-125 in a small population. Finally the (genotypic) characterization of CTCs might become even more important in the future paving the way for CTCs to a true predictive “liquid tumor biopsy”.

Introduction

Ovarian cancer is the fifth leading cause of cancer-related death in women in Europe and the leading cause of death among gynecological malignancies (1). More than 70 % of epithelial ovarian cancers (EOC) are diagnosed in advanced stages with detectable transperitoneal spread (2). Patients with EOC have a poor prognosis despite an aggressive standard therapy consisting of cytoreductive surgery and platinum-paclitaxel based chemotherapy. About 80% of patients diagnosed with EOC will relapse after this initial therapy (3). Therefore there is a high need for new markers for early detection, improved knowledge of the molecular biology, better innovative treatment options and predictive biomarkers in EOC. Nowadays treatment efficacy and prognosis after initial treatment are assessed using CA-125 as a serum marker. However, this lacks the specificity to guide treatment and does not give any predictive information considering potential second line therapy (4).

Circulating tumor cells (CTCs) are viable tumor cells that are released from the primary tumor, local recurrence or metastasis into the circulatory system. They have tumor specific phenotypic and genotypic characteristics and may be able to grow in new microenvironments. Enumeration of circulating tumor cells (CTCs) has shown to have a prognostic value in patients with breast (5), colorectal (6), gastric (7), lung (8) and prostate (9) cancer. Detection of CTCs in the peripheral blood of patients with EOC proved to be feasible but in initial studies the frequency of CTC positivity and the median number of CTCs identified were low compared with other solid malignancies such as metastatic breast cancer (10). As distant metastasis only occurs in one third of the patients with EOC and often late in the progression of the disease, one could assume that at the time of presentation in most patients there is not enough hematogenous shedding of tumor cells in EOC to make CTC detection clinically relevant. In this paper we give an overview of the various detection methods, the prognostic and predictive value of the enumeration of CTCs, and the potential future applications of CTC genotyping and phenotyping in patients with EOC.

Detection methods of CTCs in EOC

Nowadays positive immunomagnetic enrichment, based on frequently expressed surface markers, in combination with reverse transcription polymerase chain reaction (RT-PCR) or immunocytochemistry (ICC) for visualization and quantification are mostly used for the detection of CTCs (11). Detection approaches are changing continuously and have evolved a lot since the first immunocytochemical study that tried to detect disseminated tumor cells (DTCs) in EOC in 1990 (12). The focus has shifted from identification of DTCs in bone marrow to detection of CTCs in peripheral bloodstream as this seems to be more sensitive (13) and practical. However CTC assessment remains difficult because CTCs are outnumbered by white blood cells (WBC) by a factor of at least 10^6 (14). CTC researchers tried to overcome this challenge with various enrichment and purification methods. There is no

specific marker uniformly expressed by all cancer types (15) and during metastasis the expression level of epithelial markers might be subject to changes (16). Therefore it is possible that depending on the method used different cellular phenotypes, with their own prognostic value, might be identified, making comparisons of results of different studies often difficult.

The first method for detection of CTCs cleared by the Food and Drug Administration (FDA) to determine the prognosis of patients with metastatic breast, colorectal and prostate cancer (17-19) is the CellSearch™ system. Whole blood is immunomagnetically enriched for CTCs using anti EpCAM monoclonal antibodies. Then 4',6-diamidino-2-phenylindole (DAPI), a nuclear stain to define viability and cytokeratins 8/18/19 to label cells of epithelial origin are applied. Co-enriched white blood cells are in turn labeled by adding anti-CD45, a marker for hematopoietic cell lineage. Prognostic results in Studies evaluating the prognostic significance of CTCs in EOC using this technique have mostly yielded disappointing/negative results. This might be explained by a low number of EpCAM positive CTCs in EOC (20), the downregulation of EpCAM during epithelial-to-mesenchymal transition (EMT) or the lack of biological specificity of EpCAM positive CTCs in EOC (21).

In an attempt to make CTC identification not dependent on the expression of specific epithelial markers such as EpCAM, other methods for CTC detection have been developed. These enrichment protocols can be based on the Isolation by Size of Epithelial Tumor cells (ISET by screencell) (22), MetaCell™ (23) or on two-layer density gradient centrifugation (20). Many of these EpCAM independent techniques rely on CTC size, since epithelial tumor cells are bigger than the majority of leukocytes. Further analysis can be done by a variety of techniques. Kolostova et al. showed the possibility to culture CTCs on the porous polycarbonate membrane for 3 days and analyzed the cultured cells using immunohistochemistry or gene expression analysis. Another alternative technique is the cytomorphological analysis after automatized cytospin analysis (23). A negative enrichment method based on detection and depletion of CD45 positive (hematopoietic) cells en subsequent fluorescence in situ hybridization (FISH) with chromosome 8 centromere probe (CEP8) had a sensitivity of 76,2 % for detecting CTCs in patients with EOC (24). For quantification of CTCs RT-PCR was more sensitive then ICC in breast cancer (25), however ICC seems to be more specific with less false positives (26). In EOC function (21) or density based (20) enrichment methods followed by RT-PCR (20, 27, 28) seem to offer more promising results then EpCAM-based approaches and confirmed the prognostic role of CTCs in EOC as summarized in table 1.

Pearl et al. proposed a method for the enrichment and isolation of CTCs based on the avidity to bind to a type 1 collagen matrix (Cell Adhesion Matrix - CAM) and subsequently ingest this labeled matrix protein (CAM+). The isolated CTCs are called iCTCs indicating the invasive phenotype. Further

discrimination is based on ICC methods using antibodies against epithelial or tumor antigens. In a recent study antibodies against the tumor progenitor markers CD44 and seprase were used (21). The detection sensitivity with this method (83%) was much higher than other approaches that are being used (table 1). An extra advantage of this procedure is the minimal loss of viable tumor cells, which makes the culture of CTCs and additional downstream applications possible.

Furthermore new and easier diagnostic approaches are continuously being developed. Kim et al. present a one-step CTC detection based on flow cytometry. They used fluorescent silica nanoparticles conjugated with a Mucin 1 cell surface associated (MUC1) antibody to detect ovarian cancer cells in whole blood samples (11).

Enumeration of CTCs in EOC

Traditionally the predominant primary route of metastasis in EOC is considered to be direct peritoneal spread in the abdominal cavity. However recent studies suggest that hematogenous spread might be more important than previously thought. Pradeep et al. showed that the preferential way of metastasis to the omentum in ovarian cancer may in fact be hematogenous and that the ErbB3-neuregulin 1 (NRG1) axis is the responsible dominant pathway involved (29). Depending on the detection method used, detection rates of CTCs between 18 and 88% have been documented in patients with FIGO Stage I-IV EOC (Table 1). Cut-off values for CTC positivity vary between different studies: ≥ 1 CTCs, ≥ 2 CTCs or ≥ 5 CTCs (Table 1). CTC positivity is defined as one or more antigen in ICC studies using multiple antigens or a tumor associated transcript above threshold in RT-PCR studies. In most studies CTC enumeration is performed before surgery at the time of primary diagnosis, but there are some studies reporting CTC detection before and/or after adjuvant first line chemotherapy or before second line chemotherapy in patients with recurrent disease. Several smaller studies could not find a correlation between CTC positivity and progression free (PFS) or overall survival (OS). However studies on some larger series using different antigens or enrichment methods for CTC detection (often increasing the number of detected CTCs) clearly showed that the enumeration of CTCs has prognostic significance, particularly in patients with primary EOC (20, 21, 27, 28, 30). A recent meta-analysis of Zhou et al., looking at the data of 11 studies (table 1), confirmed there is a role of CTC as a prognostic marker in EOC. These authors showed that a negative CTC status is associated with longer OS (HR, 1.61; 95% CI, 1.22 – 2.13) and longer DFS (HR, 1.44; 95% CI, 1.18 – 1.75). Subgroup analysis showed a higher risk of mortality in the high RT-PCR group (HR, 2.02; 95% CI 1.34 – 3.03), which was not observed in the “Cellsearch” and “other ICC” subgroups (31). This can be explained by the previously mentioned limitations of the “Cellsearch” system. During EMT EpCAM is frequently downregulated and CTCs can be missed (32). It is also possible that CTCs of patients with EOC that underwent EMT are more aggressive and that

depending on the methodology another cellular phenotype of CTCs is detected. Additionally CTCs identified using only epithelial markers may also represent dead or dying cells that don't contribute to metastatic spread (33). In summary, approaches that isolate and/or detect CTCs without using epithelial markers seem to have a higher prognostic value.

Associations between CTCs and clinicopathological parameters have also been reported. Tumor histology (serous vs. non-serous EOC) was not significantly correlated with CTC status (13) and neither was lymph node metastasis (20, 34). This can possibly be explained by the different way of metastatic spread of EOC compared with other solid tumors. In 5 studies a significant correlation between tumor stage and CTC positivity was found (Stage I-II versus III-IV, pooled OR= 1.90; 95% CI: 1.02 – 3.56; $p = 0,044$) (13, 20, 30, 33-36). While most studies failed to show a positive correlation between optimal cytoreduction and CTC numbers after surgery (30, 34), Obermayer et al. demonstrated that CTCs significantly correlated with residual disease after suboptimal debulking surgery (20).

Some studies looked at the relation between detected CTCs and elevated CA-125, but could not find a significant correlation (33, 36). However in other series (20, 30, 34) and in a recent meta-analysis a significant correlation was described (OR, 4.07; 95% CI 1.87 – 8.85) (31). Again correlation might depend on the used detection methods. Currently CA-125 serum levels are still considered as the best parameter to monitor response to treatment and the most valuable prognostic tool in EOC patients receiving second line chemotherapy (37). However, Pearl et al. recently showed that serial enumeration of CTCs in 31 patients treated with standard taxol/carboplatin chemotherapeutic regimes predicted therapeutic responsiveness (38). In their study CTCs were more sensitive than CA-125 in predicting progressive disease or relapse and could antedate clinical status changes whereas CA-125 could not.

Despite a number of studies reporting association between the actual blood CTC load and prognosis in breast, prostate and colorectal cancer (39), the only study that evaluated the absolute enumeration of CTCs and its prognostic value in EOC could not find a significant association (40). However, this issue needs to be investigated in further detail on larger numbers of patients in a clearly defined setting (e.g. before cytoreductive surgery, before starting the first cycle of adjuvant chemotherapy and after 3 or 6 cycles of adjuvant cytostatic treatment). Beside this, the prognostic role of CTCs in EOC still suffers from some other important limitations. First the detection methods vary across studies, which can influence survival analysis. Second there is no consensus about the optimal cutoff of CTCs. In CellsearchTM based studies the cutoff of ≥ 2 CTC/7.5mL was frequently used (33, 36, 40). Furthermore there is no uniform definition for the identification of a CTC, validation

studies in EOC are still lacking and only a few studies reported CTC evolution over the course of standard therapy and the disease (20, 38, 40).

Genotypic and phenotypic characterization of CTCs in EOC

In addition to their enumeration, genotypic and phenotypic characterization of therapeutically or biologically relevant biomarkers expressed by CTCs has been shown to be feasible in different types of malignancies: EGFR gene mutations in lung cancer (41), K-ras mutations in colorectal cancer patients (42), AR gene status in prostate cancer (43) and a number of studies in advanced or metastatic breast cancer (44) that looked at Her2 (45) or Bcl-2 expression (46).

A limited number of studies proved the concept of the predictive potential of characterization CTCs in EOC. Excision repair cross complementation group 1 (ERCC1) is important in the nucleotide excision repair pathway and plays a role in the repair of platinum damaged DNA in tumor cells. ERCC1 expression in CTCs showed to be a biomarker for platinum resistance in EOC, even when immunohistochemical analysis of ERCC1 in primary tumor tissue did not have any predictive significance (28). Cyclophilin 3 (PPIC), an intracellular receptor of cyclosporine A, expression in CTCs also proved to be correlated with platinum sensitivity. Moreover the presence of PPIC positive CTCs after chemotherapy was much more common (69%) than EPCAM gene expression (4%) in patients with EOC and was associated with a significantly worse prognosis (20). Additionally overexpression of Cyclophilin A (PPIA), another cyclophilin family member, protected cancer cells against platinum induced cellular stress (47). This indicates that PPIC positive CTCs might represent a subpopulation of more aggressive CTCs that survived chemotherapy and that PPIC can be an interesting target for therapy. It also seems to confirm that EpCAM is not the optimal marker for identifying prognostic and predictive valuable CTCs in EOC. Furthermore, since the presence of CTCs is a negative prognostic feature and ERCC1 transcription in CTCs is also predictive for platinum resistance, the negative prognostic impact of CTCs may depend on specific cellular phenotypes which are sustaining the selective pressure of treatment.

At the moment the major challenge for CTC characterization encountered in most studies is that only a limited number of pre-selected genes/markers can be studied due to the presence of a still significant leukocyte background (10^2 - 10^3) after enrichment (48). Our lab recently demonstrated that it is possible to isolate and molecularly characterize single or 100% purified pools of CTCs from CellSearchTM enriched blood sample using a semi-automated dielectrophoretic cell sorting technique (DEPArray) (49). A technical update of the DEPArray system, accelerating the sorting process, and using BCT tubes (50), which stabilize intracellular RNA after blood collection, further improves RNA quality of the isolated cells. This allows the use of more advanced profiling techniques such as RNA

sequencing. Several other research groups already proved the technical feasibility of single cell RNA sequencing (51, 52). Furthermore, Cann and colleagues have shown that RNA sequencing of single CTCs is still achievable after such an elaborate workflow including magnetic enrichment, staining procedures and isolation by micropipetting (53). Single cell sequencing allows the study of sequential samples during disease progression, which may be crucial to unravel the molecular biology of therapy resistance. In addition single cell sequencing has the ability to study the heterogeneity of the CTC population and the importance of tumor cell genomic variation as some clones may be more significant than others in driving tumor recurrence (54).

Future directions

The most common approaches used to detect EOC and follow treatment efficacy are physical examination, evaluation of CA-125 levels and imaging studies. Unfortunately all have limited sensitivity and accuracy in detecting early stages. Therefore less than 30 % of EOC will be detected in an early stage nowadays (2). Metastatic disease is responsible for over 90% of cancer related death (55). Due to in depth understanding of the molecular biology of cancer, an increasing number of patients with disseminated cancer is now considered candidate for treatment with so called targeted agents in addition to standard treatment modalities, but the efficacy of these drugs largely relies on their ability to block specific molecular aberrations that usually only occur in a subset of the patient population. Therefore the need for novel diagnostic, prognostic and predictive biomarkers is high.

At present, results of targeted therapies in EOC have been largely disappointing. This can be explained by many reasons. Firstly there is a wide range in inter-patient genomic, transcriptomic and proteomic profiles of EOC, not only depending on different histology. This argues against uniform treatments, such as carboplatin/paclitaxel, which are used in all patients with EOC. (56). In clinical practice, the analysis of predictive biomarkers is routinely performed on archival tissue samples from the primary tumor. This approach is far from perfect for several reasons. Comparative analyses of established predictive biomarkers on archival primary tumor tissue and metastatic lesions in patients with MBC have documented clinically relevant discordances in up to 25% of patients only looking at hormone receptor or HER-2 status (57, 58). This is also documented for ECCR1 expression in EOC (28) and for mutations in the coding sequence of Estrogen Receptor 1 (ESR1) in metastatic ER+ breast cancer (59). Likewise, patients often exhibit multiple lesions that might be composed of different subclones of tumor cells harboring different molecular characteristics, which also may change during tumor progression (60). Furthermore in advanced staged EOC resistance to a selected treatment will eventually occur in virtually all patients, due to the activation of alternative pathways when a given one is blocked. Repetitive probing of drug targets and potential biomarkers for drug sensitivity or emerging resistance on contemporary tumor samples are essential to allow for more dynamic,

individualized treatment planning and monitoring. Sampling metastatic lesions is however often technically difficult or not without risk because of anatomical constraints. Dynamic detection of relevant markers in the bloodstream of these patients, shedding directly from the different (primary and metastatic) tumor masses, provide alternatives to obtain real-time and repeated “liquid tumor biopsies”.

It is clear that detection and characterization of CTCs but also circulating tumor DNA (ctDNA) are attractive options to guide targeted therapy and to study the behaviour of progressive EOC. The accelerated cellular turnover because of tumor growth, leads to increased amounts of circulating tumor DNA (ctDNA) into the bloodstream (61) and the quantity of cell-free DNA (cfDNA) in patient serum can therefore be used as a marker of disease presence in EOC (62). Furthermore new sequencing technologies and analytic techniques have made the detection of circulating tumor-specific DNA (ctDNA) possible. In a first study, Martignetti et al. used a tumor-specific FGFR2-FAM76A ovarian fusion for the follow-up of a single patient over a four-year period (63). They continuously detected ctDNA, hence residual disease, despite normal CA-125 levels. In a larger study the presence of ctDNA was able to detect tumor at surgery with a sensitivity of 0.81 (0.60–0.92) and a specificity of 0.99 (0.81–0.99) (64). Furthermore the study showed that undetectable levels of ctDNA after initial treatment were an independent predictor of improved PFS and OS.

CTCs are probably not ideal for early detection and screening as proteomics and circulating DNA are theoretically able to detect occult (pre)cancers in an earlier stage. However, it is interesting that a case report described the use of the CellSearch system to diagnose an occult ovarian low-grade serous carcinoma in a patient with metastases to the supraclavicular region (65) and CTCs detected EOC stage I and II better than CA-125 in a high risk group, which is important because CTC positive early stage patients had a significant worse prognosis (21).

Yet before we really can use CTCs in EOC as a diagnostic tool or as a liquid biopsy and a surrogate for repetitive tumor biopsies to make therapeutic decisions several limitations have to be overcome. First larger, less heterogeneous studies with a validation cohort are warranted, because nowadays most studies are case series with relatively small patients numbers and retrospectively designed with the risk of a selection bias. They are also very heterogeneous with variable sampling moments, follow-up and response criteria. Furthermore methodological approaches to enrich and detect CTCs differ greatly across studies, which make it difficult to implement CTCs in clinical trials or daily practice. We also have to isolate sufficient CTC numbers to obtain a representative picture, for example with apheresis of CTCs (66) and be able to characterize them on a single cell level.

Conclusion

Evidence is emerging that the presence of CTCs in EOC patients is associated with an overall worse prognosis and advanced disease stage. However this relationship depends on the used isolation and detection methods. Additionally there is no optimal cut-off value defined and the role of increasing numbers of CTCs has not yet been investigated. The presence of CTCs might have predictive value as treatment response on platinum based chemotherapy was significantly associated with CTC status in two studies and CTCs showed to be a good monitoring tool in a small population of patients. However the characterization of CTCs will probably become more important in the future and larger patient cohorts are needed. Genotypic and phenotypic characterization of CTCs allows the study of the biology, frequency, and variability of disease driving clones through the analysis of the exome-wide mutation profile and gene expression level. In the future this can lead to personalized anti-metastatic therapies, a better understanding of molecular alternations that drive the metastatic process and by comparing CTCs in drug sensitive versus resistant patients also a better knowledge about drug resistance development.

Author, year	Study type	No. of patients	Timing	Tumor stage ¹	Isolation method	Detection method	Targeted antigen/targeted gene	Cutoff of CTCs	Positivity rate	Outcome: HR, 95%CI ²	
Marth et al., 2002	Case series	90	Before adjuvant chemotherapy	I-IV (77%)	Immunomagnetic (Dynabeads®)	ICC	MOC-31	Presence of ≥2 rosettes	12.0	PFS	2.19 (0.84 - 5.74) - NS
										OS	1.99 (0.23 - 17.16) - NS
Judson et al., 2003	Case series	59	Before surgery	I-IV (79%)	Immunomagnetic beads	ICC	CK8 and 18, TFS-2, CK7, CK20, EGFR	≥1 cell positive	18.7	PFS	1.45 (0.55 - 3.83) - NS
										OS	2.14 (0.37 - 12.29) - NS
Fan et al., 2009	Case series	66	Before surgery	I-IV (79%)	CAM+ (functional enrichment)	ICC	EpCAM; CK 4,5,6,8,10, 13 and 18	≥1 CTC/3mL	60.6	PFS	1.44 (0.78 - 2.64) - p = 0.04
										OS	0.89 (0.40 - 1.95) - NS
Poveda et al., 2011	phase III clinical trial	216	Before 2nd line chemotherapy	NR	Immunomagnetic (CellSearch® system)	ICC (CellSearch® system)	EpCAM; CK8, 18 and 19	≥2 CTC/7.5mL	14.4	PFS	1.58 (0.99-2.53) - NS
										OS	1.54, (0.93-2.54) - NS
Behbakht et al., 2011	phase II clinical trial	54	Before and after Temsirolimus	NR	Immunomagnetic (CellSearch® system)	ICC (CellSearch® system)	EpCAM	≥1 CTC/7.5 mL	44.0 (before cycle 1)	PFS	1.61 (0.79 - 3.29) - NS
Aktas et al., 2011	Case series	122	Before surgery and/or after chemotherapy	NR	Immunomagnetic (Adnatest®)	RT-PCR (Adnatest®)	EpCAM, MUC-1, CA-125, HER-2	≥1 tumor-associated transcript above threshold	19.0 (before surgery)	PFS	1.58 (0.86 - 2.88) - NS
									27.0 (after CT)	OS	4.56 (1.94 - 10.73) - p=0.05
Obermayer et al., 2013	Case series	216	Before surgery and after adjuvant chemotherapy	III-IV	Density gradient centrifugation RNA extraction (Qiacube system®)	RT-PCR	PPIC, GPX8, CDH3, TUSC3, COL3A1, LAMB1, MAM, ESRP2, AGR2, BAIAP2L1, TFF1, EpCAM	≥ 1 gene over-expressed above threshold	24.5 (before surgery)	PFS	3.5 (1.8-6.9) - p < 0.001
									20.4 (after CT)	OS	2.3 (1.1-4.8) - p = 0.024
Liu et al., 2013	Case series	30 (ND) 48 (RD)	Before therapy	I-IV (92%) ³	Immunomagnetic (CellSearch® system)	ICC (CellSearch® system)	EpCAM	≥2 CTC/7.5mL	60.0 (ND)	PFS	0.71 (0.30 - 1.69) - NS
									53.8 (RD)	OS	0.53 (0.12 - 2.40) - NS
Sang et al., 2014	Xxxx	80	Xxx	I-IV (89%)	Xxx	RT-PCR	MAGE-A	xxx	47.5	OS	1.40 (0.87 - 2.27) - XXX
Pearl et al., 2014	Case series	88	Before surgery	I-IV (81%)	CAM+ (functional enrichment)	ICC	EpCAM, CA-125, DPP4 and CKs	≥5 CTC/1.0mL	88.6	PFS	1.21 (0.49 - 2.97) - p= 0.0024
										OS	1.06 (0.41 - 2.73) - p=0.0219
Kuhlmann et al., 2014	Case series	143	Before surgery	I-IV (82%)	Immunomagnetic (Adnatest®)	RT-PCR (Adnatest®)	EpCAM, MUC1, MUC6, ERCC1	≥1 tumor-associated transcript above threshold	14.0	PFS	1.50 (0.81 - 2.79) - NS
										OS	1.85 (1.03 - 3.32) - p = 0.041
Zhou et al., 2015	Meta-analysis	1129		I-IV (NR)						PFS	1.44 (1.18 - 1.75)
										OS	1.61 (1.22 - 2.13)

Table 1: Overview of used detection methods, CTC definitions and clinical outcomes in ovarian cancer. CTCs - circulating tumor cells; OS – overall survival; PFS – progression free survival; HR – hazard ratio; CI – confidence interval; NS – not significant; NR – not reported; CT – chemotherapy; ND – new disease; RD – recurrent disease; RT-PCR: Reverse transcription-polymerase chain reaction; ICC – immunocytochemistry. (1) % of advanced stage EOC (2) based on the meta-analysis of Zhou et al. when not reported. (3) 8% recurrent disease.

References

1. Ferlay J, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013 Available from: <http://globocan.iarc.fr>, accessed on 12/12/2015.
2. Goodman MT, Howe HL, Tung KH, Hotes J, Miller BA, Coughlin SS, et al. Incidence of ovarian cancer by race and ethnicity in the United States, 1992-1997. *Cancer*. 2003;97(10 Suppl):2676-85.
3. Ozols RF, Bundy BN, Greer BE, Fowler JM, Clarke-Pearson D, Burger RA, et al. Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2003;21(17):3194-200.
4. Duffy MJ, Bonfrer JM, Kulpa J, Rustin GJ, Soletormos G, Torre GC, et al. CA125 in ovarian cancer: European Group on Tumor Markers guidelines for clinical use. *International journal of gynecological cancer : official journal of the International Gynecological Cancer Society*. 2005;15(5):679-91.
5. Zhang L, Riethdorf S, Wu G, Wang T, Yang K, Peng G, et al. Meta-analysis of the prognostic value of circulating tumor cells in breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2012;18(20):5701-10.
6. Groot Koerkamp B, Rahbari NN, Buchler MW, Koch M, Weitz J. Circulating tumor cells and prognosis of patients with resectable colorectal liver metastases or widespread metastatic colorectal cancer: a meta-analysis. *Annals of surgical oncology*. 2013;20(7):2156-65.
7. Wang S, Zheng G, Cheng B, Chen F, Wang Z, Chen Y, et al. Circulating tumor cells (CTCs) detected by RT-PCR and its prognostic role in gastric cancer: a meta-analysis of published literature. *PloS one*. 2014;9(6):e99259.
8. Wang J, Wang K, Xu J, Huang J, Zhang T. Prognostic significance of circulating tumor cells in non-small-cell lung cancer patients: a meta-analysis. *PloS one*. 2013;8(11):e78070.
9. Ma X, Xiao Z, Li X, Wang F, Zhang J, Zhou R, et al. Prognostic role of circulating tumor cells and disseminated tumor cells in patients with prostate cancer: a systematic review and meta-analysis. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2014;35(6):5551-60.
10. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. *The Lancet Oncology*. 2014;15(4):406-14.
11. Kim JH, Chung HH, Jeong MS, Song MR, Kang KW, Kim JS. One-step detection of circulating tumor cells in ovarian cancer using enhanced fluorescent silica nanoparticles. *International journal of nanomedicine*. 2013;8:2247-57.
12. Cain JM, Ellis GK, Collins C, Greer BE, Tamimi HK, Figge DC, et al. Bone marrow involvement in epithelial ovarian cancer by immunocytochemical assessment. *Gynecologic oncology*. 1990;38(3):442-5.
13. Cui L, Kwong J, Wang CC. Prognostic value of circulating tumor cells and disseminated tumor cells in patients with ovarian cancer: a systematic review and meta-analysis. *Journal of ovarian research*. 2015;8:38.
14. Mostert B, Sleijfer S, Foekens JA, Gratama JW. Circulating tumor cells (CTCs): detection methods and their clinical relevance in breast cancer. *Cancer treatment reviews*. 2009;35(5):463-74.
15. Went PT, Lugli A, Meier S, Bundi M, Mirlacher M, Sauter G, et al. Frequent EpCam protein expression in human carcinomas. *Human pathology*. 2004;35(1):122-8.

16. Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, et al. Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression. *Journal of cellular physiology*. 2007;213(2):374-83.
17. Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2008;26(19):3213-21.
18. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *The New England journal of medicine*. 2004;351(8):781-91.
19. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2008;14(19):6302-9.
20. Obermayr E, Castillo-Tong DC, Pils D, Speiser P, Braicu I, Van Gorp T, et al. Molecular characterization of circulating tumor cells in patients with ovarian cancer improves their prognostic significance -- a study of the OVCAD consortium. *Gynecologic oncology*. 2013;128(1):15-21.
21. Pearl ML, Zhao Q, Yang J, Dong H, Tulley S, Zhang Q, et al. Prognostic analysis of invasive circulating tumor cells (iCTCs) in epithelial ovarian cancer. *Gynecologic oncology*. 2014;134(3):581-90.
22. Vona G, Sabile A, Louha M, Sitruk V, Romana S, Schutze K, et al. Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulating tumor cells. *The American journal of pathology*. 2000;156(1):57-63.
23. Kolostova K, Spicka J, Matkowski R, Bobek V. Isolation, primary culture, morphological and molecular characterization of circulating tumor cells in gynecological cancers. *American journal of translational research*. 2015;7(7):1203-13.
24. Ning N, Zhan T, Zhang Y, Chen Q, Feng F, Yang Z, et al. Improvement of specific detection of circulating tumor cells using combined CD45 staining and fluorescence in situ hybridization. *Clinica chimica acta; international journal of clinical chemistry*. 2014;433:69-75.
25. Ring AE, Zabaglo L, Ormerod MG, Smith IE, Dowsett M. Detection of circulating epithelial cells in the blood of patients with breast cancer: comparison of three techniques. *British journal of cancer*. 2005;92(5):906-12.
26. Krebs MG, Hou JM, Ward TH, Blackhall FH, Dive C. Circulating tumour cells: their utility in cancer management and predicting outcomes. *Therapeutic advances in medical oncology*. 2010;2(6):351-65.
27. Aktas B, Kasimir-Bauer S, Heubner M, Kimmig R, Wimberger P. Molecular profiling and prognostic relevance of circulating tumor cells in the blood of ovarian cancer patients at primary diagnosis and after platinum-based chemotherapy. *International journal of gynecological cancer : official journal of the International Gynecological Cancer Society*. 2011;21(5):822-30.
28. Kuhlmann JD, Wimberger P, Bankfalvi A, Keller T, Scholer S, Aktas B, et al. ERCC1-positive circulating tumor cells in the blood of ovarian cancer patients as a predictive biomarker for platinum resistance. *Clinical chemistry*. 2014;60(10):1282-9.
29. Pradeep S, Kim SW, Wu SY, Nishimura M, Chaluvally-Raghavan P, Miyake T, et al. Hematogenous metastasis of ovarian cancer: rethinking mode of spread. *Cancer cell*. 2014;26(1):77-91.

30. Fan T, Zhao Q, Chen JJ, Chen WT, Pearl ML. Clinical significance of circulating tumor cells detected by an invasion assay in peripheral blood of patients with ovarian cancer. *Gynecologic oncology*. 2009;112(1):185-91.
31. Zhou Y, Bian B, Yuan X, Xie G, Ma Y, Shen L. Prognostic Value of Circulating Tumor Cells in Ovarian Cancer: A Meta-Analysis. *PloS one*. 2015;10(6):e0130873.
32. Wicha MS, Hayes DF. Circulating tumor cells: not all detected cells are bad and not all bad cells are detected. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2011;29(12):1508-11.
33. Liu JF, Kindelberger D, Doyle C, Lowe A, Barry WT, Matulonis UA. Predictive value of circulating tumor cells (CTCs) in newly-diagnosed and recurrent ovarian cancer patients. *Gynecologic oncology*. 2013;131(2):352-6.
34. Sang M, Wu X, Fan X, Sang M, Zhou X, Zhou N. Multiple MAGE-A genes as surveillance marker for the detection of circulating tumor cells in patients with ovarian cancer. *Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals*. 2014;19(1):34-42.
35. Judson PL, Geller MA, Bliss RL, Boente MP, Downs LS, Jr., Argenta PA, et al. Preoperative detection of peripherally circulating cancer cells and its prognostic significance in ovarian cancer. *Gynecologic oncology*. 2003;91(2):389-94.
36. Poveda A, Kaye SB, McCormack R, Wang S, Parekh T, Ricci D, et al. Circulating tumor cells predict progression free survival and overall survival in patients with relapsed/recurrent advanced ovarian cancer. *Gynecologic oncology*. 2011;122(3):567-72.
37. Gronlund B, Hogdall C, Hilden J, Engelholm SA, Hogdall EV, Hansen HH. Should CA-125 response criteria be preferred to response evaluation criteria in solid tumors (RECIST) for prognostication during second-line chemotherapy of ovarian carcinoma? *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2004;22(20):4051-8.
38. Pearl ML, Dong H, Tulley S, Zhao Q, Golightly M, Zucker S, et al. Treatment monitoring of patients with epithelial ovarian cancer using invasive circulating tumor cells (iCTCs). *Gynecologic oncology*. 2015;137(2):229-38.
39. Coumans FA, Ligthart ST, Terstappen LW. Interpretation of changes in circulating tumor cell counts. *Translational oncology*. 2012;5(6):486-91.
40. Behbakht K, Sill MW, Darcy KM, Rubin SC, Mannel RS, Waggoner S, et al. Phase II trial of the mTOR inhibitor, temsirolimus and evaluation of circulating tumor cells and tumor biomarkers in persistent and recurrent epithelial ovarian and primary peritoneal malignancies: a Gynecologic Oncology Group study. *Gynecologic oncology*. 2011;123(1):19-26.
41. Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, et al. Detection of mutations in EGFR in circulating lung-cancer cells. *The New England journal of medicine*. 2008;359(4):366-77.
42. Yang MJ, Chiu HH, Wang HM, Yen LC, Tsao DA, Hsiao CP, et al. Enhancing detection of circulating tumor cells with activating KRAS oncogene in patients with colorectal cancer by weighted chemiluminescent membrane array method. *Annals of surgical oncology*. 2010;17(2):624-33.
43. Attard G, Swennenhuis JF, Olmos D, Reid AH, Vickers E, A'Hern R, et al. Characterization of ERG, AR and PTEN gene status in circulating tumor cells from patients with castration-resistant prostate cancer. *Cancer research*. 2009;69(7):2912-8.
44. Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2011;17(11):3600-18.

45. Ligthart ST, Bidard FC, Decraene C, Bachelot T, Delaloue S, Brain E, et al. Unbiased quantitative assessment of Her-2 expression of circulating tumor cells in patients with metastatic and non-metastatic breast cancer. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*. 2013;24(5):1231-8.
46. Smerage JB, Budd GT, Doyle GV, Brown M, Paoletti C, Muniz M, et al. Monitoring apoptosis and Bcl-2 on circulating tumor cells in patients with metastatic breast cancer. *Molecular oncology*. 2013;7(3):680-92.
47. Doyle V, Virji S, Crompton M. Evidence that cyclophilin-A protects cells against oxidative stress. *The Biochemical journal*. 1999;341 (Pt 1):127-32.
48. Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, Mostert B, Martens JW, et al. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. *Breast cancer research and treatment*. 2009;118(3):455-68.
49. Peeters DJ, De Laere B, Van den Eynden GG, Van Laere SJ, Rothe F, Ignatiadis M, et al. Semiautomated isolation and molecular characterisation of single or highly purified tumour cells from CellSearch enriched blood samples using dielectrophoretic cell sorting. *British journal of cancer*. 2013;108(6):1358-67.
50. Qin J, Alt JR, Hunsley BA, Williams TL, Fernando MR. Stabilization of circulating tumor cells in blood using a collection device with a preservative reagent. *Cancer cell international*. 2014;14(1):23.
51. Picelli S, Faridani OR, Bjorklund AK, Winberg G, Sagasser S, Sandberg R. Full-length RNA-seq from single cells using Smart-seq2. *Nature protocols*. 2014;9(1):171-81.
52. Tang F, Barbacioru C, Nordman E, Li B, Xu N, Bashkirov VI, et al. RNA-Seq analysis to capture the transcriptome landscape of a single cell. *Nature protocols*. 2010;5(3):516-35.
53. Cann GM, Gulzar ZG, Cooper S, Li R, Luo S, Tat M, et al. mRNA-Seq of single prostate cancer circulating tumor cells reveals recapitulation of gene expression and pathways found in prostate cancer. *PloS one*. 2012;7(11):e49144.
54. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature*. 2013;501(7467):338-45.
55. Mehlen P, Puisieux A. Metastasis: a question of life or death. *Nature reviews Cancer*. 2006;6(6):449-58.
56. Davidson B, Trope CG. Ovarian cancer: diagnostic, biological and prognostic aspects. *Women's health*. 2014;10(5):519-33.
57. Niikura N, Liu J, Hayashi N, Mittendorf EA, Gong Y, Palla SL, et al. Loss of human epidermal growth factor receptor 2 (HER2) expression in metastatic sites of HER2-overexpressing primary breast tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2012;30(6):593-9.
58. Higgins MJ, Jelovac D, Barnathan E, Blair B, Slater S, Powers P, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2012;18(12):3462-9.
59. Jeselsohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM, et al. Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2014;20(7):1757-67.
60. Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? *Nature reviews Cancer*. 2012;12(5):323-34.
61. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2014;32(6):579-86.

62. Kamat AA, Sood AK, Dang D, Gershenson DM, Simpson JL, Bischoff FZ. Quantification of total plasma cell-free DNA in ovarian cancer using real-time PCR. *Annals of the New York Academy of Sciences*. 2006;1075:230-4.
63. Martignetti JA, Camacho-Vanegas O, Priedigkeit N, Camacho C, Pereira E, Lin L, et al. Personalized ovarian cancer disease surveillance and detection of candidate therapeutic drug target in circulating tumor DNA. *Neoplasia*. 2014;16(1):97-103.
64. Pereira E, Camacho-Vanegas O, Anand S, Sebra R, Catalina Camacho S, Garnar-Wortzel L, et al. Personalized Circulating Tumor DNA Biomarkers Dynamically Predict Treatment Response and Survival In Gynecologic Cancers. *PloS one*. 2015;10(12):e0145754.
65. He S, Li P, Chen X, Yu Z. Fluid biopsy for circulating tumor cells in an occult ovarian cancer patient exhibiting bilateral supraclavicular lymph node metastases: A case report. *Oncology letters*. 2013;6(2):448-52.
66. de Wit S, van Dalum G, Terstappen LW. Detection of circulating tumor cells. *Scientifica*. 2014;2014:819362.