Review

Lydia Giannopoulou, Sabine Kasimir-Bauer and Evi S. Lianidou*

Liquid biopsy in ovarian cancer: recent advances on circulating tumor cells and circulating tumor DNA

https://doi.org/10.1515/cclm-2017-0019
Received January 10, 2017; accepted March 2, 2017; previously published online July 28, 2017

Abstract: Ovarian cancer remains the most lethal disease among gynecological malignancies despite the plethora of research studies during the last decades. The majority of patients are diagnosed in an advanced stage and exhibit resistance to standard chemotherapy. Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) represent the main liquid biopsy approaches that offer a minimally invasive sample collection. Both have shown a diagnostic, prognostic and predictive value in many types of solid malignancies and recent studies attempted to shed light on their role in ovarian cancer. This review is mainly focused on the clinical value of both CTCs and ctDNA in ovarian cancer and, more specifically, on their potential as diagnostic, prognostic and predictive tumor biomarkers.

Keywords: circulating tumor cells; circulating tumor DNA; liquid biopsy; ovarian cancer; tumor biomarkers.

Introduction

Ovarian cancer causes the majority of cancer-related deaths from gynecological cancers and represents the third most frequent gynecological cancer worldwide [1]. Epithelial ovarian cancer is the main type, characterized by histological and molecular heterogeneity and is considered as a highly aggressive neoplasia. It is often diagnosed at an advanced stage and little progress has been achieved in standard chemotherapy treatment and overall survival (OS) during the last 3 decades [2]. Primary disease is treated with surgical removal of the tumor, followed by standard adjuvant chemotherapy, a combination of platinum and taxane-based treatment [3, 4]. However, in more than half of the cases, chemoresistance and recurrent disease are observed [5, 6]. New therapeutic concepts now include targeted therapy applying bevacizumab or the PARP inhibitor olaparib in certain clinical situations [7, 8].

Metastasis in ovarian cancer occurs via two main routes characterized by different molecular mechanisms, the transcoelomic passive dissemination of tumor spheroids in the peritoneal fluid and ascites, and the hematogenous metastasis of cancer cells in blood circulation and their preferred seeding to the omentum. Circulating tumor cells (CTCs) contribute to the hematogenous metastatic route [9, 10]. Generally, in solid malignancies, CTCs are exceedingly rare, and in most cases, the amount of the available peripheral blood sample is limited. The development of different analytical systems for the detection, enumeration and molecular characterization of CTCs has expanded the field of liquid biopsy, providing information on patients clinical outcome and treatment efficacy [11].

Cell-free DNA (cfDNA) circulates at high concentrations in peripheral blood of cancer patients and can be used for the detection of several molecular alterations related to cancer development [12]. Circulating tumor DNA (ctDNA) represents a small percentage of cfDNA that is shed in circulation by tumor cells and carries all these molecular alterations including tumor specific mutations, microsatellite instability (MI) [12], loss of heterozygosity (LOH) [13], and DNA methylation [14]. ctDNA is a very promising non-invasive diagnostic, prognostic and predictive tool, as it provides an easily accessible source of DNA derived from the tumor [15].

In this review, we will give an overview of the published data on CTCs and ctDNA in ovarian cancer (Figure 1). We

***Corresponding author: Dr. Evi S. Lianidou,** Analysis of Circulating Tumour Cells Lab, Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, University Campus, Athens 15771, Greece, Phone: +30 210 7274311, Fax: +30 210 7274750, E-mail: lianidou@chem.uoa.gr
Lydia Giannopoulou: Analysis of Circulating Tumor Cells Lab, Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, University Campus, Athens, Greece
Sabine Kasimir-Bauer: Department of Gynecology and Obstetrics, University Hospital of Essen, University of Duisburg-Essen, Essen, Germany

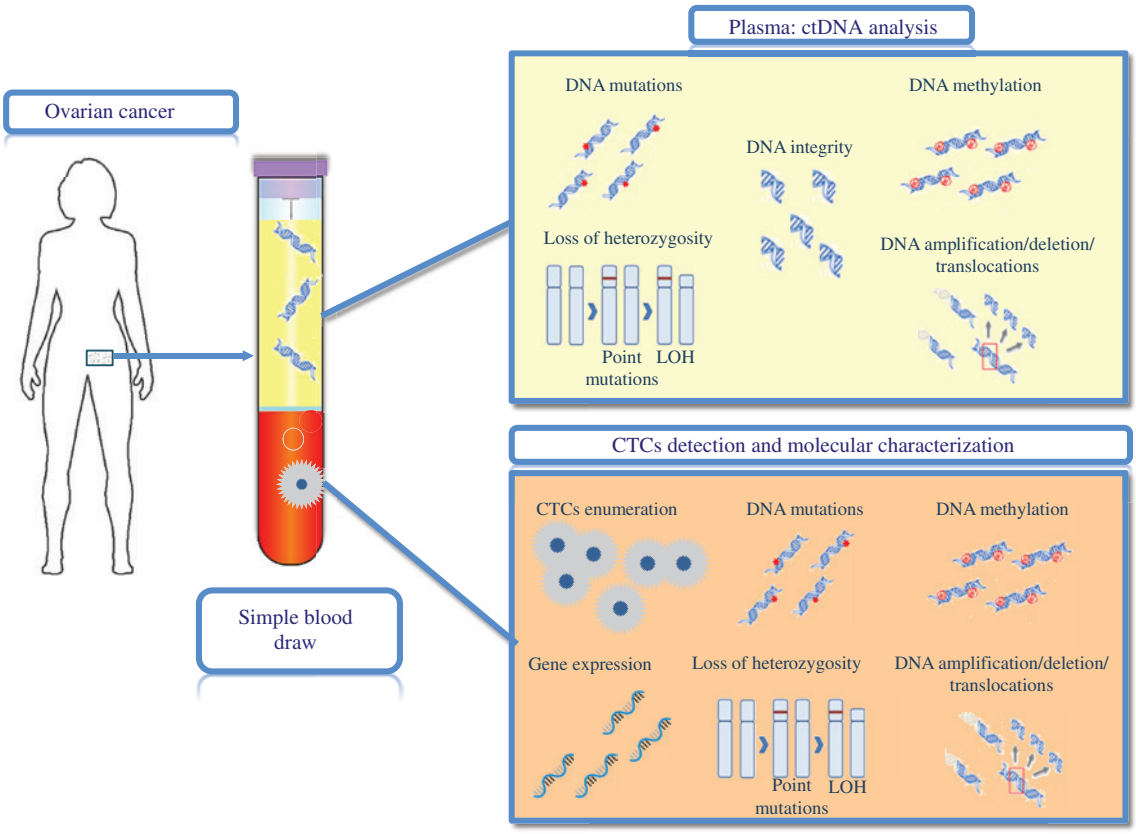

Figure 1: CTCs and ctDNA in ovarian cancer.

also focus on the possible role of liquid biopsy approaches in early diagnosis, prognosis of clinical outcome and the prediction of chemotherapy response or the development of chemoresistance in ovarian cancer patients.

Circulating tumor cells (CTCs)

Recent studies attempted to show the prognostic and predictive value of CTCs as tumor biomarkers in patients with ovarian cancer [16], and three meta-analyses report these associations using the appropriate methods for the results statistical analysis [17–19]. Different detection methods were used, mainly based on immunocytochemistry (microscopic detection or the FDA-approved CellSearch® system), RT-PCR (AdnaTest, QIAGEN, Hilden, Germany), and RT-qPCR for the quantification of CTCs levels [20, 21]. The time point of blood collection also differed, however, in the majority of studies the peripheral blood samples were obtained before surgical removal of the tumor. An overview of all research studies on CTCs in ovarian cancer patients is presented in Table 1.

The first studies on CTCs in ovarian cancer were based on the detection of CTCs using specific immunobeads [36] and an immunocytochemical (ICC) assay [35], respectively. Marth et al. [36] found carcinoma cells in the peripheral blood in 12% of ovarian cancer patients with a median follow-up of 25 months. The blood collection took place 7–20 days after surgery and before adjuvant chemotherapy. Judson et al. [35] detected CTCs in 18.7% of ovarian cancer patients with 18.7 months of a median follow-up time. They observed that most women with CTCs had grade 3 primary ovarian tumor compared to women without CTCs, and this evidence was significantly different. Both studies reported no significant association between the presence of CTCs in the peripheral blood and the clinical outcome of ovarian cancer patients [35, 36].

Fan et al. [34] first reported the prognostic significance of CTCs in primary ovarian cancer. They developed a new method for the detection of CTCs based on the ability of cancer cells to invade and ingest a cell adhesion matrix (CAM). In this study, CTC detection was based on ICC using the epithelial markers epithelial cell adhesion molecule (EpCAM), epithelial specific antigen (ESA) and a panel of seven pan-cytokeratins. They reported that the CAM + CTCs were invasive and their presence significantly correlated with decreased progression-free survival(PFS) ($p = 0.042$) [34]. The same group evaluated the prognostic

Table 1: CTCs in ovarian cancer.

Author	Year	Sampling time	Ovarian cancer patients	CTCs isolation	CTCs detection	Targeted antigen/gene	OS	PFS
Chebouti et al. [22]	2016	Before surgery (BS) and after chemotherapy (AC)	65	AdnaTest Ovarian CancerSelect	AdnaTest Ovarian CancerDetect/RT-PCR	*EpCAM*, *MUC1*, *MUC16*, *ERCC1*	p = 0.0008 (AC)	p = 0.0293 (AC)
Blassl et al. [23]	2016	Before surgery	10 (3 pts: single cell analysis)	AdnaTest Ovarian CancerSelect/ AdnaTest EMT-1/StemCellSelect	Multiplex-RT-PCR/ AdnaTest Ovarian CancerDetect	Three multi-marker panels for epithelial, EMT and stem cells associated transcipts	NR	NR
Kolostova et al. [24]	2016	Before surgery and longitudinally	56	Size-based (MetaCell®)	Cytomorphological/qPCR	12 gene panel including: *EpCAM*, *MUC1*, *MUC16*, *CK18*,*19*, *ERCC1*	NR	NR
Kolostova et al. [25]	2015	Before surgery	118 (20 pts: gene expression study)	Size-based (MetaCell®)	Cytomorphological/qPCR	*EpCAM*, *MUC1*, *MUC16*, *CK18*,*19*	NR	NR
Pearl et al. [26]	2015	Before surgery, before chemotherapy and during a 24 months follow-up	123 (31 pts: monitoring study)	Immunomagnetic CAM + method	ICC	EpCAM, ESA, CA125, DPP4	NR	p < 0.00001
Pearl et al. [27]	2014	Before surgery	76	Immunomagnetic CAM + method	ICC	EpCAM, ESA, CA125, DPP4	p = 0.0219	p = 0.0024
Kuhlmann et al. [28]	2014	Before surgery	143	AdnaTest Ovarian CancerSelect	RT-PCR (AdnaTest)	*EpCAM*, *MUC1*, *MUC16*, *ERCC1*	p = 0.026	p = 0.009
Liu et al. [29]	2013	Serial measurements during chemotherapy	78	CellSearch®	CellSearch®	EpCAM, CK8,18,19	NS	NS
Obermayr et al. [30]	2013	Before surgery and after chemotherapy	216	Density gradient centrifugation	RT-qPCR	12 gene panel including: *PPIC*, *EpCAM*	p = 0.001 (AC)	p = 0.001 (AC)
Behbakht et al. [31]	2011	Before and after temsirolimus	54	CellSearch®	CellSearch®	EpCAM, CK8,18,19, M30	NS	NS
Aktas et al. [32]	2011	Before surgery and/or after chemotherapy	122	AdnaTest	RT-PCR (AdnaTest)	*EpCAM*, *MUC1*, *HER2*, *CA125*	p = 0.0054 (BS) p = 0.047 (AC)	NS
Poveda et al. [33]	2011	After first line chemotherapy	216	CellSearch®	CellSearch®	EpCAM, CK8,18,19	p = 0.0017	p = 0.0024
Fan et al. [34]	2009	Before surgery	58	Immunomagnetic CAM + method	ICC	EpCAM, ESA, CK4,5,6,8,10,13,18	NS	p = 0.042
Judson et al. [35]	2003	Before surgery	53	Immunomagnetic microbeads	ICC	CK7,8,18,20, TFS-2, EGFR	NS	NS
Marth et al. [36]	2002	After surgery/before chemotherapy	90	Immunomagnetic (Dynabeads)	Immunomagnetic beads	MOC-31	NS	NS

NS, no significance; NR, not reported; OS, overall surviaval; PFS, progression-free survival.

significance of CTCs in a group of 129 pre-surgery ovarian cancer patients using the same method for the detection and identification of CTCs and observed statistically significant association between the presence of CTCs and both OS ($p = 0.0219$) and PFS ($p = 0.0024$) [27]. The same group also investigated the predictive value of CTC levels in a small group of 31 ovarian cancer patients that received standard taxol/carboplatin chemotherapy, where blood specimens were obtained at different time points, before and after surgery and up to 24 months after chemotherapy treatment. Using the same assay [27], they showed a statistically significant association between CTC levels and disease progression [26].

Aktas et al. investigated the prognostic value of CTCs in a large cohort of 122 ovarian cancer patients, before surgery and/or after platinum-based chemotherapy. They used the commercially available AdnaTest BreastCancer (QIAGEN, Hilden, Germany), for the isolation and detection of CTCs. AdnaTest BreastCancer is based on immunomagnetic enrichment targeting EpCAM and anti-mucin 1 (MUC1), followed by multiplex RT-PCR for EpCAM, MUC1 and human growth factor receptor 2 (HER2/neu). *CA-125* transcripts were also analyzed using a singleplex RT-PCR. CTCs were detected in 19% of patients before surgery and in 27% after platinum-based chemotherapy. According to their findings, the presence of CTCs significantly correlated with shorter OS before surgery ($p = 0.0054$) and after chemotherapy ($p = 0.047$) [32]. In a more recent study, Kuhlmann et al. investigated the predictive value of *ERCC1*-positive CTCs in 143 pre-surgery epithelial ovarian cancer patients. AdnaTest OvarianCancerSelect (QIAGEN, Hilden, Germany) was used for the immunomagnetic tumor cell enrichment in blood samples and AdnaTest OvarianCancerDetect (QIAGEN, Hilden, Germany) for the molecular characterization of CTCs. *ERCC1* transcript detection was performed using singleplex RT-PCR. The presence of CTCs was confirmed in 14% of patients and was significantly correlated with OS ($p = 0.041$). *ERCC1*-positive CTCs (*ERCC1* + CTC) were detected in 8% of patients and significantly correlated with both OS ($p = 0.026$) and PFS ($p = 0.009$). A very interesting finding in this study was the association of *ERCC1* + CTC with platinum resistance. The presence of *ERCC1* + CTC at primary diagnosis independently predicted platinum resistance ($p = 0.010$), although the ICC analysis of ERCC1 expression in primary tumor tissue did not reveal any prognostic or predictive value [28]. In their very recently published study, they were able to show that the additional assessment of *ERCC1*-transcripts enhances overall CTC detection rate in ovarian cancer patients before surgery and after chemotherapy and defines an additional highly overlapping fraction of *ERCC1*-expressing CTCs, which is potentially selected by platinum-based chemotherapy. Moreover, we describe that the assessment of CTC-derived *ERCC1*-transcripts alone is almost equivalently sufficient in detecting *ERCC1*-expressing prognostic relevant CTCs. We further showed that the presence of *ERCC1* + CTCs after chemotherapy correlates with post-therapeutic outcome of ovarian cancer and particularly, dynamics of *ERCC1* + CTCs mirror response to platinum-based chemotherapy [22].

Poveda et al. [33] also confirmed the prognostic impact of CTC detection in ovarian cancer after chemotherapy. They reported a correlation of CTC numbers with shorter OS ($p = 0.0017$) and PFS ($p = 0.0024$) in a phase III clinical trial (NCT00113607, www.clinicaltrials.gov) of pegylated liposomal doxorubicin (PLD) with trabectedin versus PLD for relapsed ovarian cancer. They used for the first time the CellSearch® system (Janssen Diagnostics) for CTC isolation and enumeration in 216 ovarian cancer patients. Behbakht et al. also used the CellSearch® system for CTC enrichment and enumeration in a phase II clinical trial (NCT00429793, www.clinicaltrials.gov) for the evaluation of the efficacy of the mTOR inhibitor temsirolimus. Fifty four recurrent ovarian cancer patients were recruited and blood specimens were obtained before and after treatment with temsirolimus. No significant association between the presence of CTCs with PFS and OS was reported [31]. Liu et al. [29] also used the CellSearch® system in 78 newly diagnosed and recurrent ovarian cancer patients. They performed serial measurements during chemotherapy, but according to their findings, the number of CTCs did not correlate with PFS or OS.

Obermayr et al. [37] developed a six-marker gene panel for the molecular detection of CTCs on female cancer patients, including ovarian cancer, using a RT-qPCR platform. The multimarker analysis using this novel panel positively identified 19% of the 23 ovarian cancer patients. The same group aimed to identify novel markers for the characterization of CTCs in ovarian cancer, using a density gradient centrifugation-based method for the isolation and RT-qPCR for CTC detection and quantification. They defined a sample as CTC positive if at least one of the 11 gene marker panels was found over-expressed. By using this gene panel, they detected CTCs in 24.3% of the baseline (before primary treatment) and 20.4% of the follow-up (6 months after chemotherapy) samples. In two-thirds of the patients, cyclophilin C gene (*PPIC*) overexpression was observed, but only a few samples were identified by EpCAM overexpression. *PPIC*-positive CTCs during follow-up were detected significantly more often in platinum-resistant than platinum-sensitive follow-up patients. This

fact also indicated poor outcome independently from other prognostic parameters [30].

Kolostova et al. [38] developed a novel size-based method (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic) for the enrichment and separation of viable CTCs, followed by in vitro CTCs culturing and cytomorphological analysis and finally, CTC molecular characterization by gene expression studies using qPCR. They isolated and cultivated CTCs in 77 (65.2%) of 118 pre-surgery advanced-stage ovarian cancer patients. Gene expression analysis was performed in 20 selected positive samples by cytomorphological analysis. They looked at possible associations between CTC presence and clinicopathological characteristics of the patients, mainly with the CA-125 status. Based on their results, they proposed a new and independent prognosis staging information. They also suggest that hematogenous metastasis route is represented by CTCs and elevated CA-125 levels indicate lymphogenic dissemination [25]. Using the same methodology, this group aimed to isolate and identify CTCs in 56 ovarian cancer patients. In this study, gene expression analysis was performed in all samples found positive by cytomorphological analysis. They reported that EpCAM relative expression is elevated in CTC-enriched fractions compared to whole peripheral blood sample and that this expression grows with in vitro cultivation time. They suggested that a seven-gene panel, including EpCAM and MUC16, could better confirm the presence of CTCs in peripheral blood of ovarian cancer patients, than a one-marker test [24]. Both studies did not provide any information on the patients clinical outcome with regard to OS and/or PFS data [24, 25].

A very recent study on CTCs in ovarian cancer proposed a multi-marker gene panel for gene expression profiling of single CTCs [23]. Blassl et al. used the AdnaTest OvarianCancerSelect (QIAGEN, Hilden, Germany) and/or the AdnaTest EMT-1/StemCellSelect (QIAGEN, Hilden, Germany) for CTC isolation and enrichment in peripheral blood samples of 10 pre-surgery epithelial ovarian cancer patients. CTCs were detected and characterized by using the AdnaTest OvarianCancerDetect (QIAGEN, Hilden, Germany) and the AdnaTest EMT-1/StemCellDetect. They isolated single cells using CellCelector (ALS GmbH, Jena, Germany) from only three ovarian cancer patients. Single CTCs were characterized by multiplex-RT-PCR, followed by capillary electrophoresis. The multiplex-RT-PCR gene panel included stem cell (CD44, ALDH1A1, Nanog, Oct 4) and EMT (N-cadherin, Vimentin, Snail2, CD117, CD146) markers. They observed inter-cellular and intra/inter-patient heterogeneity and co-expression of epithelial, mesenchymal and stem cell transcripts on the same CTC simultaneously [23].

Cell-free DNA (cfDNA)

A sufficient number of studies on cfDNA in patients with ovarian cancer pursued to clarify its clinical value [39]. For this purpose, they quantified total cfDNA and/or the circulating cell-free mitochondrial DNA (mtDNA) levels in some cases, or aimed at the detection of different genetic and epigenetic alterations, such as chromosomal abnormalities and specific tumor LOH, cancer-related somatic gene mutations and aberrant DNA methylation. Additionally, in a recent case study, Martignetti et al. [40] detected the *FGFR2-FAM76A* tumor-specific fusion in cfDNA of an advanced stage serous epithelial ovarian cancer patient.

However, in some cases, the results are still controversial. The discrepancies probably occur due to the different methods and pre-analytical conditions, the use of serum instead of plasma by some researchers and the different volumes of plasma/serum for cfDNA extraction. Many studies focused on the potential use of cfDNA as a diagnostic, prognostic and predictive biomarker in ovarian cancer and a recent meta-analysis by Zhou et al. attempted to evaluate the role of cfDNA in ovarian cancer diagnosis [41]. An overview of the research studies on cfDNA in ovarian cancer is summarized in Table 2.

The first studies on ovarian cancer circulating DNA attempted to quantify the total cfDNA amount, or the nuclear and mitochondrial DNA amounts separately, in plasma or serum of ovarian cancer patients. One of the first studies on cfDNA in ovarian cancer screening aimed to quantify plasma cfDNA using a real-time PCR assay for three reference genes and to determine the number of genome equivalents (GE) using a standard curve. Kamat et al. [42] reported that cfDNA levels in advanced ovarian cancer samples were elevated when compared to controls. A more recent study on ovarian cancer screening using cfDNA quantification showed a significant increase in serum cfDNA of advanced stage ovarian cancer patients compared to early stage ($p < 0.01$). Shao et al. [47] also reported a correlation between serum cfDNA levels and ovarian cancer occurrence using receiver operating characteristic (ROC) curves and a branched DNA (bDNA) technique for cfDNA quantification.

Kamat et al. also investigated the prognostic value of cfDNA in epithelial ovarian cancer. They quantified plasma cfDNA levels in 164 epithelial ovarian cancer patients using real-time PCR for β-globin and determined the number of GE. They reported a significant association of cfDNA > 22,000 GE/mL with decreased PFS ($p < 0.001$) and this association was shown as an independent prognostic value ($p = 0.02$) after adjusting for other clinical

Table 2: cfDNA in ovarian cancer.

cfDNA	Author	Year	Source	Ovarian cancer patients	Targeted gene	Early detection	Prognosis	Response to treatment
DNA amount (cfDNA)	Kamat et al. [42]	2006	Plasma	19	*GADPH*, *β-actin*, *β-globin*	Yes		
	Capizzi et al. [43]	2008	Plasma	22	*hTERT*			Yes
	Kamat et al. [44]	2010	Plasma	164	*GADPH*, *β-actin*		Yes	
	No et al. [45]	2012	Serum	36	*B2M*, *RAB25*, *CLDN4*, *ABCF2*		Yes	
	Steffensen et al. [46]	2014	Plasma	144	*Cyclophilin A*		Yes	
	Shao et al. [47]	2015	Serum	36	NR (bDNA technique)	Yes		
Mitochondrial (mtDNA)	Zachariah et al. [48]	2008	Plasma/serum	21	*MTATP8*	Yes		
	Choudhuri et al. [49]	2014	Plasma	100 (20 follow-up)	*MTATP8*			Yes
Chromosomal abnormalities/LOH (ctDNA)	Kuhlmann et al. [13]	2012	Serum	63	-		Yes	
	Harris et al. [50]	2016	Plasma	10	-	NR	NR	NR
	Cohen et al. [51]	2016	Plasma	32	-	Yes		
	Vanderstichele et al. [52]	2016	Plasma	57	-	Yes		
Somatic mutations (ctDNA)	Otsuka et al. [53]	2004	Plasma	27	*TP53*	Yes		
	Swisher et al. [54]	2005	Plasma/serum	69	*TP53*		Yes	
	Dobrzycka et al. [55]	2011	Plasma	126	*KRAS*		Yes	
	Forshew et al. [56]	2012	Plasma	46	*TP53*, *PTEN*, *EGFR*, *BRAF*, *KRAS*, *PIK3CA*	Yes		
	Murtaza et al. [57]	2013	Plasma	3	*RB1*, *ZEB2*, *MTOR*, *CES4A*, *BUB1*, *PARP8*			Yes
	Bettegowda et al. [58]	2014	Plasma	7	Panels including: *TP53*, *PIK3CA*, *BRAF*, *EGFR*	Yes		Yes
	Pereira et al. [59]	2015	Serum	22	Panels including: *TP53*, *PIK3CA*, *MET*, *PTEN*, *KRAS*, *BRAF*, *FBXW7*			Yes
Aberrant methylation (ctDNA)	Gifford et al. [60]	2004	Plasma	138	*hMLH1*		Yes	
	Ibanez et al. [61]	2004	Plasma/serum	50	*BRCA1*, *RASSF1A*	Yes		
	Melnikov et al. [62]	2009	Plasma	33	*BRCA1*, *HIC1*, *PAX5*, *PGR-PROX*, *THBS1*	Yes		
	Liggett et al. [63]	2011	Plasma	30	*RASSF1A*, *CALCA*, *EP300*, *PGR-PROX*, *BRCA1*, *CDKN1C*	Yes		
	Bondurant et al. [64]	2011	Serum	106	*RASSF1A*			Yes
	Giannopoulou et al. [65]	2017	Plasma	59	*RASSF1A*		Yes	
	Dong et al. [66]	2012	Serum	36	*SLIT2*	Yes		
	Zhang et al. [67]	2013	Serum	87	*APC*, *RASSF1A*, *CDH1*, *RUNX3*, *TFPI2*, *SFRP5*, *OPCML*	Yes		
	Wu et al. [68]	2014	Plasma	47	*RASSF2A*	Yes		
	Zhou et al. [69]	2014	Serum	45	*OPCML*	Yes		
	Wang et al. [70]	2015	Serum	114	*RUNX3*, *TFPI2*, *OPCML*	Yes		

characteristics [44]. On the contrary, No et al. [45] examined the prognostic value of cfDNA and reported no significant difference between cfDNA levels of cancer patients and patients with benign disease. They recruited 36 epithelial ovarian cancer samples and 16 benign tumor samples and used commercially available copy number assay kits to measure cfDNA levels of four selected genes, but they used serum as cfDNA source instead of plasma.

In a more recent study, Steffensen et al. measured plasma cfDNA levels of 144 multiresistant epithelial ovarian cancer patients treated with bevacizumab using real-time PCR for cyclophiline A gene. They found a statistically significant correlation between cfDNA levels and both PFS ($p = 0.0004$) and OS ($p = 0.005$) in both univariate and multivariate survival analyses. Thus, they concluded that plasma cfDNA is an independent prognostic factor in platinum-resistant ovarian cancer patients treated with bevacizumab [46].

Ten years ago, Kamat et al. [71] proposed the potential use of tumor-specific cfDNA levels in predicting tumor response to chemotherapy, by using an orthotopic mouse model. Capizzi et al. further investigated the predictive value of cfDNA in ovarian cancer patients. They quantified plasma cfDNA levels before and after chemotherapy in 22 epithelial ovarian cancer patients of a prospective nonrandomized clinical study and found a significant discrimination between patients and healthy controls and a correlation of cfDNA amounts with response to standard chemotherapy [43].

Altered circulating cell-free mtDNA content may serve as a potential cancer biomarker in many solid malignancies [72]. In ovarian cancer, only two studies include the determination of circulating cell-free mtDNA levels. Zachariah et al. quantified nuclear cfDNA and circulating cell-free mtDNA levels using a multiplex qPCR assay, in serum and plasma of patients with epithelial ovarian cancer, benign epithelial tumors and endometriosis, and a healthy control group. They found a significant increase in nuclear cfDNA and circulating cell-free mtDNA amounts in ovarian cancer patients compared to both healthy group and benign epithelial tumor patients. Interestingly, they reported a significant difference between ovarian cancer patients and the endometriosis group circulating cell-free mtDNA, but not in nuclear cfDNA [48]. More recently, Choudhuri et al. investigated whether nuclear cfDNA and circulating cell-free mtDNA levels can be used for advanced epithelial ovarian cancer diagnosis and for the prediction of treatment response. They recruited 100 patients and measured both levels before treatment, but in only 20 patients after the completion of chemotherapy. A significant difference was reported in nuclear cfDNA levels of the follow-up patients before and after treatment, but not in circulating cell-free mtDNA levels [49].

Circulating tumor DNA (ctDNA)

Circulating tumor DNA (ctDNA) constitutes a tiny subgroup of total cfDNA in the peripheral blood of cancer patients [73]. The following studies refer on specific aberrations characterizing ctDNA shed in the circulation from the primary ovarian tumor. They are classified according to specific genetic or epigenetic alterations detected only in ctDNA, shown as below.

Chromosomal abnormalities/LOH

It is well known that ovarian cancer and in particular the high-grade serous ovarian cancer (HGSC) subtype, is characterized by frequent chromosomal instability [5]. Recent studies aimed to detect copy number variations (CNV) [51] and to quantify specific LOH [13] or aberrant somatic chromosomal rearrangements [50] in ctDNA of ovarian cancer patients. Kuhlmann et al. quantified cfDNA of 63 primary epithelial ovarian cancer patients before surgery and after chemotherapy. They used a PCR-based fluorescence microsatellite analysis in order to measure the LOH in two fractions of cfDNA, the high- and low molecular-weight fraction (HMWF and LMWF, respectively). They reported that LOH at two markers can predict tumor grade ($p = 0.033$) and FIGO stage ($p = 0.004$) in the LMWF cfDNA. Remarkably, a LOH at another marker can significantly predict patients OS ($p = 0.030$) in both HMWF and LMWF [13].

Harris et al. introduced an algorithm for the quantification of cfDNA using a qPCR assay in order to predict relapse and treatment efficacy. They identified aberrant chromosomal junctions in primary tumors of 10 ovarian cancer patients and detected them in plasma ctDNA of eight patients before surgery. In three cases, ctDNA was also detected after surgery, indicating the presence of the disease, but in the remaining five cases, ctDNA was absent after surgery, indicating the consequential absence of the disease [50].

The first study on ovarian cancer screening using CNV detection in cfDNA was elaborated by Cohen et al. [51]. They applied a well-established non-invasive prenatal testing (NIPT) commercial platform in cfDNA of 16 pre-surgery early and 16 advanced HGSC patients. The obtained sequencing data were analyzed for the detection

of subchromosomal changes and the determination of whole chromosome gains or losses. They detected 40.6% of all HGSC cases, and more specifically, 38% of early stages, indicating a potential utility for early HGSC screening in plasma cfDNA based on specific multiple segmental chromosome gains and losses [51]. However, more validation studies along with the improvement of pre-analytical conditions and the examination of paired tumor DNA are needed before the routine application of this approach [74].

Vanderstichele et al. reported for the first time the potential of using cfDNA for primary HGSC diagnosis. They recruited 68 patients with an adnexal mass, including 57 diagnosed with invasive or borderline carcinoma and 11 with benign disease. They measured specific patterns of chromosomal instability in plasma cfDNA of all patients and reported a significantly higher quantitative measure of chromosomal instability in ovarian cancer patients compared to patients with benign disease or healthy individuals [52].

Somatic mutations

Few studies attempted to detect tumor-specific somatic mutations in ctDNA of epithelial ovarian cancer patients. Otsuka et al. [53] first identified *TP53* mutations in only two/12 pre-surgery plasma cfDNA of patients with ovarian cancer. A tumor-specific *TP53* mutation was also detected in 21 out of 69 cfDNA samples of epithelial ovarian cancer patients in a study by Swisher et al. The presence of ctDNA characterized by this mutation was significantly associated with decreased survival ($p = 0.02$) [54]. Mutations of *KRAS* gene were investigated by Dobrzycka et al. in plasma cfDNA of 126 epithelial ovarian cancer patients. They detected *KRAS* mutations in 43.7% of patients and reported a significantly decreased OS for patients with serous ovarian tumors and detectable cfDNA ($p = 0.022$) [55].

The development of very sensitive novel technologies for ctDNA detection overcomes the issue of the extremely low concentrations of ctDNA out of the total cfDNA. Based on this concept, Forshew et al. proposed a different approach for the detection and identification of cancer-specific mutations in plasma ctDNA. They established a novel method for targeted deep sequencing (Tam-Seq) of mutations at low allele frequencies (AF) with increased sensitivity and specificity, and measured mainly the frequencies of *TP53* mutant alleles at ctDNA of 46 advanced stage HGSC patients. Remarkably, an *EGFR* mutation was detected in one ctDNA sample but not in the initial ovarian tumor tissue. All results were confirmed using digital PCR [56].

Murtaza et al. performed whole exome sequencing in plasma ctDNA of three ovarian cancer patients. Serial sample measurements and quantification of allele fractions in ctDNA led to the identification of specific gene mutations related to acquired resistance to treatment. The genes with significantly increased mutant AFs are shown in Table 2. All results were confirmed using both digital PCR and Tam-Seq assay [57].

Another study by Bettegowda et al. accomplished the detection of ctDNA using digital PCR-based assays for mutation analyses in a large cohort of patients with different malignancies, including seven patients with advanced stage ovarian cancer. They detected ctDNA in most metastatic cancer patients and quantified the mutant fragments for the determination of cfDNA concentration. They reported a high mutant allele fragments (approximately 10,000 per 5 mL) for advanced ovarian cancer patients [58].

In a more recent study, Pereira et al. recruited patients with gynecological malignancies, including 22 ovarian patients, and identified specific cancer-related mutations using whole exome and targeted sequencing. They also measured and quantified ctDNA levels using droplet digital PCR (ddPCR). The detectable ctDNA after treatment significantly predicted survival for eight ovarian cancer patients, indicating a possible role of ctDNA measurements in personalized medicine [59].

Aberrant methylation

Epigenetic alterations hold an important role in cancer initiation and progression and aberrant DNA methylation patterns, mainly characterized by promoter hypermethylation, are a frequent event in most human cancers [75]. Epigenetic inactivation of a tumor suppressor gene often results from its promoter methylation and is considered as an early event during carcinogenesis [76]. Many studies have reported methylation changes in ovarian cancer [77] and a recent review summarizes the differences in the observed methylation patterns in the main histological subtypes of the disease, including HGSC [78]. DNA methylation changes have the potential to serve as biomarkers for early diagnosis of gynecological malignancies [79]. This is also observed in Table 2; only one study by Gifford et al. [60] aimed to show the prognostic value of ctDNA methylation in ovarian cancer.

In this study, the researchers investigated *hMLH1* methylation status in plasma cfDNA of 138 epithelial ovarian cancer patients enrolled in a phase III clinical trial (NCT00003998, www.clinicaltrials.gov), before

carboplatin/taxoid chemotherapy and at relapse. They reported an increase in *hMLH1* methylation at relapse and the remarkable presence of cfDNA methylation at 25% of relapse patients that was not detected before chemotherapy. This acquired methylation provided significant clinical information for patients OS ($p = 0.007$) [60].

Ibanez et al. examined *RASSF1A* and *BRCA1* hypermethylation in cfDNA of 50 epithelial ovarian cancer patients and first confirmed the detection of methylation in early stage (stage I, II) patients, using methylation specific PCR (MSP). They also observed a concordance between tumor and plasma/serum DNA methylation patterns in 82% of matched samples [61].

A microarray mediated methylation assay (MethDet test) was developed by Melnikov et al. [62] and its application in 33 serous ovarian cancer patients led to the characterization of a five genes panel for ovarian cancer detection. The same group used this assay in three cohorts of serous ovarian cancer patients, benign ovarian disease patients and healthy controls. Liggett et al. [63] now reported the distinctive promoter methylation of all three groups according to the methylation status of six selected genes.

A larger study by Bondurant et al. quantified *RASSF1A* promoter methylation in 106 serous ovarian cancer cfDNA samples, using a novel quantitative real-time PCR assay. They found *RASSF1A* promoter methylation in about half of ovarian cancer patients and observed agreement in the methylation status of 20 available paired tumor/serum samples. Interestingly, they measured *RASSF1A* methylation in nine patients over the course of treatment and found a concordance between cfDNA methylation changes and disease progression for eight patients, suggesting a possible role of cfDNA methylation in ovarian cancer prognosis [64].

Our group also reported *RASSF1A* promoter methylation in plasma ctDNA of 15/59 patients with high-grade serous ovarian cancer using a real-time MSP assay. We performed the first comparison study on *RASSF1A* promoter methylation in primary tumors, adjacent tissues and plasma samples in HGSC patients and we observed an agreement between primary tumor samples and corresponding plasma in 62.3% of cases studied [65].

Zhang et al. developed a multiplex-MSP assay for the early detection of ovarian cancer. They recruited 87 epithelial ovarian cancer patients and examined the serum cfDNA methylation status of seven selected genes simultaneously. A sample was characterized as positive, if at least one gene was found methylated [67]. In a more recent study by Wang et al., a multiplex-nested MSP was also developed for the detection of three genes methylation in 114 serum cfDNA of epithelial ovarian cancer patients. cfDNA methylation levels were significantly increased in ovarian cancer patients compared to benign disease patients and healthy control groups [70].

Furthermore, studies on *SLIT2* [66], *OPCML* [69] and *RASSF2A* [68] promoter methylation in cfDNA of epithelial ovarian cancer patients demonstrate the frequently aberrant methylation status of these genes and suggest a possible role for ovarian cancer early detection.

Methylation patterns in whole-blood DNA and white blood cell (WBC) DNA in ovarian cancer patients have been also examined using methylation arrays and bisulfite pyrosequencing. Teschendorff et al. [80] performed a methylation study in peripheral blood DNA of pre- and post-treatment ovarian cancer patients and they observed a significantly different methylation pattern in blood DNA of epithelial ovarian cancer patients compared to healthy controls. Flanagan et al. [81] investigated WBCs DNA methylation status in 880 epithelial ovarian cancer patients enrolled in a phase III clinical trial (NCT00003998, www.clinicaltrials.gov), using bisulfite pyrosequencing and reported a significant correlation between mean *SFN* methylation and PFS ($p = 0.016$). The same group analyzed blood DNA methylation patterns in 247 ovarian cancer patients enrolled in the previous clinical trial. They identified specific CpGs alterations in blood DNA at relapse after platinum-based chemotherapy and found an independent significant association with survival ($p = 2.8 \times 10^{-4}$) [82].

Conclusions

The development of a cancer biomarker and its implementation in the clinical routine requires a multistage procedure and constitutes the final result of multiannual and toilsome research approaches. However, multiple pre-analytical, analytical and post-analytical issues should be overcome and studies on the assay validations with regard to repeatability and reproducibility are also necessary [83]. The lack of effective biomarkers for early detection, prognosis of clinical outcome and response to treatment contributes to the maintenance of low survival rates for ovarian cancer patients, despite the numerous research studies on the field, the last decades. Liquid biopsy procedures are minimally invasive and allow for the easily tolerated serial sample measurements during the course of treatment. This can help towards the establishment of more efficient personalized therapeutic algorithms and real-time therapy monitoring. Nevertheless, specific challenges should be taken into account for CTCs and

ctDNA analyses, including pre-analytical issues about the sample volume, the proper tubes for sample collection, the samples storage and the time of the analysis, quality control and analytical validation of the assays.

The clinical significance of both CTCs and ctDNA has been revealed in many types of cancer [84], including ovarian cancer. However, no standard methods are used for the isolation and detection in the bloodstream and few studies recruited large cohorts of ovarian cancer patients. Further studies towards the validation, standardization and quality control of the assays used are a matter of utmost importance before the implementation of liquid biopsy approaches in the clinical routine.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015;65:5–29.
2. Du Bois A, Pfisterer J. Future options for first-line therapy of advanced ovarian cancer. Int J Gynecol Cancer 2005;15 Suppl 1:42–50.
3. du Bois A, Reuss A, Pujade-Lauraine E, Harter P, Ray-Coquard I, Pfisterer J. Role of surgical outcome as prognostic factor in advanced epithelial ovarian cancer: a combined exploratory analysis of 3 prospectively randomized phase 3 multicenter trials: by the Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR) and the Groupe d'Investigateurs Nationaux Pour les Etudes des Cancers de l'Ovaire (GINECO). Cancer 2009;115:1234–44.
4. Wimberger P, Wehling M, Lehmann N, Kimmig R, Schmalfeldt B, Burges A, 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. Network. TCGAR. Integrated genomic analyses of ovarian carcinoma. Nature 2011 Jun 29;474:609–15.
6. Patch AM, Christie EL, Etemadmoghadam D, Garsed DW, George J, Fereday S, et al. Whole-genome characterization of chemoresistant ovarian cancer. Nature 2015;521:489–94.
7. Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H, et al. Incorporation of bevacizumab in the primary treatment of ovarian cancer. N Engl J Med 2011;365:2473–83.
8. Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a pre-planned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. Lancet Oncol 2014;15:852–61.
9. Tan DS, Agarwal R, Kaye SB. Mechanisms of transcoelomic metastasis in ovarian cancer. Lancet Oncol 2006;7:925–34.
10. Yeung TL, Leung CS, Yip KP, Au Yeung CL, Wong ST, Mok SC. Cellular and molecular processes in ovarian cancer metastasis. A Review in the Theme: Cell and Molecular Processes in Cancer Metastasis. Am J Physiol Cell Physiol 2015;309:444–56.
11. Lianidou ES, Strati A, Markou A. Circulating tumor cells as promising novel biomarkers in solid cancers. Crit Rev Clin Lab Sci 2014;51:160–71.
12. Marzese DM, Hirose H, Hoon DS. Diagnostic and prognostic value of circulating tumor-related DNA in cancer patients. Expert Rev Mol Diagn 2013;13:827–44.
13. Kuhlmann JD, Schwarzenbach H, Wimberger P, Poetsch M, Kimmig R, Kasimir-Bauer S. LOH at 6q and 10q in fractionated circulating DNA of ovarian cancer patients is predictive for tumor cell spread and overall survival. BMC Cancer 2012;12:325.
14. Warton K, Samimi G. Methylation of cell-free circulating DNA in the diagnosis of cancer. Front Mol Biosci 2015;2:13.
15. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426–37.
16. Romero-Laorden N, Olmos D, Fehm T, Garcia-Donas J, Diaz-Padilla I. Circulating and disseminated tumor cells in ovarian cancer: a systematic review. Gynecol Oncol 2014;133:632–9.
17. 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. J Ovarian Res 2015;8:38.
18. Zeng L, Liang X, Liu Q, Yang Z. The predictive value of circulating tumor cells in ovarian cancer: a meta analysis. Int J Gynecol Cancer 2017;27:1109–17.
19. 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:e0130873.
20. Gasparri ML, Savone D, Besharat RA, Farooqi AA, Bellati F, Ruscito I, et al. Circulating tumor cells as trigger to hematogenous spreads and potential biomarkers to predict the prognosis in ovarian cancer. Tumour Biol 2016;37:71–5.
21. Van Berckelaer C, Brouwers AJ, Peeters DJ, Tjalma W, Trinh XB, van Dam PA. Current and future role of circulating tumor cells in patients with epithelial ovarian cancer. Eur J Surg Oncol 2016;30160–3.
22. Chebouti I, Kuhlmann JD, Buderath P, Weber S, Wimberger P, Bokeloh Y, et al. ERCC1-expressing circulating tumor cells as a potential diagnostic tool for monitoring response to platinum-based chemotherapy and for predicting post therapeutic outcome of ovarian cancer. Oncotarget 2017;8:24303–13.
23. Blassl C, Kuhlmann JD, Webers A, Wimberger P, Fehm T, Neubauer H. Gene expression profiling of single circulating tumor cells in ovarian cancer - Establishment of a multi-marker gene panel. Mol Oncol 2016;10:1030–42.
24. Kolostova K, Pinkas M, Jakabova A, Pospisilova E, Svobodova P, Spicka J, et al. Molecular characterization of circulating tumor cells in ovarian cancer. Am J Cancer Res 2016;6:973–80.

25. Kolostova K, Matkowski R, Jedryka M, Soter K, Cegan M, Pinkas M, et al. The added value of circulating tumor cells examination in ovarian cancer staging. Am J Cancer Res 2015;5:3363–75.
26. 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). Gynecol Oncol 2015;137:229–38.
27. 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. Gynecol Oncol 2014;134:581–90.
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. Clin Chem 2014;60:1282–9.
29. 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. Gynecol Oncol 2013;131:352–6.
30. 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. Gynecol Oncol 2013;128:15–21.
31. 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. Gynecol Oncol 2011;123:19–26.
32. 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. Int J Gynecol Cancer 2011;21:822–30.
33. 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. Gynecol Oncol 2011;122:567–72.
34. 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. Gynecol Oncol 2009;112:185–91.
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. Gynecol Oncol 2003;91:389–94.
36. Marth C, Kisic J, Kaern J, Trope C, Fodstad O. Circulating tumor cells in the peripheral blood and bone marrow of patients with ovarian carcinoma do not predict prognosis. Cancer 2002;94:707–12.
37. Obermayr E, Sanchez-Cabo F, Tea MK, Singer CF, Krainer M, Fischer MB, et al. Assessment of a six gene panel for the molecular detection of circulating tumor cells in the blood of female cancer patients. BMC Cancer 2010;10:666.
38. Kolostova K, Spicka J, Matkowski R, Bobek V. Isolation, primary culture, morphological and molecular characterization of circulating tumor cells in gynecological cancers. Am J Transl Res 2015;7:1203–13.
39. Esposito A, Bardelli A, Criscitiello C, Colombo N, Gelao L, Fumagalli L, et al. Monitoring tumor-derived cell-free DNA in patients with solid tumors: clinical perspectives and research opportunities. Cancer Treat Rev 2014;40:648–55.
40. 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;16:97–103.
41. Zhou Q, Li W, Leng B, Zheng W, He Z, Zuo M, et al. Circulating cell free DNA as the diagnostic marker for ovarian cancer: a systematic review and meta-analysis. PLoS One 2016;11:e0155495.
42. 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. Ann N Y Acad Sci 2006;1075:230–4.
43. Capizzi E, Gabusi E, Grigioni AD, De Iaco P, Rosati M, Zamagni C, et al. Quantification of free plasma DNA before and after chemotherapy in patients with advanced epithelial ovarian cancer. Diagn Mol Pathol 2008;17:34–8.
44. Kamat AA, Baldwin M, Urbauer D, Dang D, Han LY, Godwin A, et al. Plasma cell-free DNA in ovarian cancer: an independent prognostic biomarker. Cancer 2010;116:1918–25.
45. No JH, Kim K, Park KH, Kim YB. Cell-free DNA level as a prognostic biomarker for epithelial ovarian cancer. Anticancer Res 2012;32:3467–71.
46. Steffensen KD, Madsen CV, Andersen RF, Waldstrom M, Adimi P, Jakobsen A. Prognostic importance of cell-free DNA in chemotherapy resistant ovarian cancer treated with bevacizumab. Eur J Cancer 2014;50:2611–8.
47. Shao X, He Y, Ji M, Chen X, Qi J, Shi W, et al. Quantitative analysis of cell-free DNA in ovarian cancer. Oncol Lett 2015;10:3478–82.
48. Zachariah RR, Schmid S, Buerki N, Radpour R, Holzgreve W, Zhong X. Levels of circulating cell-free nuclear and mitochondrial DNA in benign and malignant ovarian tumors. Obstet Gynecol 2008;112:843–50.
49. Choudhuri S, Sharma C, Banerjee A, Kumar S, Kumar L, Singh N. A repertoire of biomarkers helps in detection and assessment of therapeutic response in epithelial ovarian cancer. Mol Cell Biochem 2014;386:259–69.
50. Harris FR, Kovtun IV, Smadbeck J, Multinu F, Jatoi A, Kosari F, et al. Quantification of somatic chromosomal rearrangements in circulating cell-free DNA from ovarian cancers. Sci Rep 2016;6:29831.
51. Cohen PA, Flowers N, Tong S, Hannan N, Pertile MD, Hui L. Abnormal plasma DNA profiles in early ovarian cancer using a non-invasive prenatal testing platform: implications for cancer screening. BMC Med 2016;14:126.
52. Vanderstichele A, Busschaert P, Smeets D, Landolfo C, Van Nieuwenhuysen E, Leunen K, et al. Chromosomal instability in cell-free DNA as a highly specific biomarker for detection of ovarian cancer in women with adnexal masses. Clin Cancer Res 2017;23:2223–31.
53. Otsuka J, Okuda T, Sekizawa A, Amemiya S, Saito H, Okai T, et al. Detection of p53 mutations in the plasma DNA of patients with ovarian cancer. Int J Gynecol Cancer 2004;14:459–64.
54. Swisher EM, Wollan M, Mahtani SM, Willner JB, Garcia R, Goff BA, et al. Tumor-specific p53 sequences in blood and peritoneal fluid of women with epithelial ovarian cancer. Am J Obstet Gynecol 2005;193:662–7.
55. Dobrzycka B, Terlikowski SJ, Kinalski M, Kowalczuk O, Niklinska W, Chyczewski L. Circulating free DNA and p53 antibodies in plasma of patients with ovarian epithelial cancers. Ann Oncol 2011;22:1133–40.

56. Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 2012;4:136ra68.
57. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497:108–12.
58. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014;6:224.
59. 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:e0145754.
60. Gifford G, Paul J, Vasey PA, Kaye SB, Brown R. The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. Clin Cancer Res 2004; 10:4420-6.
61. Ibanez de Caceres I, Battagli C, Esteller M, Herman JG, Dulaimi E, Edelson MI, et al. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. Cancer Res 2004;64:6476–81.
62. Melnikov A, Scholtens D, Godwin A, Levenson V. Differential methylation profile of ovarian cancer in tissues and plasma. J Mol Diagn 2009;11:60–5.
63. Liggett TE, Melnikov A, Yi Q, Replogle C, Hu W, Rotmensch J, et al. Distinctive DNA methylation patterns of cell-free plasma DNA in women with malignant ovarian tumors. Gynecol Oncol 2011;120:113–20.
64. Bondurant AE, Huang Z, Whitaker RS, Simel LR, Berchuck A, Murphy SK. Quantitative detection of RASSF1A DNA promoter methylation in tumors and serum of patients with serous epithelial ovarian cancer. Gynecol Oncol 2011;123:581–7.
65. Giannopoulou L, Chebouti I, Pavlakis K, Kasimir-Bauer S, Lianidou ES. RASSF1A promoter methylation in high-grade serous ovarian cancer: a direct comparison study in primary tumors, adjacent morphologically tumor cell free tissues and paired circulating tumor DNA. Oncotarget 2017;8:21429–43.
66. Dong R, Yu J, Pu H, Zhang Z, Xu X. Frequent SLIT2 promoter methylation in the serum of patients with ovarian cancer. J Int Med Res 2012;40:681–6.
67. Zhang Q, Hu G, Yang Q, Dong R, Xie X, Ma D, et al. A multiplex methylation-specific PCR assay for the detection of early-stage ovarian cancer using cell-free serum DNA. Gynecol Oncol 2013;130:132–9.
68. Wu Y, Zhang X, Lin L, Ma XP, Ma YC, Liu PS. Aberrant methylation of RASSF2A in tumors and plasma of patients with epithelial ovarian cancer. Asian Pac J Cancer Prev 2014;15:1171–6.
69. Zhou F, Ma M, Tao G, Chen X, Xie W, Wang Y, et al. Detection of circulating methylated opioid binding protein/cell adhesion molecule-like gene as a biomarker for ovarian carcinoma. Clin Lab 2014;60:759–65.
70. Wang B, Yu L, Yang GZ, Luo X, Huang L. Application of multiplex nested methylated specific PCR in early diagnosis of epithelial ovarian cancer. Asian Pac J Cancer Prev 2015;16:3003–7.
71. Kamat AA, Bischoff FZ, Dang D, Baldwin MF, Han LY, Lin YG, et al. Circulating cell-free DNA: a novel biomarker for response to therapy in ovarian carcinoma. Cancer Biol Ther 2006;5:1369–74.
72. Yu M. Circulating cell-free mitochondrial DNA as a novel cancer biomarker: opportunities and challenges. Mitochondrial DNA 2012;23:329–32.
73. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 2014;32:579–86.
74. Kulasingam V, Diamandis EP. Genomic profiling for copy number changes in plasma of ovarian cancer patients – a new era for cancer diagnostics? BMC Med 2016;14:186.
75. Esteller M. Epigenetics in cancer. N Engl J Med 2008;358: 1148–59.
76. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
77. Barton CA, Hacker NF, Clark SJ, O'Brien PM. DNA methylation changes in ovarian cancer: implications for early diagnosis, prognosis and treatment. Gynecol Oncol 2008;109:129–39.
78. Earp MA, Cunningham JM. DNA methylation changes in epithelial ovarian cancer histotypes. Genomics 2015;106:311–21.
79. Wittenberger T, Sleigh S, Reisel D, Zikan M, Wahl B, Alunni-Fabbroni M, et al. DNA methylation markers for early detection of women's cancer: promise and challenges. Epigenomics 2014;6:311–27.
80. Teschendorff AE, Menon U, Gentry-Maharaj A, Ramus SJ, Gayther SA, Apostolidou S, et al. An epigenetic signature in peripheral blood predicts active ovarian cancer. PLoS One 2009;4:e8274.
81. Flanagan JM, Wilhelm-Benartzi CS, Metcalf M, Kaye SB, Brown R. Association of somatic DNA methylation variability with progression-free survival and toxicity in ovarian cancer patients. Ann Oncol 2013;24:2813–8.
82. Flanagan JM, Wilson A, Koo C, Masrour N, Gallon J, Loomis E, et al. Platinum-based chemotherapy induces methylation changes in blood DNA associated with overall survival in patients with ovarian cancer. Clin Cancer Res 2017;23:2213–22.
83. Pavlou MP, Diamandis EP, Blasutig IM. The long journey of cancer biomarkers from the bench to the clinic. Clin Chem 2013;59:147–57.
84. Ignatiadis M, Lee M, Jeffrey SS. Circulating Tumor Cells and Circulating Tumor DNA: Challenges and Opportunities on the Path to Clinical Utility. Clin Cancer Res 2015;21:4786–800.