

1-1-2020

Liquid biopsy in ovarian cancer using circulating tumor DNA and cells: Ready for prime time?

Du-Bois Asante

Edith Cowan University, duboisa@ecu.edu.au

Leslie Calapre

Edith Cowan University, l.calapre@ecu.edu.au

Melanie Ziman

Edith Cowan University, m.ziman@ecu.edu.au

Tarek M. Meniawy

Edith Cowan University, t.meniawy@ecu.edu.au

Elin S. Gray

Edith Cowan University, e.gray@ecu.edu.au

Follow this and additional works at: <https://ro.ecu.edu.au/ecuworkspost2013>



Part of the [Medicine and Health Sciences Commons](#)

Recommended Citation

Asante, D., Calapre, L., Ziman, M., Meniawy, T. M., & Gray, E. S. (2020). Liquid biopsy in ovarian cancer using circulating tumor DNA and cells: Ready for prime time?. DOI: <https://doi.org/10.1016/j.canlet.2019.10.014>

[10.1016/j.canlet.2019.10.014](https://doi.org/10.1016/j.canlet.2019.10.014)

Asante, D. B., Calapre, L., Ziman, M., Meniawy, T. M., & Gray, E. S. (2019). *Liquid biopsy in ovarian cancer using circulating tumor DNA and cells: Ready for prime time?*. *Cancer Letters*, 468, 59-71. <https://doi.org/10.1016/j.canlet.2019.10.014>

This Other is posted at Research Online.

<https://ro.ecu.edu.au/ecuworkspost2013/7436>



Mini-review

Liquid biopsy in ovarian cancer using circulating tumor DNA and cells: Ready for prime time?



Du-Bois Asante^a, Leslie Calapre^a, Melanie Ziman^{a,d}, Tarek M. Meniawy^{a,b,c}, Elin S. Gray^{a,*}

^a School of Medical and Health Sciences, Edith Cowan University, Australia

^b School of Medicine, University of Western Australia, Crawley, Western Australia, Australia

^c Department of Medical Oncology, Sir Charles Gairdner Hospital, Nedlands, Western Australia, Australia

^d School of Biomedical Science, University of Western Australia, Crawley, Western Australia, Australia

ARTICLE INFO

Keywords:

Cell-free DNA (cfDNA)
Circulating tumor DNA (ctDNA)
Circulating tumor cells (CTC)
Ovarian cancer
Prognosis
Diagnosis

ABSTRACT

Liquid biopsies hold the potential to inform cancer patient prognosis and to guide treatment decisions at the time when direct tumor biopsy may be impractical due to its invasive nature, inaccessibility and associated complications. Specifically, circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) have shown promising results as companion diagnostic biomarkers for screening, prognostication and/or patient surveillance in many cancer types. In ovarian cancer (OC), CTC and ctDNA analysis allow comprehensive molecular profiling of the primary, metastatic and recurrent tumors. These biomarkers also correlate with overall tumor burden and thus, they provide minimally-invasive means for patient monitoring during clinical course to ascertain therapy response and timely treatment modification in the context of disease relapse. Here, we review recent reports of the potential clinical value of CTC and ctDNA in OC, expatiating on their use in diagnosis and prognosis. We critically appraise the current evidence, and discuss the issues that still need to be addressed before liquid biopsies can be implemented in routine clinical practice for OC management.

1. Introduction

Ovarian cancer (OC) is the 7th most common cancer and the 8th leading cause of cancer mortality in women world-wide [1]. OC can be cured in up to 90% of cases, if diagnosed while still limited to the ovaries. However, due to lack of effective screening tests and absence of clinical symptoms in early stage disease, approximately 70% of patients with OC are diagnosed at advanced stages (stage III and IV). Late stage OC is associated with poor prognosis despite best therapeutic efforts with cytoreductive surgery and systematic chemotherapy [2,3]. Thus, an efficient method for early detection and monitoring of disease progression or relapse may improve the survival outcomes and management of OC patients.

Neoplasms of the ovary are diagnosed and monitored by conventional tissue biopsy method, transvaginal ultrasound (TVUS), computerised tomography (CT) scan, positron emitting tomography (PET), and a blood test for the detection of the membrane glycoprotein, Muc-16, also known as cancer antigen-125 (CA-125) [4]. Imaging studies can help identify observable cell mass in the ovary or other sites, but do not provide a clear diagnosis nor differentiate between malignant or benign lesions [5]. Moreover, the detection of possible metastasis at

other sites may be elusive or undetectable until reaching a sufficient size. Tissue biopsy on the other hand can be challenging, invasive and does not reveal tumor heterogeneity [6]. CA-125 is the best characterized biomarker for OC, and currently the clinical standard for disease monitoring [4]. However, CA-125 lacks specificity as a screening tool, since it can be elevated in other benign (endometriosis and pelvic inflammatory diseases) and malignant tumors (breast, lung and gastrointestinal cancers) [7–10]. Thus, CA-125 is useful for disease and treatment monitoring, but unreliable for screening or initial diagnosis of OC. The use of novel alternative biomarkers to support and complement CA-125 use is an unmet need.

In the last decade, several studies have demonstrated the potential use of liquid biopsies for cancer detection and management. In OC circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) appears to be a promising diagnostic and/or prognostic biomarkers.

Here we carried out a literature search in NCBI PubMed from January 2011 to May 2019, using ‘ovarian cancer’ together with ‘circulating tumor cells’ or ‘circulating tumor DNA’. A total of 24 and 33 published articles were identified describing the analysis of CTCs and ctDNA, respectively, for diagnosis, prognosis and/or monitoring in OC patients. We summarized the findings of these studies, present the

* Corresponding author. School of Medical Sciences, Edith Cowan University, 270 Joondalup Drive, Joondalup, Perth, WA, 6027, Australia.

E-mail address: e.gray@ecu.edu.au (E.S. Gray).

<https://doi.org/10.1016/j.canlet.2019.10.014>

Received 12 September 2019; Accepted 6 October 2019

0304-3835/© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Table 1
CTC studies in ovarian cancer.

Subtype/Stage	No. of Patients	Sampling Time	Isolation Method	Detection Technique	Genetic marker/antigen	Detection rate (%)	Diagnostic Sensitivity and/ Specificity	Prognostic Significance	Year	Ref
EOC (I-IV)	30 patients	Before and after surgery	Tapered-slit membrane filters	ICC	CK-9, EpCAM	Before surgery = 76.7%; After surgery = 57.1%	NR	NS	2019	[18]
EOC/I-IV	109	Before chemotherapy/surgery, and after chemotherapy/surgery	Immunomagnetic beads (EpCAM, HER2 and MUC1)	Multiplex RT-PCR	EpCAM, HER2, MUC1, WTI, P16 and PAX8	90 Newly diagnosed; 91 after treatment	NR	OS ($p = 0.041$)	2018	[24]
Advanced OC/III and IV	20	After chemotherapy	Immunomagnetic beads (EpCAM and N-cadherin)	ICC	EpCAM, N-cadherin, CK, VE-cad and Vimentin	90	NR	NR	2018	[32]
Six different subtypes of OC, including, HGSOC	30	Before surgery	Size based microfluidic technique	ICC	EpCAM, HE4, panCK, CK7 and Vimentin	73.3	Sn = 76.7% Sp = 63% AUC = 0.715 ($p = 0.005$) Sp > 80%	NR	2018	[15]
NR Primary and recurrent/II, III and IV	20	NR	Magnetic separation (Folic acid) Microfluidic Parsortix™	ICC	HE4	80	NR	NR	2018	[27]
EOCs (II-IV)	20	Before and after treatment	Density gradient centrifugation	RT-qPCR	29 gene markers including: EpCAM, PPIA, MAL2, LAMB1, SERPINE2, TUSC3	70	NR	NR	2018	[14]
EOC/I-IV	At diagnosis (102); After treatment (78)	Before surgery and after adjuvant chemotherapy	Density gradient centrifugation	ICC and FISH	ICC: EpCAM, Cytokeratins, EGFR, MUC1 and HER2 FISH: MECOM and HHLAI	26.5 at baseline; 7.7 after therapy	NR	OS ($p = 0.007$); PFS ($p = 0.008$)	2017	[20]
EOC/I-IV	23	Before treatment	Microfluidics plus immunomagnetic beads (EpCAM)	ICC	EpCAM, CK3–6HS, panCK	87	NR	NR	2017	[31]
EOC/I-IV	54	Before and after surgery	Nanoroughened microfluidic platform	ICC	EpCAM, TROP-2, EGFR, Vimentin, N-cadherin	98.1	NR	HR = 4.3 (3.08–32.15; $p = 0.003$)	2017	[16]
Serous (24) and non-serous (63)/I-IV	87	Before surgery	Tapered-slit membrane filters	ICC	EpCAM, CK9	56.3	Sn = 77.4%; Sp = 55.8% AUC = 0.655 ($p = 0.025$)	NR	2017	[17]
NR EOC	10	NR	Magnetic separation (Folic acid) AdnaTest OvarianCancerSelect, AdnaTest EMT and StemCell Select	ICC	HE4 and FITC AffiniPure	50	NR	NR	2016	[28]
EOC/II-IV	10 (3 for single-cell analysis)	Before surgery	AdnaTest OvarianCancer and EMT-1 Select/Detect	Multiplex RT-PCR (AdnaTest)	19 gene transcripts, including epithelial, EMT and stem cell markers	30 (100 for the 3 single cell analysis)	NR	NR	2016	[33]
EOC/II-IV	95	Before surgery and after chemotherapy	AdnaTest OvarianCancer and EMT-1 Select/Detect	Multiplex RT-PCR (AdnaTest)	EpCAM, ERCC1, MUC1, MUC16, PI3Ka, Akt-2, Twist	82	Sn and Sp > 90%	PFS ($p = 0.042$) OS ($p = 0.001$)	2016	[36]
NR	56	Before surgery	MetaCell®	ICC/qPCR	ICC: NucBlue™, Celltracker™. 12 gene markers including: EpCAM, MUC1, MUC16, KRT18, KRT19, ERCC1, WTI	58	NR	NR	2016	[13]

(continued on next page)

Table 1 (continued)

Subtype/Stage	No. of Patients	Sampling Time	Isolation Method	Detection Technique	Genetic marker/antigen	Detection rate (%)	Diagnostic Sensitivity and/ Specificity	Prognostic Significance	Year	Ref
EOC/I-IV	118	Before surgery	MetaCell*	ICC/qPCR	ICC: NucBlue™, Celltracker™. Gene expression: <i>EpcAM</i> , <i>MUC1</i> , <i>MUC16</i> , <i>KRT18</i> , <i>KRT19</i> . ICC: <i>EpcAM</i> , <i>CA-125</i> , <i>CD44</i> , <i>seprase</i> Gene expression: <i>EpcAM</i> , <i>CD44</i> , <i>MUC16</i> and <i>FAP</i> CK, CEP8	65.2	NR	NR	2015	[12]
EOC/I-IV	31	Before and after surgery, during 24months follow up	CAM uptake-cell enrichment	ICC/RT-qPCR		100	Sn = 83%; Sp = 97%	Risk for PD ($p = 0.00001$; OR = 121.3)	2015	[26]
EOC/III and IV	21	After surgery	Immunomagnetic beads (CD45)	FISH, ICC		76.2	Sn = 76.2% Sp > 80% AUC = 0.963 ($p = 0.000$) Sn = 83% Sp = 95.1%	NR	2014	[30]
EOC/I-IV	129	Before surgery	CAM uptake -cell enrichment	ICC	<i>EpcAM</i> , <i>ESA</i> , <i>CA-125</i> , <i>DPP4</i>	88.6		HR = 1.06 (0.41–2.73; $p = 0.022$) OS ($p = 0.026$) PFS ($p = 0.009$) NS	2014	[25]
EOC/IV	143	Before surgery	Immunomagnetic (AdnaTest) <i>EpcAM</i> and <i>MUC1</i>	RT-PCR (AdnaTest)	<i>EpcAM</i> , <i>MUC1</i> , <i>MUC16</i> , <i>HRCC1</i>	14	NR		2014	[34]
EOC/IV	78	Before and during chemotherapy	Immunomagnetic (CellSearch*) <i>EpcAM</i>	ICC	<i>EpcAM</i> , <i>CK8</i> , <i>19</i> , <i>18</i>	60.0 primary, 53.8 recurrent	NR		2013	[23]
Serous (I/73) and non-serous (27)/II-IV	216	Before surgery and after adjuvant chemotherapy	Density gradient centrifugation	RT-qPCR	12 gene markers including: <i>PPIC</i> , <i>EpcAM</i> , <i>LAMB1</i> , <i>GPX8</i> , <i>TUSC3</i> , <i>TFPI</i> . Also, <i>HE4</i>	24.5 before surgery; 20.4 after chemotherapy	Sn = 22% Sp = 85%	HR = 2.3 (1.1–4.8; $p = 0.024$)	2013	[19]
EOC/I-IV	122	Before surgery and after chemotherapy	Immunomagnetic (AdnaTest) GA 73.3 and <i>MUC1</i>	RT-PCR (AdnaTest)	<i>HER-2</i> , <i>MUC1</i> , <i>MUC16</i> , <i>EpcAM</i>	19 before surgery; 27 after chemotherapy	NR	HR = 4.56 (1.94–10.73; $p < 0.05$) NS	2011	[35]
EOC	54	Before and after chemotherapy	Immunomagnetic (CellSearch*) <i>EpcAM</i>	ICC	<i>EpcAM</i> , <i>M30</i> , <i>CK8</i> , <i>18</i> , <i>19</i>	44	NR		2011	[21]
NR	216	After chemotherapy	Immunomagnetic (CellSearch*) <i>EpcAM</i>	ICC	<i>EpcAM</i> , <i>CK8</i> , <i>18</i> , <i>19</i>	14.4	NR	PFS ($p = 0.0024$)	2011	[22]

NR: Not reported; NS: Not Significant; CR: Chromosomal rearrangement; HR: Hazard ratio; OR: Odds ratio; RT-PCR: Real-time PCR technology; qPCR: Quantitative PCR; PFS: Progression free survival; CAM: Cell adhesion matrix; OS: Overall survival; ICC: Immunocytochemistry staining; FISH: fluorescence in situ hybridization; CAM: Cell adhesion matrix; VE-cad: Anti-vascular endothelial-cadherin; PD: Progression disease; Sn: Sensitivity; Sp: Specificity.

current technologies utilised for analysis and the potential clinical applications and limitations of these techniques. We also discuss emerging technologies and concepts, as well as the existing challenges for liquid biopsy applications. Finally, we offer our perspective as to when the information from liquid biopsies may be considered reliable and clinically applicable.

2. Ovarian cancer CTCs

CTCs are tumor cells that are released from primary, metastatic or recurrent tumors and can be identified in the peripheral blood of cancer patients [11]. In OC, the presence of these CTCs in the blood of patients provide useful diagnostic and prognostic information associated with both primary and metastatic tumors [12–14].

2.1. Enrichment and detection methods

The ratio of CTCs to other cells in the blood is extremely low. Thus, it is quite challenging to detect and differentiate CTCs from non-tumor cells in whole blood. CTC enrichment techniques are employed in combination with subsequent detection methods for evaluation of CTC presence [11]. Various methods reported for the isolation of CTCs from OC patients rely on either the; (1) physical properties of tumor cells such as density, size, deformability or (2) biological properties such as positive or negative label-dependent immunoaffinity enrichment targeting specific surface proteins (Table 1).

Isolation methods based on physical properties include the use of micro-fluidic platforms such as Parsortix™ [14], the Biotin/Ppy-microfluidic and others [15,16]. Other methods include filter based size exclusion like MetaCell® [12,13] and the tapered-slit membrane filters (TSF) [17,18]. Other studies reported the use of density gradient centrifugation for the isolation of OC CTCs [19,20].

The most common immunoaffinity-based CTC isolation method used in OC is the FDA-approved CellSearch® system, which capture CTCs of epithelial origin using EpCAM [21–23]. Similar methods employing immunomagnetic beads have also been utilised targeting a variety of ligands such as EpCAM, HER2 and MUC1 [24]. Other novel techniques such as the functional cell adhesion molecule (CAM) uptake-cell enrichment method, have also been reported for CTC isolation in OC [25,26] (Table 1). Moreover, the use of magnetic nanoparticles targeting folic acid receptors on OC CTCs have also been reported [27,28].

CTCs are detected in the enriched sample either by immunocytochemistry (ICC) [15–18,20–23,27–32] or by gene expression analysis, mostly by real-time PCR (RT-PCR) [14,19,24,33–36]. Fluorescent in situ hybridization (FISH), has also been reported as a confirmatory test for identifying OC CTC with stem-cell like fusion genes [20]. Studies using only ICC (14/24) for CTC quantification reported detection rates ranging from 7.7 to 98%, while those that only used RT-PCR (7/24) had a 14–91% detection rate, irrespective of sampling time (Table 1). However, other studies (3/24) used a combination of the two for the identification of potential CTCs, and had detection rates ranging from 65 to 100% [12,13,26]. Thus, a combination of ICC and RT-PCR techniques may increase the detection of CTCs.

Both epithelial (EpCAM/cytokeratin) and mesenchymal markers (N-cadherin and vimentin) are used for detection of CTCs in OC [15,16,32], which consistently show higher detection rates ($\geq 90\%$) compared to the use of epithelial markers only (CellSearch®) ($\leq 60\%$) [21–23]. However, the use of epithelial mesenchymal transition (EMT) markers such as N-cadherin [32] and vimentin [15] to complement EpCAM for CTC detection in OC may result in “false positives”, since circulating endothelial cells also express these markers. Hence, efficient negative exclusion need to be adopted to avoid false positives [32]. Similarly, leucocytes in the CTC enriched samples (Fig. 1), may also express mesenchymal and stem cell markers [33]. Thus, single-cell molecular characterization could be more accurate for assessing CTC

gene expression more broadly [33].

Overall, most studies did not report on sensitivity and specificity of the assay used, which affects the comparison of the diagnostic performance of different platforms employed for the detection and analysis of OC CTCs. Therefore, the robustness and clinical validity of these techniques across different platforms beyond the initial proof of concept, warrants further study using larger sample sizes.

2.2. Molecular profiling of CTCs

Molecular profiling of CTCs in OCs has revealed a myriad of potential biomarkers of diagnostic importance [12,13,33–36]. A study by Kolostova et al., evaluated the heterogeneity of CTCs in OC patients and identified *EpCAM*, *WT1*, *MUC16*, *MUC1*, *KRT7*, *KRT18* and *KRT19* as genes that are highly specific for CTCs from OC, which are also associated with tumorigenic characteristics [13].

Blassl et al. [33] performed a single-cell quantitative transcriptomic profiling of a CTC from an OC patient, utilising gradient density for initial cell enrichment, followed by CD45 depletion. They identified EMT (Vimentin, N-cadherin, *Snai2*, CD117, and CD146) and stem cell (CD44, ALDH1A1, Oct4 and Nanog) gene transcripts on the single CTC. Similarly, other EMT-gene transcripts (*PI3Ka*, *Akt-2* and *Twist*) were demonstrated in OC CTCs [36]. Detection of EMT gene transcripts from CTCs from patients can help inform clinicians about potential tumor resistance, and may ultimately aid in developing personalized approaches to combat these mesenchymal tumor cells.

More applicable to clinical care, Kuhlmann et al. [34] indicated that platinum-resistance could be predicted through the detection of *ERCC1* (excision repair cross-complementation group 1) gene expression in CTCs. *ERCC1* which aids in the repairing of DNA-platinum adducts, was an independent predictor of platinum resistance (OR = 8.5; 95% CI, 1.7–43.6; $p = 0.010$) in this study.

The above studies underscore that beyond CTC enumeration, molecular characterization may provide additional biological information that could be used for prognostication or treatment decisions.

2.3. Cluster of CTCs

CTCs, especially epithelial CTCs, normally do not survive in peripheral blood due to anoikis, a form of programmed cell death in anchorage-dependent cells, and haemodynamic shear stress [37]. Nevertheless, EMT may circumvent death-inducing signals, allowing mesenchymal cancer cells to thrive and metastasize even in the presence of therapy [36]. EMT transcription factors (EMT-TFs) such as Slug and Snail, have been demonstrated to directly contribute to resistance to platinum-based drugs such as cisplatin in OC patients [38].

Mesenchymal and epithelial CTCs can also form clusters and aid collective migration to better enhance their chances of survival [39]. This has been widely accepted as a critical stage of CTC dissemination through research studies in breast cancer CTCs [39,40]. In OC, microemboli or clusters of CTCs (2–30 cells) were identified in 59% of patients in one study, and were significantly associated with platinum resistance ($p = 0.0001$), shorter progression free-survival (PFS) and time to progression (TTP) [16]. The presence of CTC clusters were also reported in another OC study, but limited details were provided regarding the association of CTC clusters and clinical outcomes [26].

Given the paucity of data on CTC clusters in OC, further investigation is warranted to address and precisely clarify the molecular pathways involved in this process. Nevertheless, EMT-TFs such as Snail and ZEB [41], may be a potential target for the treatment of those peculiar, resistance-prone and invasive CTC-hybrid phenotypes.

2.4. Diagnostic significance of CTCs in ovarian cancer

Studies have consistently shown that detection of CTCs in OC is significantly associated with more advanced disease stage (III and IV)

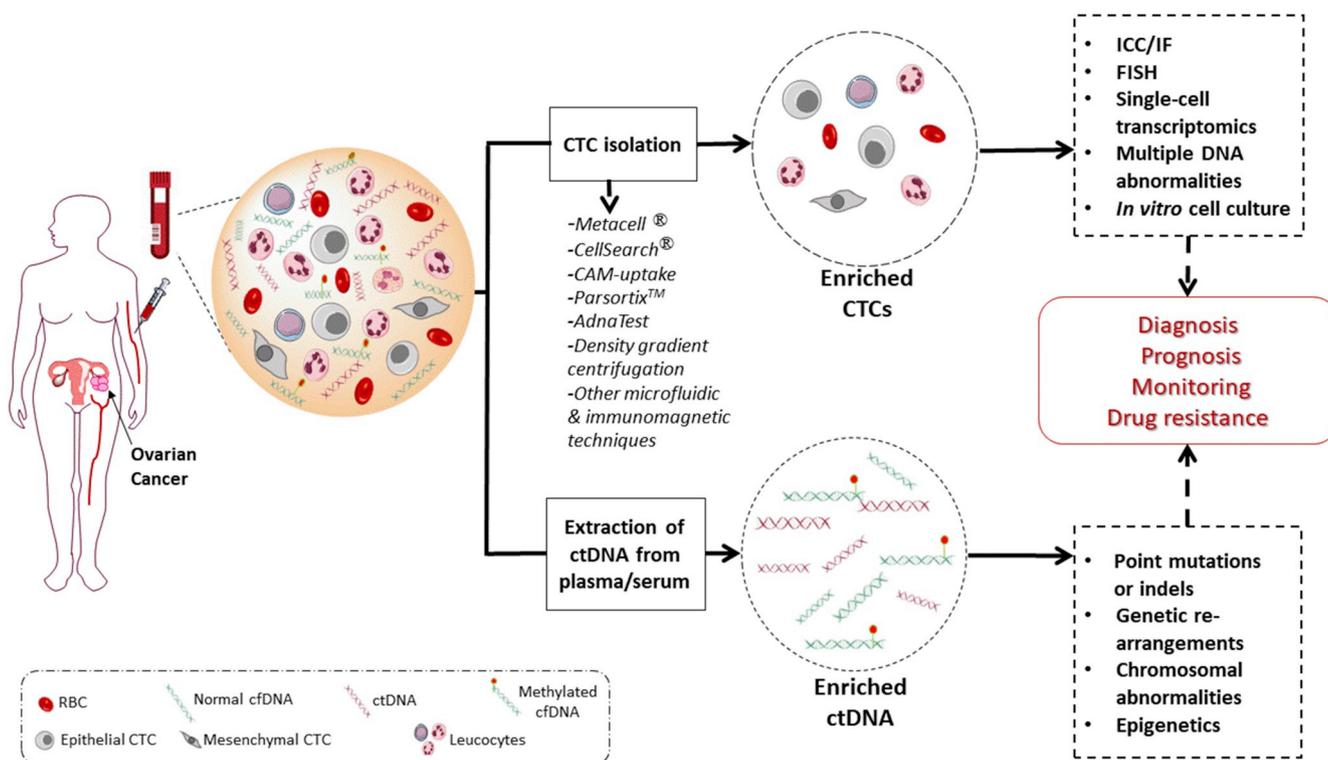


Fig. 1. CTC and ctDNA detection methods and clinical applications in ovarian cancer.

[18,19,24–26,34]. In a recent study by Zhang et al., the number of CTCs found in stage I patients was significantly lower than those with stage III and IV ($p < 0.05$) [24]. Similarly, a significant association ($p < 0.0001$) was demonstrated between CTC number and tumor stage in patients with advanced disease [25]. Compared to benign controls, early stage (I and II) and late stage (III and IV) disease were 8.4 and 16.9 times more likely to have CTCs respectively [25]. More recently, post-operative OC CTC count was significantly associated with the advanced stages than the early stage ($p = 0.010$) [18]. Advanced stages have higher tumor burden and thus, are able to shed higher numbers of CTCs into circulation, which have the potential to establish metastatic colonies at near or distant sites from the area of release. This may also explain why patients at late stages have a high incidence of recurrence and worse survival despite maximal efforts at debulking surgery.

The diagnostic performance of CTC detection methods through ICC have a sensitivity ranging from 76 to 83% and a specificity of 55–95% [15,17,25,30]. Amongst the six studies that used RT-qPCR for CTC detection, only one reported on the diagnostic sensitivity and specificity (22% and 85% respectively [19]), which were surprisingly lower than that of ICC methods. A study that combined both detection methods (ICC/RT-qPCR) reported a high sensitivity (83%) and specificity (97%) [26]. To note, the discussed studies varied regarding the use of benign [15,17,25,30] or healthy donors [19,26] as control groups. Nonetheless, ICC methods seem to provide a reliable and accurate diagnosis of OC, particularly at later stages.

2.5. CTC as a predictor of response to therapy and prognosis in ovarian cancer

A number of studies also evaluated the utility of CTCs as a biomarker of chemotherapy response in OC (Table 1), and demonstrated that CTC numbers significantly decline after chemotherapy ($p < 0.05$) [19,22–24,26]. For example, a study demonstrated that the overall CTC count decreased over time at a linear rate of 0.1 cell per month ($p < 0.0001$) during chemotherapy [23]. Another study reported a higher rate of CTC positivity after chemotherapy (19 and 27% pre- and

post-treatment, respectively) [35]. Additionally, a study showed that after chemotherapy, CTCs were more likely ($p = 0.015$) to be identified in patients classified as non-responders than responders [20]. Moreover, platinum resistant patients had significantly ($p < 0.05$) higher numbers of CTCs as compared to platinum sensitive patients [19]. Overall, these results suggest that CTCs may be a predictive marker for response in OC to platinum-based chemotherapy. CTC release into circulation could be hindered by chemotherapy, and this may differ among individuals due to patient heterogeneity and tumor stage. However, the lack of standardisation of methodology, and the absence of large prospective studies are significant barriers for its implementation into clinical practice.

Multiple studies have reported significant association between the presence of OC CTCs and PFS or overall survival (OS), using specific cut-off levels for CTC positivity [16,20,22,26,34,35]. For example, previously untreated patients with advanced OC and high CTC count (≥ 3) prior to chemotherapy had a significantly shorter PFS, compared with patients with < 3 CTCs ($p < 0.05$) [16]. Similarly, patients with persistent elevated CTC counts ≥ 2 at baseline and follow-up, had shorter PFS ($p = 0.0024$) and OS ($p = 0.0017$), compared with patients with < 2 CTCs [22]. Likewise, another study has also shown that increase in CTCs has a high association with increased risk of progression [26]. Other report, however, did not obtain significant association with clinical outcome (PFS/OS) [18].

Overall, these studies demonstrate that patients with persistently high CTCs have worse prognosis compared to those with negative CTCs using either ICC or RT-PCR methods (Table 1). While different thresholds for CTC positivity and detection methodologies were used, it is clear that irrespective of the methodology utilised for detection, the presence of CTCs may help in the prediction of survival outcomes in OC.

3. Circulating tumor DNA

Cell-free DNA (cfDNA) is released via necrosis or apoptosis of tumor cells [42]. Currently, the exact mechanism of cfDNA release is unclear but its levels has been shown to increase after excessive physical

Table 2
Studies on ctDNA in ovarian cancer.

Subtype/Stage	No. of Patients	Vol. of Whole Blood/Plasma or serum	Sampling Time	Genetic Marker	Detection Method	Detection Rate (%)	Analytical Sensitivity	Diagnostic Sensitivity and/ Specificity	Prognostic Significance	Year	Refs
EOCs (96% HGSOC)	97	Blood (9 mL)/Plasma (2–3 mL)	Before and after therapy	<i>BRAC1, BRAC2, TP53</i>	Targeted-NGS	TP53 = 96	0.13%	NR	PFS, HR = 0.12 ($p < 0.0001$)	2019	[61]
EOCs/I, III and IV	4	Plasma (NR)	Before and after treatment	<i>TP53, APC, BRCA1 and KRAS</i>	Targeted-NGS	TP53 (75%), <i>APC</i> (50%), <i>BACAI</i> (50%) and <i>KRAS</i> (25%)	NR	NR	NR	2019	[53]
HGSOC/II-IV	12	Blood (5–6 mL)/Plasma (1–2 mL)	Before, during and after therapy	CNV and > 500 cancer related genes including <i>TP53, PTEN, BRCA2</i> etc.	Targeted-NGS	100 for TP53 and variable for the other genes	NR	NR	PFS ($p < 0.01$)	2019	[52]
EOCs (II and III)	10 drug-resistant recurrent; 11 drug-sensitive recurrent	Plasma (1 mL)	Before and after therapy	CNV and mutant genes including <i>TP53, BRCA1, NOTCH2, DNMT3A</i> etc.	Targeted-NGS	~90% for only TP53. 100 for all mutant genes.	NR	Sp = 100%; Sn = 74–75%	OS ($p = 0.025$); PFS ($p < 0.001$)	2018	[54]
Clear cell carcinoma	29	Plasma (NR)	Before and after treatment	<i>KRAS</i> and <i>PIK3CA</i>	ddPCR	10	NR	NR	PFS ($p = 0.004$)	2018	[95]
HGSOC, dysgerminoma and carcinosarcoma (I-IV)	36	Blood (10 mL)/Plasma (4 mL)	Before and after treatment	CNV	WGS	16.7	NR	NR	OS, HR = 3.87 ($p = 0.015$); PFS, HR: 7.98 ($p = 0.045$)	2018	[59]
HGSOC/II, III and IV	61	Blood (15 mL)/Plasma (1–5 mL)	Before and after therapy	<i>TP53</i>	ddPCR	93	NR	NR	PFS, Reduced TTP ($p = 0.038$)	2018	[80]
SOC/III and IV	4	Blood (Average = 20 mL)/Plasma (5 mL)	Before and after surgery	<i>TP53</i>	ddPCR	100	NR	NR	NR	2018	[49]
EOCs/I-III	54	Plasma (7.5 mL)	Before surgery	16 gene panel	CancerSEEK (Targeted-NGS)	98	NR	Sn: 98%; Sp: > 99% AUC = 0.91 (0.90–0.92) Sn: 27–100%	NR	2018	[76]
HGSOC/III and IV	14	Plasma (NR)	Before and after therapy	50 gene panel	Targeted-NGS	100	1%	NR	NR	2018	[51]
HGSOC/III and IV	121	Plasma (NR)	NR	Germline and somatic <i>BRAC1/BRAC2</i>	Targeted-NGS	25	NR	NR	NR	2017	[60]
EOCs/I-IV	42	Plasma (NR)	At the time of diagnosis and before tumor resection	55 gene panel including <i>TP53, KIT, ALK, APC, ERBB4</i> etc.	Targeted-NGS (TEC-Seq) and ddPCR	71	< 0.1	Sn:97.4%; Sp:100%	NR	2017	[77]
HGSOC/I-IV	30	Plasma (1 mL)	After debulking and after recurrence	<i>BRAC1 BRAC2</i>	Targeted-NGS	<i>BRCA1 & 2</i> Reversion = 17	NR	NR	Reversion of <i>BRAC1/2</i>	2017	[79]

(continued on next page)

Table 2 (continued)

Subtype/Stage	No. of Patients	Vol. of Whole Blood/Plasma or serum	Sampling Time	Genetic Marker	Detection Method	Detection Rate (%)	Analytical Sensitivity	Diagnostic Sensitivity and/Specificity	Prognostic Significance	Year	Refs
18 HGSOC & Endometrioid/III and IV	19	Plasma (1 mL)	Before therapy (After resistance)	<i>BRCA1, BRCA2, TP53</i>	TAm-RSeq, dPCR	<i>BRCA1 & 2</i> reversion = 21; <i>TP53</i> = 79	0.031–0.085%	NR	Reversion of <i>BRCA1/2</i>	2017	[50]
HGSOC	18	Plasma (NR)	Before and after therapy	<i>TP53</i>	Targeted-NGS	100	NR	NR	NR	2016	[55]
HGSOC/III and IV	10	Plasma (3 mL)	Before and after surgery	CR	qPCR, Targeted-NGS	100	NR	NR	NR	2016	[43]
HGSOC/III and IV	40	Blood (7.5 mL)/Plasma (Average = 2.1 mL)	Before and during treatment	<i>TP53</i>	TAm-Seq, dPCR	Before treatment = 82 Newly Diagnosed = 86	0.15%	Sn: 86%	Reduced TTP HR = 0.22 (0.07–0.67; <i>p</i> = 0.008)	2016	[48]
HGSOC/I-IV	32	Plasma (NR)	Before surgery	CNV	WEG (WISECONDOR)	40.6	NR	Sn: 40.6%; Sp: 93.8%	NR	2016	[58]
Invasive and borderline OC/I-IV	57	Plasma (NR)	Before surgery or treatment	CNV	WGS	NR	0.3%	Sn: 67%; Sp: 99.6%	NR	2016	[57]
HGSOC/I-IV	22	Serum (0.2 mL)	Before surgery and during treatment	<i>TP53, PTEN, PIK3CA, MET, KRAS, FBXW7, BRAF</i>	WES, ddPCR, TGS	93.8	0.01–0.002%	Sn: 81–91%; Sp: 60–99%	PFS (<i>p</i> = 0.001) OS (<i>p</i> = 0.0194)	2015	[47]
Stage III	7	Plasma (5 mL)	NR	markers include <i>PIK3CA, BRAF, EGFR</i>	dPCR, BEAMing, PCR-ligation	> 75	NR	NR	NR	2014	[78]
NR	3	Plasma (NR)	Before and after treatment	<i>RBI, ZEB2, BUB1, G54A, MTOR, PARP8</i>	Targeted-NGS, qPCR	100	50%	Sn: 97.5%	Acquired resistance	2013	[75]
HGSOC/III and IV	38	Plasma (~0.15 mL)	Before and after treatment	<i>TP53</i> , Other markers include <i>PTEN, BRAF, KRAS, EGFR, PIK3CA</i>	TAm-Seq, dPCR	53	0.6%	Sn and Sp > 97%	Acquired resistance	2012	[46]

NR: Not reported; CR: Chromosomal Rearrangement; dPCR: droplet Polymerase chain reaction; ddPCR: Droplet digital PCR; CNV: Copy number variation; qPCR: quantitative PCR; NGS: Next generation sequencing; WES: Whole exome sequencing; WGS: Whole genome sequencing; qPCR: Allele-specific quantitative PCR; WISECONDOR: Within-Sample copy number aberration Detector; TGS: Targeted gene sequences; TAm-Seq: Tagged-amplicon deep sequencing; TAm-RSeq: Targeted amplicon re-sequencing; PFS: Progression free survival; OS: Overall survival; TTP: Time to progression; EOC: Epithelial ovarian cancer; SOC: Serous ovarian cancer; HGSO: High grade serous ovarian cancer.

activity, inflammatory conditions and sepsis [42]. Nonetheless, cfDNA levels in cancer patients is higher than in healthy individuals [6,42]. Plasma cfDNA emanating from the tumor is considered as circulating tumor DNA (ctDNA) (Fig. 1).

Levels of ctDNA in OC are significantly associated with the conventional serum biomarker CA-125 [43]. However, ctDNA levels were found to be more specific, accurate and depicts a real-time picture of tumor burden due to its short half-life [42]. Conversely, CA-125 can be elevated in benign and non-specific inflammatory conditions [9], and has a relatively longer half-life estimated to range from 9 to 44 days [44]. Therefore, ctDNA may be a more reliable biomarker of disease status and treatment response.

Most research involving ctDNA analysis in OC are currently focused on high grade serous ovarian carcinoma (HGSOC) patients (Table 2). Molecular analysis conducted by the TCGA network revealed a high prevalence (90%) of somatic *TP53* mutations in HGSOC tumors [45]. Previous studies targeting mutant *TP53* in HGSOC patients have demonstrated high sensitivity (> 75%) and specificity (> 80%) for mutant ctDNA detection [46–48]. Furthermore, *TP53* mutant-ctDNA detection rates in HGSOC patients have been reported to be relatively high, ranging from 75 to 100% [47–55] (Table 2). Other studies examined gene fusion [56] and somatic copy number variations (CNV) [52,54,57–59] as alternative targets for OC ctDNA detection. Lastly, plasma ctDNA detection via *BRCA1/2* mutation in OC, which has a detection rate of ~25%, could also be exploited for analysis (Table 2) [60].

3.1. ctDNA detection methods and analysis

Plasma ctDNA analysis capitalizes on the identification of cancer-specific mutations identified by technologies such as digital PCR (dPCR) and next generation sequencing (NGS). In OC, ctDNA analysis using droplet dPCR has been shown to have a limit of detection at 0.002%, with a high specificity (81%) and sensitivity (99%), accompanied by a high detection rate (> 93%) [47]. However, pre-identification of mutant gene targets is required. NGS on the other hand, relies on the analysis of multiple DNA regions and their comparison to germline sequence [51,60]. NGS-based approaches used in the analysis of ctDNA in OC includes whole genome sequencing (WGS), whole exome sequencing (WES) and tagged-amplicon deep sequencing (TAm-Seq). The limit of detection (~1%–0.03%) depends on the platform used (Table 2). TAm-Seq is the most sensitive, detecting allelic frequencies as low as 0.03% [61]. NGS methods can interrogate several loci, providing comprehensive mutational profiling and may reveal clonal evolution as patients undergoing treatment.

Analysis of ctDNA can also be used to identify cancer specific methylation patterns in OC patients using techniques such as methylation-specific PCR (MSP), microarray mediated methylation assay (M^3 -assay), reduced representation bisulfite sequencing (RRBS), targeted ultra-high coverage bisulfite sequencing (TUC-BS), real-time methylation specific PCR (RT-MSP) and multiplex nested methylated specific PCR (MN-PCR) [62–71] (Table 3).

MSP is the main detection method employed for detecting methylated ctDNA in serum of OC patients [62,64,66,68–70,72]. Its limit of detection has been reported to be as low as 0.01% [70], with high sensitivity (> 85%) and specificity (> 90%) for the detection of differentially methylated ctDNA [65,66,68]. However, bisulphite conversion could potentially miss targeted DNA [68], especially loci that are prone to random fragmentation during apoptosis such as the promoter gene regions [73]. This is an important consideration for designing assays for analysis of methylation in cfDNA.

Overall, different methods have been utilised to detect and quantify ctDNA with high diagnostic sensitivity and specificity, as well as high detection rates in advanced disease. However, a limited number of studies have carried out orthogonal validation to corroborate the results obtained.

3.2. ctDNA as a diagnostic tool

Plasma ctDNA has the potential to serve as a minimally invasive diagnostic tool of cancer. In particular for OC, ctDNA have been demonstrated to have a better diagnostic performance than the conventional biomarker CA-125 [48,51,57,74]. We identified nine studies showing significantly higher rates of positivity and/or higher quantity of ctDNA than non-malignant individuals, with corresponding high sensitivity and specificity (Table 2). These studies demonstrated that quantitative analysis of ctDNA has relatively high specificity (> 88%) but varied in sensitivity (27–100%) for diagnosis of OC [46–48,54,57,58,75–77]. However, some of these studies had small sample size (≤ 10) [43,49,53,54,75,78], and others had highly selected populations such as patients with *BRCA1/2* mutations treated with PARP inhibitors [50,79]. Large studies conducted by Phallen et al. [77] and Cohen et al. [76] underscore the potential of ctDNA as a diagnostic tool for OC. These studies reflect on the utility of the CancerSEEK [76] and TEC-Seq [77] technologies to diagnose OC at ~97% sensitivity and > 99% specificity.

In summary, ctDNA is a promising tool for evaluating tumor burden in OC patients. However, further clarification is needed to define the minimum tumor size detectable through ctDNA analysis. Moreover, large prospective studies are needed to determine the clinical utility of ctDNA detection for early diagnosis of OC and its impact on patient outcomes.

3.3. Utility of ctDNA for prognostication, detecting residual disease and monitoring of response to treatment

A few studies have evaluated the utility of ctDNA as a prognostic biomarker for HGSOC. These studies have also demonstrated that ctDNA dynamics correlate with response to adjuvant chemotherapy in OC, and may predict progression or response earlier than CA-125 [46,48,80] or imaging [47]. Pereira et al. demonstrated that undetectable levels of ctDNA at 6 months following initial primary treatment was associated with significantly improved PFS ($p = 0.001$) and OS ($p < 0.05$). The authors also showed that ctDNA detection had a predictive lead time 7 months over CT scan [47].

In an exploratory analysis of *TP53* mutations in the ctDNA of relapsed HGSOC patients, Parkinson et al. reported that ctDNA was detected at no less than 20 amplifiable copies per milliliter of plasma in nearly all relapsed patients with disease volume > 32 cm³. The study also highlighted the prognostication ability of *TP53* in HGSOC, demonstrating that a decrease of $\leq 60\%$ in *TP53* mutant allele fraction after one cycle of chemotherapy was associated with poor response and shorter time to progression (TTP), whereas a decrease of > 60% was predictive of longer TTP [48].

Recent reports have also shown that persistence of *TP53* gene variants in ctDNA after neoadjuvant chemotherapy could be used to determine minimal residual disease [51] and treatment response monitoring [52,80] in OC patients. A case study by Martignetti et al., highlighted the possibility of detecting rare tumor-specific gene fusion (*FGFR2-FAM76A*), using ctDNA [56]. The authors concluded that ctDNA analysis was more sensitive and specific than the conventional biomarker CA-125 when used for monitoring response to therapy in advanced OC patients. Similarly, Harris et al. [43] demonstrated the feasibility of using somatic chromosomal re-arrangements to identify plasma ctDNA. Persistently detectable ctDNA levels post-surgery was consistent with disease burden and risk of recurrence in OC patients, while undetectable ctDNA levels were consistent with absence of detectable disease. More recently, plasma ctDNA was found to be an independent factor of OS ($p = 0.025$) and PFS ($p = 0.001$) in a cohort of OC patients with disease recurrence [54].

The strongest evidence to date on clinical utility of ctDNA in OC is the identification of residual disease and the monitoring of treatment response more accurately than the conventional imaging and CA-125

Table 3
Epigenetic modifications detected in ctDNA from ovarian cancer.

Subtype/Stage	No. of Patients	Vol. of Whole Blood/Plasma or serum	Sampling Time	Genetic Marker	Detection Method	Detection Rate (%)	Diagnostic Sensitivity and/ Specificity	Prognostic Significance	Year	Ref
HGSOC /I,II,III and IV	50	Blood (5 mL)/Plasma (2 mL)	Before and after therapy	<i>ESR1</i>	RT-MSP	38	NR	NS	2018	[62]
HGSOC /I,II,III and IV	151	Blood (20–40 mL)/Serum (at least 4 mL)	During diagnosis and after treatment	Regions linked to <i>COL23A1, C2CD4D</i> and <i>WNT6</i>	TUC-BS & RRBS	41	Sn = 41.4% Sp = 90.7%	NR	2017	[63]
HGSOC /I,II,III and IV	59	Blood (5 × 2 mL)/Plasma (2 mL)	Before and after surgery	<i>RASSF1A</i>	RT-MSP	33	NR	NS	2017	[64]
EOC /I,II,III and IV	149	Blood (3 mL)/Serum (0.2 mL)	NR	<i>OPCML, RUNX3, TFP12</i>	MSP	90	Sn = 90.14% Sp = 91.87%	NR	2017	[65]
EOC /I,II,III and IV	114	Serum (NR)	Before surgery	<i>OPCML, RUNX3, TFP12</i>	MN-MSP	90.1	Sp = 91.06%	NR	2015	[66]
EOC /I,II,III and IV	47	Plasma (NR)	Before surgery	<i>RASSF2A</i>	MSP	36.2	NR	NR	2014	[67]
EOC /I,II,III and IV	87	Serum (0.2 mL)	Before surgery	<i>APC, RASSF1A, CDH1, RUNX3, TFP12, SFRP5, OPCML</i>	Multiplex-MSP	Early stage: 83.3 Advanced stage: 93.9	Sn = 85.3% Sp = 90.5% AUC (early stage): 0.892 AUC (advanced stage): 0.931	NR	2013	[68]
iPrimary ovarian tumors/ stage I,II,III and IV	36	Blood (3 mL)/Serum	Before treatment	<i>SLIT2</i>	MSP	93.1	NR	NR	2012	[69]
HGSOC /I,II,III and IV	21	Serum (0.5 mL)	Before and after surgery or chemotherapy	<i>RASSF1A</i>	MSP	85.7	NR	NR	2011	[70]
EOC, mostly SOC	30	Plasma (NR)	Before surgery or chemotherapy	<i>RASSF1A, CALCA, EP300, PGRPROX, BRCA1, CDKN1C</i>	M ² -assay	90	Sn = 90.0% Sp = 86.7%	NR	2011	[71]

NR: Not Reported; NS: Not Significant; M²-assay: Microarray mediated methylation assay; MSP: Methylation specific PCR; RRBS: Reduced representation bisulphite sequencing; TUC-BS: Targeted ultra-high coverage bisulphite sequencing; RT-MSP: Real-Time methylation specific PCR; MN-MSP: Multiplex nested methylated specific PCR; EOC: Epithelial ovarian cancer; SOC: Serous ovarian cancer.

methods. Implementation of liquid biopsy in this context could aid in the selection of individuals who may be at greater risk of relapse, and may be considered for alternative management approaches and potential inclusion in appropriate clinical trials [51,80].

3.4. ctDNA for identification of treatment resistance

Selective pressure induced by treatment, whether platinum-based chemotherapy or targeted therapy such as PARP-inhibitors, can cause cancer cells to evolve, via additional gain or reversion of genetic alterations. For example, one study [46] reported the emergence of a *de novo* mutation in the tyrosine kinase domain of *EGFR* in a HGSOc patient at disease relapse that was not present 15 months earlier in the same patient, despite of a *TP53* mutation at 85% allelic frequency. Similarly, WES of plasma ctDNA in three OC patients revealed specific gene mutations (*RB1*, *MTOR*, *ZEB2*, *CES4A*, *BUB1*, *PARP8*) that may confer resistant to treatment with drugs such as cisplatin, paclitaxel and liposomal doxorubicin. Serial sampling measurements and quantification of allele fractions in ctDNA led to the identification of these gene mutations associated with the acquired resistance [75]. In a recent study by Oikkonen et al., *ERBB2* amplification was identified as a potential mediator of resistance to platinum chemotherapy and ctDNA-guided therapy using trastuzumab, increased response and aided tumor shrinkage [52]. This study highlights a mark improvement in the management of recurrent solid cancers such as OC, where repeat biopsies may cause potential risk or complications.

Reversion mutations of *BRCA1/2* detected in the plasma of OC patients were also associated with resistance to therapy (platinum/PARP inhibitors) [50,61,79]. Weigelt et al. assessed the feasibility of analyzing cfDNA for detecting *BRCA1/2* reversion mutations in advanced OC patients previously treated with platinum and/or PARP inhibitors. Immunoprecipitation and functional assay analysis demonstrated that the reversion mutations restored the DNA repair functions previously inactivated by the original *BRCA1/2* mutation, leading to the observed resistance in these patients [50]. Christie et al. also indicated that *BRCA1/2* reversion mutations can be detected in cfDNA of HGSOc patients (3/16), albeit at lower frequency than through the analysis of tumor samples (5/16). All cases with reversions had become resistant to platinum/PARP-inhibitors at the time of blood collection. The authors highlighted a patient who had a *BRCA1/2* reversion mutation and did not respond to platinum and a PARP inhibitor but that subsequently had partial response to gemcitabine and bevacizumab treatment [79], to underscore that detection of reversions can aid selection of more suitable treatments.

Together, these data demonstrate the potential of ctDNA to detect the emergence of escape mutations and reversion of *BRCA1/2* mutations. Regular monitoring through a liquid biopsy could aid the early detection of resistance and the selection of alternative drugs. It may also lead to personalized combinatorial therapy, including chemotherapies, targeted therapies or immunotherapy that may target different oncogenic drivers or reduce the emergence of resistance.

3.5. Diagnostic potential of promoter methylation in cfDNA

Promoter methylation resulting in epigenetic inactivation of tumor suppressor genes has been demonstrated as an early event during the pathogenesis of OC [68,69]. These changes such as hypermethylation in promoter regions of ctDNA have been reported to possess diagnostic potential [64–68,70,71]. Essentially, ctDNA methylation status in the serum/plasma of OC patients could be useful for early detection and help guide personalized treatment.

Patients with advanced disease (III-IV) have shown significantly ($p < 0.05$) higher serum CA-125 than the early stages (I-II) but comparison of methylation patterns in both stages showed no significant difference ($p < 0.05$). However, both early and advanced stage disease

showed a significant association ($p < 0.05$) with abnormal methylation of tumor suppressor genes when compared to normal healthy and benign controls [65,68,69]. In another report, though CA-125 levels were not computed, methylation patterns were still significantly different between OC patients and benign or healthy control groups [67]. These reports suggest that aberrant gene promoter methylation is an early event in the development of OC that could aid the early detection of malignancy.

Studies have shown the feasibility of detecting hypermethylation in the promoters of the tumor suppressor genes *RUNX3*, *TFP12*, *RASSF1A* and *2A* from serum and plasma samples for diagnosis of OC patients [64–68,70,71]. These studies have alluded to the high diagnostic sensitivity and specificity of these hypermethylated genes in patients compared to normal healthy controls [64–68,70,71], indicating its diagnostic potential. However, it is important to note that markers analysed for these studies have been previously reported in other cancers. In addition, Widschwendter et al. [57] found that their set of epigenetic markers, including *COL23A1*, *C2CD4D* and *WNT6*, measured in serum DNA had superior specificity compared to CA-125 (90.7% vs 87.1%) but had lower sensitivity (82.8% vs 41.4%). From these studies, it appears that variations in diagnostic specificity and sensitivity can be attributed to the epigenetic marker used for analysis. Identification of highly specific ovarian cancer-specific methylated could improve the utility of methylated ctDNA for early diagnosis in OC patients.

Of note, epigenetic analysis reported thus far mostly utilised serum, compared to mutational ctDNA analysis which primarily uses plasma (Tables 2 and 3). The disparity in the choice of biological material used for epigenetic studies and mutant circulating DNA analysis in OC is yet to be elucidated. Compared to that of plasma [64,67,71], serum-based studies [63,65,66,68] showed consistently higher specificity (> 90%), with sensitivity ranging from 41 to 91% with a corresponding high detection rate.

Overall, methylation of promoter regions of tumor suppressor genes, which consequently brings about their inactivation and repression, is a potential important aspect of OC pathogenesis that warrants further study. Furthermore, elucidating epigenetic abnormalities by cfDNA analysis may be used to select patients for targeted therapies such as DNA methyltransferase inhibitors.

3.6. Value of methylated cfDNA for patient monitoring and prognostication

Existing data evaluated aberrant methylation patterns in OC cfDNA for diagnostic efficiency and very few studies have evaluated its utility for patient monitoring [57] and prognostication [58]. A study by Widschwendter et al. [57] have demonstrated that reduction of methylated ctDNA levels in OC patients after 2 cycles of chemotherapy was associated with treatment response. Hypermethylation of promoter regions of *RASSF1A*, have been reported to be associated with reduced OS and disease relapse in the tumor but not in ctDNA [64]. Overall, there is paucity of studies addressing the potential utility of methylated markers for monitoring treatment response and prognostication of OC patients.

4. Future directions

Our review of studies on ctDNA and CTC in OC patients identified significant variability between studies, in terms of sample size, sampling time, isolation and detection methods. Particularly concerning is the lack of consensus for CTC analysis. Cut-off values used for confirming CTCs positivity varied across studies and platforms, which could impact on the derived conclusions. Therefore, a more uniform approach for CTC characterization and definitions of positivity is needed to evaluate intra- and inter-laboratory reproducibility. This will ultimately improve standardization across different CTC enrichment and detection platforms. However, unlike ctDNA, CTCs can provide

detailed information about the tumors at RNA and protein level, enabling further research to better our understanding of the metastatic process.

Further basic research is needed to identify the role of CTC clusters and mesenchymal/stem cells CTCs in disease progression. Isolation platforms need to be optimized to minimize the shear stress that may bring about the detachment of individual cells in the CTC cluster or loss of EMT CTCs. In particular, it will be important to determine how stromal interactions and cluster cell formation may aid CTC release, especially in reactive stromal subtypes of OCs [81]. This could potentially help unravel chemo-resistance molecular trajectories in the EMT CTCs phenotypes which may lay a foundation for developing personalized therapeutic approaches.

In addition to blood, peritoneal fluid [82,83] and uterine lavage [84] have been used to obtain cell pellets that are used for the identification of common mutant genes (*TP53*, *BRCA1* and *BRCA2*). Several other bodily fluids, including urine, uterine lavage, ascites and peritoneal fluids, have also been shown as source of tumor-derived material that can be used to differentiate between OC patients and healthy or benign individuals [84–88]. Further studies are needed to determine the sensitivity and specificity of other non-invasive tests for diagnosis of OC.

Early detection of malignancy is critical in reducing mortality and morbidity. Technologies such as NGS and digital PCR allows the detection of very low frequency events. However, sensitivity remains a limiting factor for early stage disease, not only in terms of assay sensitivity but also by the rate or potentially low amount of ctDNA shed by small asymptomatic tumors that may not be easily detectable in a reasonable volume of blood drawn [89]. In addition, early detection through ctDNA assumes that all mutant DNA in blood is tumor derived. However, clonal hematopoiesis, mainly the formation of a distinct subset of blood cells with genetic alterations, has been observed in 10% of cancer free individuals aged over 65 years and may persist for many years [90]. While 40–60% of mutations arise randomly outside of known driver genes, several genes are commonly mutated in clonal hematopoiesis [77], amongst them is *TP53* which is also relevant for the diagnosis of HGSOc. Though earlier reports utilising samples from uterine lavage demonstrated absence of mutations in women without cancer [84], current studies have highlighted high background of driver gene mutations in healthy controls [91,92]. Therefore, non-cancer derived mutations may constitute a significant cause of false positivity for mutant DNA detection. Thus, algorithms that account for clonal hematopoiesis (in blood) and regenerative defects (tissues) in NGS data is required to achieve high specificity for mutant DNA analysis in gynecological studies.

Several initiatives have emerged for early diagnosis through ctDNA analysis. The CancerSEEK study [76] combines a machine-learning algorithm with the analysis of specific mutations in cfDNA and of circulating protein markers known to increase in certain cancers, to derive an accurate diagnosis of eight cancer types. In particular, OC was detected with a sensitivity of 98% and specificity of > 99%. The biotechnology company GRAIL reported at the 2018 ESMO Conference [93] results on 2402 prospectively collected samples from non-cancer controls and newly diagnosed untreated patients. Amongst them, seven OC patients were included, with 71% of them being positively identified. More recently, in ASCO 2019, GRAIL reported on their prototype methylation technology that can detect the tumor tissue of origin with high accuracy [94]. However, no data on ovarian cancer patients was presented. As discussed above, hypermethylated promoter regions of tumor suppressor genes could be used as a marker for detecting OC at an early stage, since these genes have been shown to be significantly methylated in both early (I and II) and advanced stage (III and IV) [65,68,69]. Hence, future large prospective studies in early stage ovarian cancer are key to demonstrate clinical validity of ctDNA analysis before they may be incorporated in routine clinical practice.

5. Conclusions

In conclusion, CTC enumeration coupled with molecular characterization is a non-invasive tool that hold promise as a prognostic marker for OC, identifying patients at greater risk of recurrence or death. It may also have a role for monitoring response and post-treatment surveillance after surgery or systemic therapies. Similarly, ctDNA analysis demonstrates high concordance of mutation and epigenetic changes seen in tumor biopsies, and can potentially track minimal residual disease during treatment with a higher sensitivity than CTCs.

Furthermore, both CTCs and ctDNA may help determine the emergence of novel mutations or reversions that manifest as resistance tumor subclones, ultimately leading to disease recurrence. Future efforts are required for standardization of analysis platforms, and the incorporation of liquid biopsies as a companion biomarker in large therapeutic clinical trials. Finally, improvements in patient outcomes are key to demonstrate the utility of these liquid biopsy modalities for personalized cancer medicine.

Funding

D.A. is supported by an Edith Cowan University PhD Scholarship. E.G. is supported by fellowships from the Cancer Research Trust and Cancer Council Western Australia. This study was funded by a grant from the Australia New Zealand Gynaecological Oncology Group (ANZGOG) to T.M., E.G and L.C.

Author contributions

Conceptualisation: D.A., L.C., E.G; data curation: D.A, L.C; funding acquisition: L.C., T.M., M.Z., E.G.; writing - original draft: D.A.; writing - review & editing: all authors.

Declaration of competing interest

None declared.

References

- [1] L.A. Torre, et al., Global cancer statistics, 2012, *CA A Cancer J. Clin.* 65 (2) (2015) 87–108.
- [2] S. Anuradha, et al., Survival of Australian women with invasive epithelial ovarian cancer: a population-based study, *Med. J. Aust.* 201 (5) (2014) 283–288.
- [3] A. Heintz, et al., Carcinoma of the ovary, *Int. J. Gynecol. Obstet.* 95 (2006) S161–S192.
- [4] J.T. Henderson, E.M. Webber, G.F. Sawaya, Screening for ovarian cancer: updated evidence report and systematic review for the US preventive services task force, *Jama* 319 (6) (2018) 595–606.
- [5] J.V. Frangioni, New technologies for human cancer imaging, *J. Clin. Oncol.* 26 (24) (2008) 4012–4021.
- [6] L.A. Diaz Jr., A. Bardelli, Liquid biopsies: genotyping circulating tumor DNA, *J. Clin. Oncol.* 32 (6) (2014) 579–586.
- [7] G. Sölkert, et al., Clinical use of cancer biomarkers in epithelial ovarian cancer: updated guidelines from the European Group on Tumor Markers, *Int. J. Gynecol. Cancer* 26 (1) (2016) 43.
- [8] V. Nossov, et al., The early detection of ovarian cancer: from traditional methods to proteomics. Can we really do better than serum CA-125? *Am. J. Obstet. Gynecol.* 199 (3) (2008) 215–223.
- [9] P. Bagan, et al., Value of cancer antigen 125 for diagnosis of pleural endometriosis in females with recurrent pneumothorax, *Eur. Respir. J.* 31 (1) (2008) 140–142.
- [10] R. Bast Jr. et al., CA 125: the past and the future, *Int. J. Biol. Mark.* 13 (4) (1998) 179–187.
- [11] K. Pantel, C. Alix-Panabières, Liquid biopsy and minimal residual disease—latest advances and implications for cure, *Nat. Rev. Clin. Oncol.* (2019) 1.
- [12] K. Kolostova, et al., The added value of circulating tumor cells examination in ovarian cancer staging, *Am. J. Canc. Res.* 5 (11) (2015) 3363.
- [13] K. Kolostova, et al., Molecular characterization of circulating tumor cells in ovarian cancer, *Am. J. Canc. Res.* 6 (5) (2016) 973.
- [14] E. Obermayr, et al., Efficient leukocyte depletion by a novel microfluidic platform enables the molecular detection and characterization of circulating tumor cells, *Oncotarget* 9 (1) (2018) 812–823.
- [15] Y.-x. Guo, et al., Diagnostic value of HE4+ circulating tumor cells in patients with suspicious ovarian cancer, *Oncotarget* 9 (7) (2018) 7522.
- [16] M. Lee, et al., Predictive value of circulating tumor cells (CTCs) captured by

- microfluidic device in patients with epithelial ovarian cancer, *Gynecol. Oncol.* 145 (2) (2017) 361–365.
- [17] D.H. Suh, et al., Circulating tumor cells in the differential diagnosis of adnexal masses, *Oncotarget* 8 (44) (2017) 77195.
- [18] M. Kim, et al., Post-debulking circulating tumor cell as a poor prognostic marker in advanced stage ovarian cancer: a prospective observational study, *Medicine* 98 (20) (2019).
- [19] E. Obermayr, 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.* 128 (1) (2013) 15–21.
- [20] E. Obermayr, et al., Circulating tumor cells: potential markers of minimal residual disease in ovarian cancer? a study of the OVCAD consortium, *Oncotarget* 8 (63) (2017) 106415.
- [21] K. Behbakht, 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.* 123 (1) (2011) 19–26.
- [22] A. Poveda, et al., Circulating tumor cells predict progression free survival and overall survival in patients with relapsed/recurrent advanced ovarian cancer, *Gynecol. Oncol.* 122 (3) (2011) 567–572.
- [23] J.F. Liu, et al., Predictive value of circulating tumor cells (CTCs) in newly-diagnosed and recurrent ovarian cancer patients, *Gynecol. Oncol.* 131 (2) (2013) 352–356.
- [24] X. Zhang, et al., Analysis of circulating tumor cells in ovarian cancer and their clinical value as a biomarker, *Cell. Physiol. Biochem.* 48 (5) (2018) 1983–1994.
- [25] M.L. Pearl, et al., Prognostic analysis of invasive circulating tumor cells (ICTCs) in epithelial ovarian cancer, *Gynecol. Oncol.* 134 (3) (2014) 581–590.
- [26] M.L. Pearl, et al., Treatment monitoring of patients with epithelial ovarian cancer using invasive circulating tumor cells (ICTCs), *Gynecol. Oncol.* 137 (2) (2015) 229–238.
- [27] L. Nie, et al., Folic acid targeting for efficient isolation and detection of ovarian cancer CTCs from human whole blood based on two-step binding strategy, *ACS Appl. Mater. Interfaces* 10 (16) (2018) 14055–14062.
- [28] W. Liu, et al., Folic acid conjugated magnetic iron oxide nanoparticles for non-destructive separation and detection of ovarian cancer cells from whole blood, *Biomater. Sci.* 4 (1) (2016) 159–166.
- [29] L. Zhu, et al., Analysis of the gene expression profile in response to human epididymis protein 4 in epithelial ovarian cancer cells, *Oncol. Rep.* 36 (3) (2016) 1592–1604.
- [30] N. Ning, et al., Improvement of specific detection of circulating tumor cells using combined CD45 staining and fluorescence in situ hybridization, *Clin. Chim. Acta* 433 (2014) 69–75.
- [31] Q. Rao, et al., Detection of circulating tumour cells in patients with epithelial ovarian cancer by a microfluidic system, *Int. J. Clin. Exp. Pathol.* 10 (9) (2017) 9599–9606.
- [32] J.W. Po, et al., Improved ovarian cancer EMT-CTC isolation by immunomagnetic targeting of epithelial EpCAM and mesenchymal N-cadherin, *J. Circ. Biomarkers* 7 (2018) 1849454418782617.
- [33] C. Blassl, et al., Gene expression profiling of single circulating tumor cells in ovarian cancer—Establishment of a multi-marker gene panel, *Mol. Oncol.* 10 (7) (2016) 1030–1042.
- [34] J.D. Kuhlmann, et al., ERCC1-positive circulating tumor cells in the blood of ovarian cancer patients as a predictive biomarker for platinum resistance, *Clin. Chem.* 60 (10) (2014) 1282–1289.
- [35] B. Aktas, et al., 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* 21 (5) (2011) 822–830.
- [36] I. Chebouti, et al., EMT-like circulating tumor cells in ovarian cancer patients are enriched by platinum-based chemotherapy, *Oncotarget* 8 (30) (2017) 48820.
- [37] M.J. Mitchell, M.R. King, Computational and experimental models of cancer cell response to fluid shear stress, *Front. Oncol.* 3 (2013) 44.
- [38] A.M. Haslehurst, et al., EMT transcription factors snail and slug directly contribute to cisplatin resistance in ovarian cancer, *BMC Canc.* 12 (1) (2012) 91.
- [39] N. Aceto, et al., Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis, *Cell* 158 (5) (2014) 1110–1122.
- [40] S. Gkountela, et al., Circulating tumor cell clustering shapes DNA methylation to enable metastasis seeding, *Cell* 176 (1–2) (2019) 98–112 e14.
- [41] E.S. Cho, et al., Therapeutic implications of cancer epithelial-mesenchymal transition (EMT), *Arch Pharm. Res. (Seoul)* 42 (1) (2019) 14–24.
- [42] J.C. Wan, et al., Liquid biopsies come of age: towards implementation of circulating tumour DNA, *Nat. Rev. Cancer* 17 (4) (2017) 223.
- [43] F.R. Harris, et al., Quantification of somatic chromosomal rearrangements in circulating cell-free DNA from ovarian cancers, *Sci. Rep.* 6 (2016) 29831.
- [44] S. Čolaković, et al., Prognostic value of CA125 kinetics and half-life in advanced ovarian cancer, *Int. J. Biol. Mark.* 15 (2) (2000) 147–152.
- [45] C.G.A.R. Network, Integrated genomic analyses of ovarian carcinoma, *Nature* 474 (7353) (2011) 609.
- [46] T. Forshew, et al., Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA, *Sci. Transl. Med.* 4 (136) (2012) 136ra68–136ra68.
- [47] E. Pereira, et al., Personalized circulating tumor DNA biomarkers dynamically predict treatment response and survival in gynecologic cancers, *PLoS One* 10 (12) (2015) e0145754.
- [48] C.A. Parkinson, et al., Exploratory analysis of TP53 mutations in circulating tumour DNA as biomarkers of treatment response for patients with relapsed high-grade serous ovarian carcinoma: a retrospective study, *PLoS Med.* 13 (12) (2016) e1002198.
- [49] Y.R. Park, et al., Optimization to detect TP53 mutations in circulating cell-free tumor DNA from patients with serous epithelial ovarian cancer, *Obstet. Gynecol. Sci.* 61 (3) (2018) 328–336.
- [50] B. Weigelt, et al., Diverse BRCA1 and BRCA2 reversion mutations in circulating cell-free DNA of therapy-resistant breast or ovarian cancer, *Clin. Cancer Res.* 23 (21) (2017) 6708–6720, <https://doi.org/10.1158/1078-0432.CCR-17-0544>.
- [51] R.C. Arend, et al., Molecular response to neoadjuvant chemotherapy in high-grade serous ovarian carcinoma, *Mol. Cancer Res.* 16 (2018) 813–824.
- [52] J. Oikonen, et al., Prospective longitudinal ctDNA workflow reveals clinically actionable alterations in ovarian cancer, *JCO Precis. Oncol.* 3 (2019) 1–12.
- [53] N. Iwahashi, et al., Liquid biopsy-based comprehensive gene mutation profiling for gynecological cancer using CAncer Personalized Profiling by deep Sequencing, *Sci. Rep.* 9 (1) (2019) 10426.
- [54] Z.H. Du, et al., Next-generation sequencing unravels extensive genetic alteration in recurrent ovarian cancer and unique genetic changes in drug-resistant recurrent ovarian cancer, *Mol. Genet. Genom. Med.* 6 (4) (2018) 638–647.
- [55] A. Piskorz, et al., Feasibility of Monitoring Response to the PARP Inhibitor Rucaparib with Targeted Deep Sequencing of Circulating Tumor DNA (ctDNA) in Women with High-Grade Serous Carcinoma on the ARIEL2 Trial, *American Society of Clinical Oncology*, 2016.
- [56] J.A. Martignetti, et al., Personalized ovarian cancer disease surveillance and detection of candidate therapeutic drug target in circulating tumor DNA, *Neoplasia* 16 (1) (2014) W28–W29.
- [57] A. Vanderstichele, 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.* 23 (9) (2016) 2223–2231, <https://doi.org/10.1158/1078-0432.CCR-16-1078>.
- [58] P.A. Cohen, et al., Abnormal plasma DNA profiles in early ovarian cancer using a non-invasive prenatal testing platform: implications for cancer screening, *BMC Med.* 14 (1) (2016) 126.
- [59] M. Nakabayashi, et al., Massively parallel sequencing of cell-free DNA in plasma for detecting gynaecological tumour-associated copy number alteration, *Sci. Rep.* 8 (1) (2018) 11205.
- [60] M. Ratajska, et al., Detection of BRCA1/2 mutations in circulating tumor DNA from patients with ovarian cancer, *Oncotarget* 8 (60) (2017) 101325.
- [61] K.K. Lin, et al., BRCA reversion mutations in circulating tumor DNA predict primary and acquired resistance to the PARP inhibitor rucaparib in high-grade ovarian carcinoma, *Cancer Discov.* 9 (2) (2019) 210–219.
- [62] L. Giannopoulou, et al., ESR1 methylation in primary tumors and paired circulating tumor DNA of patients with high-grade serous ovarian cancer, *Gynecol. Oncol.* 150 (2) (2018) 355–360.
- [63] M. Widschwendter, et al., The potential of circulating tumor DNA methylation analysis for the early detection and management of ovarian cancer, *Genome Med.* 9 (1) (2017) 116.
- [64] L. Giannopoulou, et al., 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* 8 (13) (2017) 21429.
- [65] B. Wang, et al., Detection of OPCML methylation, a possible epigenetic marker, from free serum circulating DNA to improve the diagnosis of early-stage ovarian epithelial cancer, *Oncol. Lett.* 14 (1) (2017) 217–223.
- [66] B. Wang, et al., Application of multiplex nested methylated specific PCR in early diagnosis of epithelial ovarian cancer, *Asian Pac. J. Cancer Prev. APJCP* 16 (7) (2015) 3003–3007.
- [67] Y. Wu, et al., Aberrant methylation of RASSF2A in tumors and plasma of patients with epithelial ovarian cancer, *Asian Pac. J. Cancer Prev. APJCP: Asian Pac. J. Cancer Prev. APJCP* 15 (3) (2014) 1171–1176.
- [68] Q. Zhang, et al., A multiplex methylation-specific PCR assay for the detection of early-stage ovarian cancer using cell-free serum DNA, *Gynecol. Oncol.* 130 (1) (2013) 132–139.
- [69] R. Dong, et al., Frequent SLIT2 promoter methylation in the serum of patients with ovarian cancer, *J. Int. Med. Res.* 40 (2) (2012) 681–686.
- [70] A.E. BonDurant, et al., Quantitative detection of RASSF1A DNA promoter methylation in tumors and serum of patients with serous epithelial ovarian cancer, *Gynecol. Oncol.* 123 (3) (2011) 581–587.
- [71] T.E. Liggitt, et al., Distinctive DNA methylation patterns of cell-free plasma DNA in women with malignant ovarian tumors, *Gynecol. Oncol.* 120 (1) (2011) 113–120.
- [72] R. Wang, et al., Cell-free circulating tumor DNA analysis for breast cancer and its clinical utilization as a biomarker, *Oncotarget* 8 (43) (2017) 75742.
- [73] L. Calapre, et al., Locus-specific concordance of genomic alterations between tissue and plasma circulating tumor DNA in metastatic melanoma, *Mol. Oncol.* 13 (2) (2019) 171–184.
- [74] J.D. Kuhlmann, et al., LOH at 6q and 10q in fractionated circulating DNA of ovarian cancer patients is predictive for tumor cell spread and overall survival, *BMC Canc.* 12 (1) (2012) 325.
- [75] M. Murtaza, et al., Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA, *Nature* 497 (7447) (2013) 108.
- [76] J.D. Cohen, et al., Detection and localization of surgically resectable cancers with a multi-analyte blood test, *Science* 359 (6378) (2018) 926–930.
- [77] J. Phallen, et al., Direct detection of early-stage cancers using circulating tumor DNA, *Sci. Transl. Med.* 9 (403) (2017) ean2415.
- [78] C. Bettgowda, et al., Detection of circulating tumor DNA in early- and late-stage human malignancies, *Sci. Transl. Med.* 6 (224) (2014) 224ra24–224ra24.
- [79] E.L. Christie, et al., Reversion of BRCA1/2 germline mutations detected in circulating tumor DNA from patients with high-grade serous ovarian cancer, *J. Clin. Oncol.* 35 (12) (2017) 1274–1280.

- [80] Y.-M. Kim, et al., Prospective study of the efficacy and utility of TP53 mutations in circulating tumor DNA as a non-invasive biomarker of treatment response monitoring in patients with high-grade serous ovarian carcinoma, *J. Gynecol. Oncol.* 30 (3) (2018).
- [81] R.W. Tothill, et al., Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome, *Clin. Cancer Res.* 14 (16) (2008) 5198–5208.
- [82] M. Schwartz, et al., Applying precision medicine to ovarian cancer: proof-of-principle for a “molecular second look”, *Int. J. Gynecol. Cancer* 28 (3) (2018) 479.
- [83] M. Barquín, et al., Peritoneal washing is an adequate source for somatic BRCA1/2 mutation testing in ovarian malignancies, *Pathol. Res. Pract.* 215 (2) (2019) 392–394.
- [84] E. Maritschnegg, et al., Lavage of the uterine cavity for molecular detection of Müllerian duct carcinomas: a proof-of-concept study, *J. Clin. Oncol.* 33 (36) (2015) 4293.
- [85] J. Zhou, et al., Urinary microRNA-30a-5p is a potential biomarker for ovarian serous adenocarcinoma, *Oncol. Rep.* 33 (6) (2015) 2915–2923.
- [86] L. Závěský, et al., Evaluation of cell-free urine microRNAs expression for the use in diagnosis of ovarian and endometrial cancers. A pilot study, *Pathol. Oncol. Res.* 21 (4) (2015) 1027–1035.
- [87] C.M. Yamamoto, et al., Comparison of benign peritoneal fluid-and ovarian cancer ascites-derived extracellular vesicle RNA biomarkers, *J. Ovarian Res.* 11 (1) (2018) 20.
- [88] Y. Wang, et al., Evaluation of liquid from the Papanicolaou test and other liquid biopsies for the detection of endometrial and ovarian cancers, *Sci. Transl. Med.* 10 (433) (2018) eaap8793.
- [89] C. Fiala, E.P. Diamandis, Utility of circulating tumor DNA in cancer diagnostics with emphasis on early detection, *BMC Med.* 16 (1) (2018) 166.
- [90] A.J. Silver, S. Jaiswal, Clonal hematopoiesis: pre-cancer PLUS, *Adv. Cancer Res.* 141 (2019) 85–128.
- [91] N. Nair, et al., Genomic analysis of uterine lavage fluid detects early endometrial cancers and reveals a prevalent landscape of driver mutations in women without histopathologic evidence of cancer: a prospective cross-sectional study, *PLoS Med.* 13 (12) (2016) e1002206.
- [92] J.J. Salk, et al., Ultra-sensitive TP53 sequencing for cancer detection reveals progressive clonal selection in normal tissue over a century of human lifespan, *Cell Rep.* 28 (1) (2019) 132–144 e3.
- [93] M.C. Liu, et al., Plasma cell-free DNA (cfDNA) assays for early multi-cancer detection: the Circulating Cell-Free Genome Atlas (CCGA), *Ann. Oncol.* 29 (8) (2018) ESMO 2018 Congress.
- [94] M. Liu, et al., Genome-wide cell-free DNA (cfDNA) methylation signatures and effect on tissue of origin (TOO) performance, in ASCO, *J. Clin. Oncol.* 37 (15_suppl) (2019) 3049-3049.
- [95] A. Morikawa, et al., PIK3CA and KRAS mutations in cell free circulating DNA are useful markers for monitoring ovarian clear cell carcinoma, *Oncotarget* 9 (20) (2018) 15266.