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Review Article

Epithelial ovarian cancer and the use of circulating tumor DNA: A systematic review

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HIGHLIGHTS

- Circulating tumor DNA as a biomarker in epithelial ovarian cancer is promising in several settings.
- The methods used to analyze circulating tumor DNA in epithelial ovarian cancer need to be optimized.
- Further studies are needed to establish the use of circulating tumor DNA in the clinic.

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ABSTRACT

Objective. One way to improve the survival rate of epithelial Ovarian Cancer (EOC) is by identifying effective biomarkers useful at different stages and time points of the disease. A potential biomarker is circulating tumor DNA (ctDNA) in plasma or serum. In this systematic review, we provide an overview of applications of ctDNA in EOC to discuss the direction of future research in this field.

Methods. We performed a systematic search in Pubmed, Embase, and Scopus to identify relevant clinical studies eligible for inclusion. Furthermore, the references in the identified studies and relevant reviews were assessed to identify additional studies. The PRISMA guideline was employed to perform the systematic review, and data from the studies were extracted using piloted data extraction forms.

Results. A total of 36 observational studies were included. The concordance between tumor and ctDNA was assessed in 19 studies, early diagnosis in 1, diagnosis in 23, monitoring of treatment response in 7, detection of reversion mutations in 3, prognosis in 9, but no studies assessed early detection of recurrence. Data from the studies were reported descriptively. The studies had a large variation in the methods used for ctDNA analysis and limited sample sizes of 10–126 patients. Overall, the studies show that ctDNA is a potential biomarker for EOC useful in several settings during assessment and treatment of these patients.

Conclusions. Although the identified studies are limited in number and their methods for ctDNA analysis vary, it is clear that ctDNA as a biomarker for EOC is promising for several applications in diagnostics, monitoring of treatment response, and prognostics. However, more studies are needed to establish the ideal methods and settings for the clinical use of ctDNA in EOC.

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Contents

1. Introduction

Ovarian cancer (OC) is the third most common gynecologic cancer worldwide and has the highest mortality rate among gynecologic cancers [1]. The term OC refers to cancers arising in the ovaries, fallopian tubes, or peritoneum as these types of cancers are similar regarding pathology, symptoms, and treatment [2]. The most common type of OC is epithelial ovarian cancer (EOC), which accounts for 90% of the cases [1,3]. It has several subtypes with differences in aggressiveness and prognosis [2,3]. The 5-year survival rate of OC in advanced stages (FIGO stage III-IV) is 29%. However, FIGO stage I disease has a 5-year survival of 93% [4]. Despite relevant treatment, the majority of patients with advanced ovarian cancer relapse within a few years [2].

CA-125 is the best-characterized biomarker in OC and has been widely studied to improve its specificity and sensitivity [2]. Two screening studies used the dynamics of CA-125, combined with other modalities to screen for OC. Although both studies improved the proportion of patients diagnosed in early stages, their level of specificity, sensitivity, and survival benefit was not sufficient to recommend the introduction of a screening program for OC [2,5,6]. Additionally, intensive monitoring of CA-125 during follow-up does not improve the overall survival rate [2]. These shortcomings of CA-125 has resulted in a continued search for more specific and sensitive biomarkers to improve the survival rate for these patients.

During the last decades, there has been a considerable focus on the need for biomarkers for various cancer types. This has led to the discovery of liquid biopsies [7,8], which covers the approach of using tumor-derived circulating cell-free DNA (cfDNA) and RNA, circulating tumor cells, and exosomes for the identification of disease [8]. CfDNA is found in plasma and released to the blood passively by necrotic or apoptotic cells or secreted actively from cancer cells [7,8]. The presence of cfDNA in the bloodstream of humans was first described in 1948 by Mandel and Metais [9]. In 1977 Leon and co-workers found a higher amount of cfDNA in cancer patients than individuals without cancer [10]. Later researchers established that cancer-specific DNA originating from the malignant tumor is detectable in cfDNA in cancer patients [8]. This is named circulating tumor DNA (ctDNA). Cancers harbor somatic genetic alterations unique for the individual tumor which means that ctDNA is distinguishable from noncancerous cfDNA because it contains these genetic alterations [7,8]. One of the major advantages of ctDNA is that it potentially holds genetic material from the entire tumor as well as metastases (if present) [7]. Furthermore, the use of ctDNA enables repeated and noninvasive testing of the molecular landscape of cancers. This is not the case for tissue biopsies that are invasive and painful [7,8]. Previous research has reported a high concordance between mutational profiles in matched tumor and ctDNA, especially in breast, colorectal, and non-small-cell lung carcinomas [8]. The fraction of ctDNA in cfDNA typically ranges between 0.01% [11] and 93% [12]. Therefore, highly sensitive methods to detect ctDNA are essential [7]. Several technologies to detect ctDNA are described, including quantitative PCR, digital PCR-based technologies, and next-generation sequencing (NGS) with a targeted approach or non-targeted as is seen in whole-exome and whole-genome sequencing (WGS) [8]. The analysis of ctDNA is potentially useful in several diagnostic applications [7,8], which is illustrated in Fig. 1.

CtDNA has been studied as a biomarker for various other types of cancer and seems promising for EOC. It is possibly the biomarker needed to improve the survival rate of the disease. The objective of this systematic review is to provide an overview of applications of ctDNA analyses as a new biomarker in EOC. The focus is on the analysis of tumor-specific aberrations and methylation patterns in ctDNA. The methods for ctDNA detection and its applications will be discussed along with the direction of future research in this field.

2. Method

The systematic review was reported according to the PRISMA guidelines [13].

2.1. Eligibility criteria

The systematic review included clinical studies assessing the use of circulating tumor DNA as a biomarker in EOC. Inclusion and exclusion criteria were predetermined and based on study design, publication date, population, and intervention.

Clinical studies were defined as randomized controlled trials, observational studies (cohort, case-control, and cross-sectional studies) presenting original research data. The population of interest were patients with EOC or any of its subtypes. The chosen intervention was the use of ctDNA in serum or plasma focusing on at least one of the following applications: Concordance between tumor and ctDNA; early diagnosis; diagnosis; monitoring of treatment response; detection of reversion mutations; prognosis, and early detection of recurrence. To avoid bias linked to small sample sizes, studies were excluded if

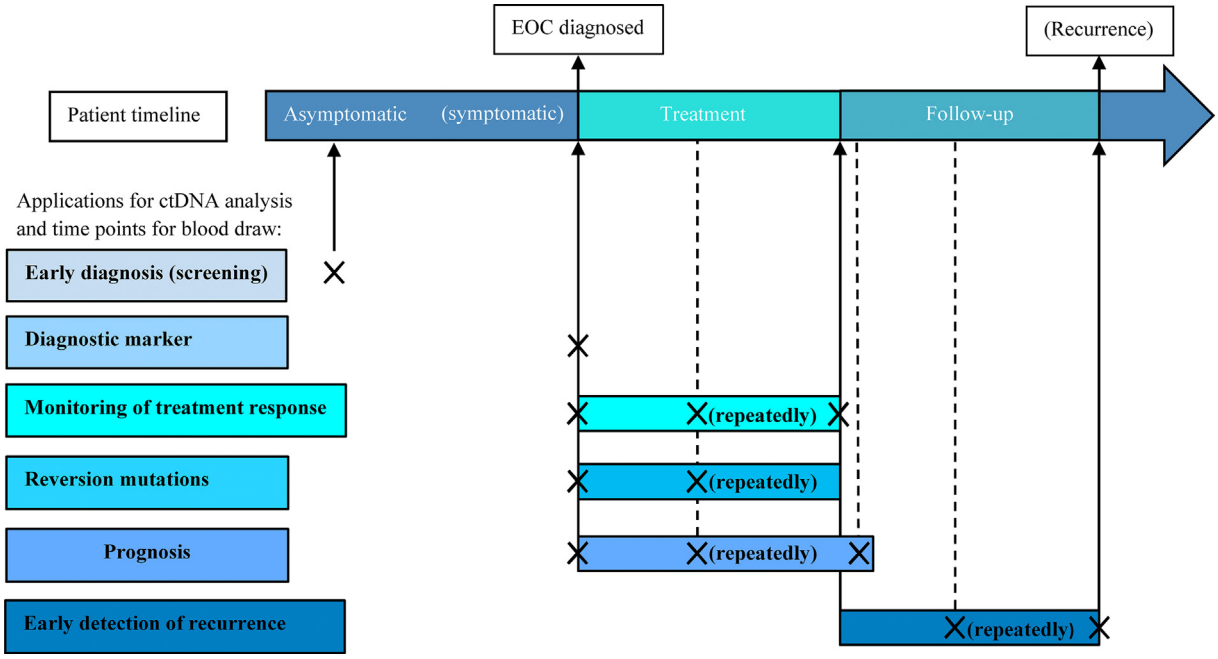

Fig. 1. A timeline for ctDNA analysis during patient care. For each application, the most relevant time points for blood draw are marked with an "X". For some applications, the blood might be drawn repeatedly during patient care. The timespan for repeated blood draw is shown in colors matching the applications. Prognosis can be assessed by ctDNA analysis of a sample collected once (at diagnosis, during treatment, or after the end of treatment) or by the change in ctDNA in samples collected at several time points during and after completion of treatment. Once a recurrence is diagnosed, and a different treatment is started, the applications used during treatment are relevant again.

ctDNA analysis was performed on less than 10 patients. Studies were included if data for EOC patients were clearly distinguishable from that of other patients or if these data were retrievable. In studies where this was not the case, the authors were contacted via e-mail for additional information, and if no reply was received the study was excluded.

Studies were excluded if their assessment of ctDNA was limited to total cfDNA level, concentration, or fragment lengths because this is not cancer-specific. Publication date before 2004 was also an exclusion criterion to ensure that the included studies used the most relevant methods for ctDNA analysis. Non-English articles, articles with no original data, and studies with no full-text article available were additionally discarded.

2.2. Search strategy

The author CFT created a systematic search strategy for a comprehensive literature search in three electronic databases; PubMed, Embase, and Scopus. The search strategy was designed in collaboration with the research group and a research librarian. The free text words "ovarian cancer", "epithelial ovarian cancer" and its subtypes along with "circulating tumor DNA", "cell-free DNA" and "circulating DNA" were used for the search. Furthermore, the search included relevant MESH terms (Pubmed) or subject headings (Embase) linked to the keywords (see Supplementary Table S1 for the search strings used in the databases). We also examined reference lists of included studies and relevant reviews for additional studies eligible for inclusion.

2.3. Study selection

Searches in the three databases were performed on June 22, 2020. The search results from the databases were imported to Endnote X9 (Thomson Reuters, New York, NY, USA) and doublets were removed. Two authors (CFT and MK) independently screened the titles and abstracts. The same two authors subsequently performed a full-text review of the selected studies. Any inconsistency in the selection process was resolved by discussion until consensus.

2.4. Data extraction

Data extracted from the eligible articles include the first author's name, year of publication, title, sample type, number of participants and their type of EOC, FIGO stage, number of controls (if any), the method used to analyze ctDNA, the focus of the study, and the results. The data were extracted using a piloted data collection form.

3. Results

After reviewing the databases a total of 36 studies were included in this systematic review (Fig. 2). All the studies were observational. The different applications were assessed in the following number of studies: the concordance between tumor and ctDNA in 19 studies (Table 1); early diagnosis in 1 (Table 2); diagnosis in 23 (Table 2); monitoring of treatment response in 7 (Table 3); detection of reversion mutations in 3 (Table 3), and prognosis in 9 studies (Table 4). No studies assessed ctDNA as a tool for early detection of recurrence.

4. Discussion

The number of studies on ctDNA and its use in various types of cancer has increased tremendously during the last decade. We performed this systematic review to provide an overview of studies assessing different applications of ctDNA in patients with EOC. As seen in Tables 1–4, the 36 included studies apply a wide range of different methods and outcome measures. A discussion of the different applications is presented in the next paragraphs.

4.1. Concordance between tumor DNA and ctDNA

How aberrations identified in ctDNA correspond to tumor DNA are important knowledge, when developing methods for the use of ctDNA as a biomarker. A summary of the articles assessing this is presented in Table 1.

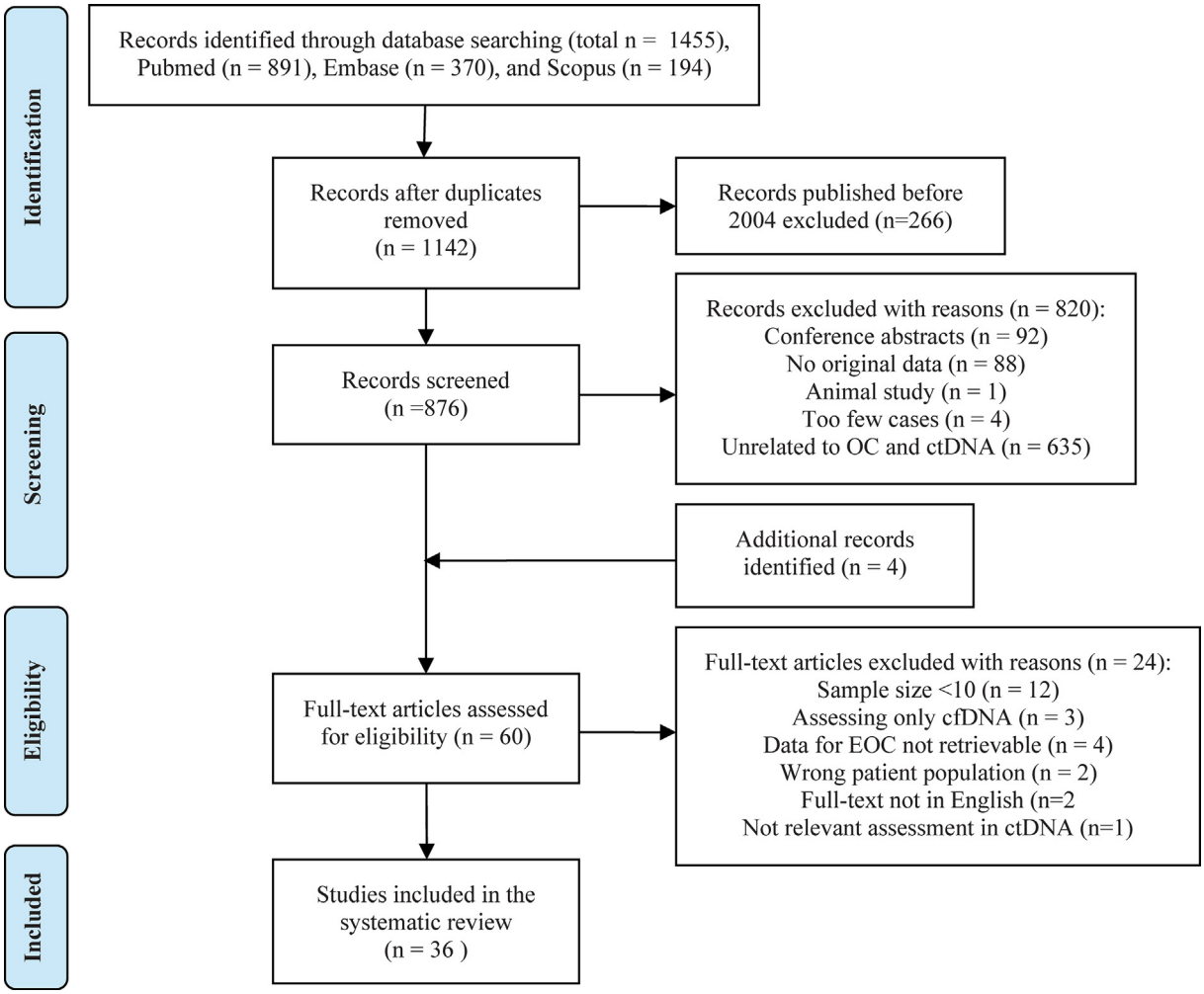

Fig. 2. PRISMA flow diagram.

Methylations in tumor and ctDNA were assessed by methylation-specific PCR (MSP) in eight studies. Four studies focused on the methylation status of one gene and identified an agreement in methylation status between tumor and ctDNA in 62–100% of cases [14–17]. Interestingly, the lowest and highest agreements were found in two studies assessing methylation status in *RASSF1A* [14,15]. The remaining four studies focused on methylations in tumor and ctDNA in more than one gene. Ibanez de Caceres et al. mainly focused on two genes and detected concordant methylation status in tumor and ctDNA in 93% of the patients [18], and Dvorska et al. identified a significant correlation coefficient in methylation status of *CHD1* and *PAX1* between tumor and ctDNA [19]. Two studies determined the methylation profile in six and seven genes, respectively. One study identified at least one concordant hypermethylation in tumor and ctDNA in 62% of the patients [20] and the other in all of the patients [21]. The concordance of the methylation status between tumor and ctDNA varies between the assessed genes but also between studies assessing the same gene. This was seen for *RASSF1A* [14, 15] and may be explained by a difference in sample type since Bondurant et al. used serum and fresh frozen tumor tissue [14] while Giannopoulou et al. used plasma and formalin-fixed paraffin-embedded tumor tissue [15]. The two studies also use different types of MSP and Giannopoulou et al. included more than twice as many patients [15]. In the above-mentioned studies, the concordance between tumor and ctDNA appears to be better in serum than plasma. This is surprising because plasma has a lower risk of contamination with genomic DNA released from white blood cells during the clotting process and is therefore generally recommended rather than serum. Contamination from the white blood cells may cause an elevated amount of cfDNA, which then again reduces the concentration of ctDNA [22]. We would therefore expect a better concordance in plasma than serum.

Targeted NGS for assessment of single nucleotide variants (SNV) and indels was used in five studies. The studies applied different panels, assessing varying numbers of genes, and identified concordant mutations in tumor and ctDNA in between 50% and 95% of the patients [23–28]. Another study with a targeted approach used a panel with 500 genes for targeted NGS to identify SNV, indels, and Copy Number Variants (CNV), and they detected concordant mutations in tumor and ctDNA in all patients [29]. Forshew et al. used tagged amplicon deep sequencing to identify SNV in five genes, and in 19 out of 20 patients, mutations in ctDNA were also detected in matched tumor DNA [30]. All studies with a targeted approach found a high level of concordance, except for one; Han et al. identified concordant mutations in only half of the patients. It is not possible to compare the studies, because it differs whether the concordance was assessed based on tumor, ctDNA, or both. Therefore, it is unclear, why the fraction of patients with concordant mutations is lower in one study than the others. All studies used plasma and the sequencing depth is similar. The explanation may be a combination of a panel with a lower number of genes than the others, and that the panel was limited to coding regions [24]. This was not the case for Arend et al., where the panel, although comprising fewer genes, assessed for mutations in both coding and non-coding regions [23]. The potentially low ctDNA concentration may also affect the

Table 1
Concordance between tumor and ctDNA.

References	Samples	No. of pts.	Stage	Method	Focus	Results
Bondurant et al. 2011 [14]	S, Tff	20 EOC	I-IV	Multiplex, real-time methylation-specific PCR	*RASSF1A* promoter methylation	In all pts., methylation status of tumor and ctDNA was concordant
Giannopoulou et al. 2017 [15]	P, Tfp	53 HGSC	I-IV	Real-time methylation-specific PCR	*RASSF1A* promoter methylation	In 33/53 pts., methylation status of tumor and ctDNA was concordant
Giannopoulou et al. 2018 [16]	P, Tfp	53 HGSC	I-IV	Real-time methylation-specific PCR	*ESR1* promoter methylation	In 36/48 pts., methylation status of tumor and ctDNA was concordant
Dong et al. 2012 [17]	S, Tff	36 EOC	I-IV	Methylation-specific PCR	*SLIT2* promoter methylation	In 34/36 pts., methylation status of tumor and ctDNA was concordant
Ibanez de Caceres et al. 2004 [18]	S, Tfp	EOC 40	I + III + IV	Methylation-specific PCR	Hypermethylation of *RASSF1A* and *BRCA1*	In 37/40 pts., methylation status of tumor and ctDNA was concordant
		EOC 12			Hypermethylation of *RASSF1A* and *BRCA1*, *APC*, *p14*, *p16*, and *DAPK*	In 11/12 pts., methylation status of tumor and ctDNA was concordant
Dvorska et al. 2019 [19]	P, Ts	33 EOC	I-IV	Methylation-specific PCR followed by pyrosequencing	MI for *PAX1*, *PTEN*, *CDH1*, and *RASSF1A*	Significant correlation coefficient in methylation status between tumor and ctDNA in *CDH1* ($r = 0.43$, $p = 0.015$) and *PAX1* ($r = 0.77$, $p < 0.01$)
Su et al. 2009 [20]	S, Tff	26 EOC	I-IV	Methylation-specific PCR	Methylation status of *SRP1*, *2*, *5*, *SOX1*, *PAX1*, and *LMX1A*	In 16 pts., at least 1 concordant hypermethylation was identified in tumor and ctDNA
Zhang et al. 2013 [21]	S, T	20 EOC	I-IV	Multiplex methylation-specific PCR	Methylation status of *APC*, *RASSF1A*, *CDH1*, *RUNX3*, *TFPI2*, *SFRP5*, and *OPCML*	In all pts., at least 1 concordant hypermethylation was detected in tumor and ctDNA
Arend et al. 2018 [23]	P, Tff	14 HGSC	IIIC-IV	Targeted NGS (50 genes), amplicon-based	SNV and indels	In 12/14 pts., at least 1 identical mutation was identified in tumor DNA and ctDNA
Han et al. 2020 [24]	P, Tff	10 EOC	III-IV	Targeted NGS (88 genes), hybridization-based	SNV, and indels	5 pts. had 0 and 5 pts. had 1–2 concordant mutations in tumor and ctDNA
Lin et al. 2019 [25]	P, Tfp	41 EOC	II-IV	Targeted NGS (54 genes), hybridization-based	Primary somatic *BRCA* mutations (SNV and indels)	In 30/41 pts., identical *BRCA* mutations were detected in tumor and ctDNA
		110 EOC			*TP53* mutations (SNV and indels)	In 98/110 pts., identical *TP53* mutations were detected in tumor and ctDNA
Weigelt et al. 2017 [26]	P, Tfp	19 EOC	III-IV	Targeted NGS (143 genes), hybridization-based	*TP53*, *BRCA1*, *BRCA2*, and 140 other genes (SNV, indels, and CNV)	In 18/19 pts., identical somatic mutations were identified in tumor and ctDNA
Phallen et al. 2017 [27]	P, Tff, Tfp	14 EOC	I-IV	Targeted NGS (58 genes), hybridization-based	SNV, small insertions, and deletions	In 13/14 pts., mutations in ctDNA were identified in matched tumor DNA
Wang Y et al. 2018 [28]	P, T	36 EOC	I-IV	Targeted NGS (16 genes), amplicon-based	SNV and indels in 16 genes	In 29/36 pts., at least 1 of the mutations identified in ctDNA was identified in tumor DNA
Oikkonen et al. 2019 [29]	P, Tff	12 HGSC	IIB-IVB	Targeted NGS (508 or 635 genes), hybridization-based	SNV, indels, and CNV	Concordant mutations were identified in all pts. Median concordance for mutations in tumor and ctDNA: 79%. Median concordance for CNV with ctDNA tumor content >30%: 74% (7/12 pts.).
Forshew et al. 2012 [30]	P, Tfp	37 HGSC	III-IV	Targeted NGS (6 genes), amplicon-based	SNV in *TP53*, *PTEN*, *EGFR*, *BRAF*, *KRAS*, and *PIK3CA*	In 19/20 pts., mutations identified in ctDNA were detected in matched tumor DNA
Harris et al. 2016 [31]	P, Tff	10 HGSC	III-IV	WGS on tumor DNA and qPCR of ctDNA	Structural rearrangements	In 8/10 pts., junctions identified in tumor DNA were also detected in ctDNA
Kim et al. 2019 [32]	P, Tff, Tfp	61 HGSC	II-IV	Sanger sequencing on tumor DNA and ddPCR of ctDNA	*TP53* mutations (SNV)	In 38/41 pts., the tumor-specific *TP53* mutations were detected in ctDNA.
Swisher et al. 2005 [33]	P, S, T	69 EOC	I-IV	Nested PCR, followed by ligase detection reaction	Tumor-specific *TP53* mutations (SNV and indels)	Tumor-specific *TP53* mutations detected in ctDNA in 30% of pts.

CNV Copy Number Variants, ctDNA circulating tumor DNA, ddPCR droplet digital PCR, EOC Epithelial Ovarian Carcinoma, HGSC High-Grade Serous Carcinoma, MI Methylation indices, NGS Next-Generation Sequencing, No. Number, P plasma, pts. Patients, qPCR quantitative PCR, S serum, SNV single nucleotide variants, T tumor tissue, Tff Fresh Frozen tumor tissue, Tfp formalin-fixed and paraffin-embedded tumor tissue, Ts RNA stabilized tumor tissue, WGS Whole-Genome Sequencing.

observed concordance. Oikkonen et al. found that the concordance in CNV between tumor and plasma was lower in plasma samples with low tumor content, but still they identified concordant mutations in all patients [29].

Three studies assessed the detection rate of tumor-specific mutations in ctDNA. Harris et al. used mate-paired WGS of tumor DNA to detect breakpoints of structural variants. Then, based on the findings in the tumor they developed a personalized assay for quantitative PCR on ctDNA for each patient. In eight out of ten patients, junctions identified in the tumor were also identified in ctDNA [31]. Similarly, Kim et al. used Sanger sequencing of tumor DNA to identify mutations in *TP53* in twelve target regions, and then personalized droplet digital PCR assays were applied to quantify the mutations in ctDNA. In 38 out of 41 patients the tumor-specific *TP53* mutations were detectable in ctDNA [32]. The last study, by Swisher et al. used a ligase detection reaction and identified tumor-specific *TP53* mutations in ctDNA in 30% of the patients [33].

Overall, the studies in this section have demonstrated concordance between mutations identified in tumor and ctDNA. However, tumor heterogeneity is one of the major challenges when comparing findings in tumor and ctDNA.

4.2. Early diagnosis

A biomarker for EOC effective in a screening setting would be highly valuable. Currently, two-thirds of EOC cases are diagnosed in advanced stages and the prognosis is significantly better when diagnosed early. Only one study had a screening setting (See Table 2). They applied

Table 2
Early diagnosis (screening) and diagnosis (symptomatic patients).

References	Samples	No. of pts.	Stage	Controls	Method	Focus	Results
ctDNA and early diagnosis							
Widschwendter et al. 2017 [34]	S	43 EOC	I-IV	129 healthy	Targeted bisulfite sequencing	Methylation in 3 markers linked to *COL23A1*, *C2CD4D*, and *WNT6*	Detected EOC 0–2 years before diagnosis with SN: 23% and SP: 97%. For the low DNA sample: SN: 58% and SP: 88% (CA-125: SN: 42%, SP: 96%)
ctDNA as a diagnostic marker							
Giannopoulou et al. 2017 [15]	P, Tfp	59 HGSC	I-IV	51 healthy	Real-time methylation-specific PCR	*RASSF1A* promoter methylation	SN: 25%, no results for controls
Giannopoulou et al. 2018 [16]	P, Tfp	53 HGSC	I-IV	51 healthy	Real-time methylation-specific PCR	*ESR1* promoter methylation	SN: 26%, SP: 98%
Dong et al. 2012 [17]	S, Tff	36 EOC	I-IV	25 healthy	Methylation-specific PCR	*SLIT2* promoter methylation	SN: 75% and SP: 100%. SN stage I/II: 100% and III/IV: 72%
Wu et al. 2014 [37]	P	47 EOC	I-IV	10 healthy, 14 benign	Methylation-specific PCR	*RASSF1A* hypermethylation	SN: 36%, SP: 100%. SN stage I/II: 23% and III/IV: 48%.
Zhou et al. 2014 [38]	S, Tff	45 EOC	I-IV	20 healthy, 40 benign	Methylation-specific restriction enzyme PCR	Promoter methylation in *OPCML*	SN: 80%, SP: 100%. SN depending on stage; I: 43%, II: 68%, III: 86%, IV: 100%
Wang B et al. 2017 [39]	P	71 EOC	I-IV	80 healthy, 43 benign	Nested methylation-specific PCR	Promoter methylation in *OPCML*	SN: 90% and SP: 92%. SN stage I/II: 87% and III/IV: 94%
Ibanez de Caceres et al. 2004 [18]	S, Tfp	EOC 40	I + III + IV	20 healthy, 10 benign	Methylation-specific PCR	Hypermethylation of *RASSF1A* and *BRCA1*	SN: 63% and SP: 100%. SN stage I: 88% and III/IV: 56%
		EOC 12				Hypermethylation of *RASSF1A*, *BRCA1*, *APC*, *p14*, *p16*, and *DAPK*	SN: 93% and SP: 100%. SN stage I: 100% and III/IV: 91%
Sandeep Kumar et al. 2019 [40]	P	53 EOC	I-IV	15 healthy, 12 benign	Methylation-specific PCR	Promoter methylation in *RASSF1A* and *BRCA1*	Diagnosed EOC with SN: 58%. SP for healthy and benign was 100% and 83%. SN increased with stage, *RASSF1A* I/II: 27%, III/IV: 50%, *BRCA1*: I/II: 40%, III/IV: 92%
Dvorska et al. 2019 [19]	P, Ts	33 EOC	I-IV	7 healthy, 5 benign	Methylation-specific PCR followed by pyrosequencing	MI of *PAX1*, *PTEN*, *CDH1*, and *RASSF1A* combined with patients' age	SN: 91%, SP: 56%
Liggett et al. 2011 [41]	P	30 EOC	III-IV	30 healthy	Multiplex, nested methylation-specific PCR	Methylation status of *RASSF1A*, *CALCA*, and *EP300*	SN: 90%, SP: 87%,
				30 benign		Methylation status of *RASSF1A* and *PGR-PROX*	SN: 73%, SP: 80%
Melnikov et al. 2009 [42]	P, Tfp	33 serous OC	III-IV	33 healthy	Multiplex, nested methylation-specific PCR	Methylation status of *BRCA1*, *HIC1*, *PAX5*, *PGR-PROX*, and *THBS1*	SN: 85%, SP: 61%
Su et al. 2009 [20]	S	26 EOC	I-IV	20 benign	Methylation-specific PCR	Methylation status of *SRP1*, *2*, *5*, *SOX1*, *PAX1*, and *LMX1A*	Methylation in *SOX1*, *PAX1*, or *SFRP1* gave the best result. SN: 73%, SP: 75%
Zhang et al. 2013 [21]	S, T	126 EOC	I-IV	62 healthy, 82 benign	Multiplex methylation-specific PCR	Methylation status of *APC*, *RASSF1A*, *CDH1*, *RUNX3*, *TFPI2*, *SFRP5*, and *OPCML*	SN: 90%. SP for healthy was 100% and benign 88%. SN depending on stage; I: 85%, II: 82%, III: 96%, IV: 100% (only 1 patient)
Singh et al. 2020 [43]	S	44 EOC	I-IV	25 healthy	Multiplex methylation-specific qPCR	Promoter methylation in *HIC1* and *HOXA9*	SN: 84%, SP: 100%.
Widschwendter et al. 2017 [34]	S	48 HGSC	I-IV	41 healthy, 153 benign	Targeted bisulfite sequencing	Methylation in 3 markers linked to *COL23A1*, *C2CD4D*, and *WNT6*	SN: 58%. SP: 92%. SN stage I/II: 55% and III/IV: 59%
Cohen PA et al. 2016 [44]	P	32 HGSC	I-IV	32 benign	WGS low coverage (NIPT platform)	CNV	SN: 41% and SP: 94%. SN stage I/II: 38% and III/IV: 44%
Vanderstichele et al. 2017 [45]	P, Tff	45 HGSC	I-IV	44 healthy, 11 benign	WGS low coverage	CNV	Discriminated HGSC from healthy with SN: 84% and SP: 91%
Forshew et al. 2012 [30]	P, Tff, Tfp	38 HGSC	III-IV	None	Targeted NGS (6 genes), amplicon-based	SNV in *TP53*, *PTEN*, *EGFR*, *BRAF*, *KRAS*, *and PIK3CA*	SN: 53%
Phallen et al. 2017 [27]	P, Tff, Tfp	37 EOC	I-IV	44 healthy	Targeted NGS (58 genes), hybridization-based	SNV, small insertions and deletions	SN: 73%, SP: 100%, SN dependent on stage; I: 70%, II: 75%, III: 71%, IV: 83%
Cohen JD et al. 2018 [46]	P, Tfp	54 EOC	AJCC I-III	812 healthy	Targeted NGS (16 genes), amplicon-based and single immuno-assay (CancerSEEK)	SNV and indels in the genes and 8 protein biomarkers	SN: 98%, SP: 99%. No results for ctDNA alone.
Wang Y et al. 2018 [28]	P, T	82 EOC	I-IV	192 healthy	Targeted NGS (16 genes), amplicon-based	SNV and indels	SN: 44%, SP 100%. SN stage I/II: 35% and III/IV: 56%
No et al. 2012 [47]	S	36 EOC	I-IV	16 benign	qPCR	CNV in *B2M*, *ABCF2*, *RAB25*, and *CLDN4*.	No significant difference in CNV level between EOC and benign disease, and stage of the disease
Kuhlmann et al. 2012 [48]	S	63 EOC	I-IV	20 healthy	cfDNA divided into HMWF and LMWF followed by microsatellite analysis	LOH at 4 loci in *PTEN*, *BRCA1*, *BRCA2*, and *IGF2R*	LOH in at least one locus could diagnose cancer with SN: 49% and SP: 100%

AJCC American Joint Committee on Cancer staging system, cfDNA cell-free DNA, CNV Copy Number Variants, ctDNA circulating tumor DNA, EOC Epithelial Ovarian Carcinoma, HGSC High-Grade Serous Carcinoma, HMWF high molecular-weight fraction, LMWF low molecular-weight fraction, LOH Loss of heterozygosity, MI Methylation indices, NIPT Non-Invasive Prenatal Testing, NGS Next-Generation Sequencing, No. Number, OC Ovarian Carcinoma, P plasma, pts. patients, qPCR quantitative PCR, S serum, SN sensitivity, SNV single nucleotide variants, SP specificity, T tumor tissue, Tff Fresh Frozen tumor tissue, Tfp formalin-fixed and paraffin-embedded tumor tissue, Ts RNA stabilized tumor tissue, WGS Whole-Genome Sequencing.

Table 3
Monitoring of treatment response and detection of revesion mutations.

References	Samples	No. of pts.	Stage	Method	Focus	Results
ctDNA and monitoring of treatment response						
Rusan et al. 2020 [49]	P	32 EOC, platinum-resistant	I-IV	Methylation-specific ddPCR	*HOXA9* promoter methylation status after 3rd treatment cycle	92% of pts. with undetectable methylation had an imaging response, compared to 62% of pts. with detectable methylation. Difference non-significant.
Widschwendter et al. 2017 [34]	S	25 EOC	IIIC-IV	Targeted bisulfite sequencing	Methylation in 3 markers linked to *COL23A1*, *C2CD4D*, and *WNT6* during NAC	78% and 86% of responders and non-responders to treatment were classified correctly. For CA-125 it was 20% and 75%.
Han et al. 2020 [24]	P, Tff	10 EOC	III-IV	Targeted NGS (88 genes), hybridization-based	SNV and indels. Change in MAF in ctDNA pre- and post-surgery	In the 6 pts. with mutations detected in ctDNA before surgery, MAF decreased after debulking surgery. Not linked to clinical response.
Oikkonen et al. 2019 [29]	P, Tff	12 HGSC	IIB-IVB	Targeted NGS (508 or 635 genes), hybridization-based	SNV, indels, and CNV. Change in MAF during treatment	Good-responding pts. had a significantly higher proportion of mutations with decreasing MAF than poor-responding pts.
Parkinson et al. 2016 [50]	P, Tfp	27 HGSC	I, III-IV	Sanger sequencing of tumor, then dPCR on ctDNA	*TP53* mutations (SNV and short indels). *TP53*-MAF during treatment	A faster decrease during treatment was seen in *TP53*-MAF than CA-125. Stable or rising *TP53*-MAF and decreasing CA-125 were observed in 2 pts. In both cases, CT confirmed progressive disease
Kim et al. 2019 [32]	P, Tff, Tfp	28 HGSC	II-IV	Sanger sequencing on tumor DNA and ddPCR of ctDNA	*TP53* mutations (SNV). *TP53*-MAC during treatment	*TP53*-MAC decreased significantly during treatment. Not linked to clinical response. The *TP53*-MAC reduction rate was 93% in pts. with optimal and 74% in pts. with suboptimal debulking surgery.
Kuhlmann et al. 2012 [48]	S	63 EOC	I-IV	cfDNA was divided into HMWF and LMWF. Then microsatellite analysis	LOH at 4 loci in *PTEN*, *BRCA1*, *BRCA2*, and *IGF2R* pre- and post-treatment	No correlation of LOH in the 4 loci to platinum-resistance
ctDNA and detection of reversion mutations						
Christie et al. 2017 [53]	P, Tfp	30 HGSC, 16 with recurrence	I-IV	Targeted NGS, amplicon-based	*TP53*, *BRCA1*, and *BRCA2* reversion mutations (SNV and indels)	Reading frame restoring reversion mutations detected in ctDNA with SN: 60% and SP: 100% (tumor DNA is the reference). They all had a recurrence.
Lin et al. 2019 [25]	P, Tfp	78 EOC	Unclear	Targeted NGS, hybridization-based	*BRCA1* and *BRCA2* reversion mutations (SNV and indels) pretreatment	8 pts. had detectable reversion mutations in ctDNA pretreatment. They had significantly shorter PFS after PARP-inhibitor treatment than those without reversion mutations (1.8 vs 9.0 months). In 4/4 pts. with matched tumor biopsies available, at least one reversion mutation detected in ctDNA was also detected in the tumor.
		112 EOC			*BRCA1* and *BRCA2* reversion mutations (SNV and indels) acquired during therapy	In 4/8 pts., acquired *BRCA* reversion mutations were identified before progression (median 3.4 months). In the remaining 4 pts., they were detected at the time of clinical progression.
Weigelt et al. 2017 [26]	P, Tfp	19 EOC, platinum-resistant	III-IV	Targeted NGS, hybridization-based	*BRCA1* and *BRCA2* reversion mutations and resistance genes (SNV, indels, and CNV)	6 putative *BRCA1* reversion mutations were identified in 4 pts.

cfDNA cell-free DNA, CNV Copy Number Variants, ctDNA circulating tumor DNA, dPCR digital PCR, ddPCR droplet digital PCR, EOC Epithelial Ovarian Carcinoma, HGSC High-Grade Serous Carcinoma, HMWF high molecular-weight fraction, LMWF low molecular-weight fraction, LOH Loss of heterozygosity, MAC mutant allele count, MAF mutant allele frequency, NAC Neo-adjuvant Chemotherapy, NGS Next-Generation Sequencing, No. Number, P plasma, PARP poly ADP-ribose polymerase, PFS progression-free survival, pts. patients, S serum, SN sensitivity, SNV single nucleotide variants, SP specificity, Tff Fresh Frozen tumor tissue, Tfp formalin-fixed and paraffin-embedded tumor tissue.

targeted bisulfite sequencing to assess for methylation in three markers. Setting up a screening study is demanding for EOC because of the relatively low incidence of the disease. Therefore, it was necessary to collect samples from 100,000 women to obtain 40–50 OC patients within the study period. They detected EOC zero to two years before diagnosis with 23% sensitivity and 97% specificity. Because of an extended time from sample collection to serum separation the samples were divided into low and high DNA concentration to adjust for the risk of contamination of serum by white blood cell leakage. For the low DNA samples (19 EOC and 67 from healthy control), the sensitivity was improved to 58% and the specificity was reduced to 88% [34]. Even then, the samples with low DNA concentration might still be contaminated with DNA from white blood cells, and this poses a great limitation to the study. Using blood collection tubes that stabilize the white blood cells in future studies would circumvent this risk of contamination [35].

A recent prospective clinical trial used ctDNA detection for early diagnosis of cancer in women and detected cancer with a similar sensitivity and specificity [36] as Widscwendter et al. [34]. For OC it is crucial that a new biomarker in early detection and diagnosis achieves a high specificity and positive predictive value. This is due to the low prevalence of OC and because a positive test result will lead to surgery under general anesthesia with the risk of complications. The positive predictive value for samples with low DNA in the study by Widschwendter et al. means that for each EOC patient detected one healthy woman would undergo surgery [34]. Similarly, the positive predictive value of CA-125 combined with other modalities in the UKCTOCS and UKFOCSS studies would mean that for each OC cancer detected, two healthy women would have surgery [5,6]. It is evident that ctDNA as a biomarker in early diagnosis has a promising potential, but further studies are needed to improve the level of sensitivity, specificity and positive predictive value.

4.3. Diagnosis

A biomarker in EOC with improved sensitivity and specificity compared to CA-125 would be very valuable. It could potentially increase the number of patients diagnosed early and reduce the number of women with benign ovarian cysts undergoing laparotomy. CtDNA as a diagnostic marker in EOC have been assessed in several studies (Table 2).

Table 4
ctDNA as a prognostic tool.

References	Samples	No. of pts.	Stage	Method	Focus	Results
Giannopoulou et al. 2017 [15]	P, Tfp	47 HGSC	I-IV	Real-time methylation-specific PCR	*RASSF1A* promoter methylation at diagnosis	No significant correlation between *RASSF1A* promoter methylation and OS or PFS
Giannopoulou et al. 2018 [16]	P, Tfp	42 HGSC	I-IV	Real-time methylation-specific PCR	*ESR1* promoter methylation at diagnosis	No significant correlation between *ESR1* promoter methylation in ctDNA and OS or PFS
Rusan et al. 2020 [49]	P	32 EOC, platinum-resistant	I-IV	Methylation-specific ddPCR	*HOXA9* promoter methylation status:	No significant difference. Median PFS: 5.5 vs 5.7 months, median OS: 13.1 vs 17.3 months
					• At baseline, pts. with and without methylation	
					• After 1 treatment cycle, pts. with and without methylation	Significant difference. Median PFS: 5.2 vs 6.7 months, median OS: 12.6 vs 18.7 months
					• After 3 treatment cycles, pts. with and without methylation	Significant difference. Median PFS: 5.1 vs 8.3 months, median OS 9.4 vs 19.4 months
					• Changes during 2 treatment cycles, pts. with persistent methylation and those where it disappeared	Significant difference. Median PFS: 5 vs 9 months, median OS: 9 vs 19 months
Thomsen et al. 2019 [54]	P	23 EOC, platinum-resistant	I, III-IV	Methylation-specific dPCR	*HOXA9* methylation changes during 1 treatment cycle, pts. with increasing levels vs pts. with stable levels	Significant difference. Median PFS: 1.4 vs 7.8 months, median OS: 4.3 vs 12 months
Kim et al. 2019 [32]	P, Tff, Tfp	28 HGSC	II-IV	Sanger sequencing on tumor DNA and ddPCR of ctDNA	*TP53*-MAC pre-operative	No correlation between pre-operative *TP53*-MAC and TTP.
					TP53-MAC level 3 months after completed ct	Significantly shorter TTP in pts. with a high level of *TP53*-MAC (> 0.2 copies/μl) than those with low *TP53*-MAC
					TP53-MAC change from immediately after completed ct to 3 months after completed ct	7% in *TP53*-MAC non-doubling group and 58% in the doubling group got a recurrence within 12 months after the end of ct. The difference in TTP was significant.
Parkinson et al. 2016 [50]	P, Tfp	40 HGSC	I, III-IV	Sanger sequencing of tumor, then dPCR on ctDNA	*TP53*-MAF at diagnosis, pts. with *TP53*-MAF below and above the median	Significant difference. Median TTP of 168 vs 245 days.
					TP53-MAF decrease ≤60% after 1 cycle of ct	Predicted TTP <6 months with SN: 71% and SP: 88%.
Kuhlmann et al. 2012 [48]	S	63 EOC	I-IV	cfDNA was divided into HMWF and LMWF. Then microsatellite analysis	LOH at 4 loci in *PTEN, BRCA1, BRCA2,* and *IGF2R* at diagnosis	LOH in the *IGF2R*-locus was predictive for reduced OS (25 vs. 53 months). No correlation to PFS.
No et al. 2012 [47]	S	36 EOC	I-IV	qPCR	CNV in *B2M, ABCF2, RAB25,* and *CLDN4* at diagnosis	Deletions in *RAB25* was predictive for poor DFS and among advanced-stage disease also for poor OS
Swisher et al. 2008 [33]	P, S, T	69 EOC	I-IV	Nested PCR, followed by ligase detection reaction	Tumor-specific *TP53* mutations (SNV, indels) at diagnosis	OS significantly reduced in pts. with detectable ctDNA, median OS: 28 vs 56 months

cfDNA cell-free DNA, CNV Copy Number Variants, ct chemotherapy, ctDNA circulating tumor DNA, DFS disease-free survival, dPCR digital PCR, ddPCR droplet digital PCR, EOC Epithelial Ovarian Carcinoma, HGSC High-Grade Serous Carcinoma, HMWF high molecular-weight fraction, LMWF low molecular-weight fraction, LOH Loss of heterozygosity, MAC mutant allele count, MAF mutant allele frequency, No. Number, OS Overall Survival, P plasma, PFS progression-free survival, pts. patients, qPCR quantitative PCR, S serum, SN sensitivity, SNV single nucleotide variants, SP specificity, T tumor tissue, Tff Fresh Frozen tumor tissue, Tfp formalin-fixed and paraffin-embedded tumor tissue, TTP time to progression.

The use of methylation status in ctDNA to diagnose EOC was assessed in fifteen studies. Six of these studies applied MSP to assess methylation status in one gene with varying sensitivities and high specificities [15–17,37–39]. The two studies assessing the methylation status of *OPCML* demonstrated the highest sensitivity which was 80% [38] and 90% [39]. The methylation status of *RASSF1A* and *ESR1* had the lowest sensitivities of around 25% [15,16]. Another eight studies used MSP to assess the methylation status of two to seven genes, which improved the sensitivity to between 58% and 93% [18–21,40–43]. One of the studies with the highest sensitivity also had the lowest specificity (56%). This study used a combination of methylation level in four genes and patients' age, but the number of controls was limited to 13 [19]. Widschwendter et al. applied targeted bisulfite sequencing to assess methylation status in three markers and identified EOC with 58% sensitivity and 92% specificity [34]. Eight studies estimated sensitivity depending on the stage of the disease. As anticipated six studies confirmed that sensitivity increased with advancing stages [21,34,37,38,40,41], but two studies found a decline in sensitivity with higher stage [17,18]. As expected, sensitivity improved in studies assessing methylations in more than one gene, whereas the studies assessing only one gene had stronger specificities. The two studies with the lowest specificities assessed methylation status in four [19] and five genes [42], which implies that methylation of the genes included in those studies were not cancer-specific. The specificities were generally higher when discriminating cancer patients from healthy controls than patients with benign diseases.

WGS was used in two studies with different approaches. Cohen et al. used WGS with a platform for Non-Invasive Prenatal Testing to identify CNV in ctDNA. This identified EOC in 13/32 patients at 94% specificity [44]. More convincing was the study by Vanderstichele et al. where WGS with low coverage was used to identify chromosomal instability. They discriminated EOC from healthy controls with 84% sensitivity and 91% specificity [45].

Two previously described studies used targeted NGS to identify EOC. Forshew et al. identified ctDNA in 20 out of 38 patients [30], and Phallen et al. identified EOC with 73% sensitivity and 100% specificity. The sensitivity improved with advancing stage of disease [27]. Additionally, Cohen et al. applied the CancerSEEK approach, which is comprised of a combination of amplicon-based targeted NGS to detect mutations in 16 genes along with an immunoassay to identify protein biomarkers in plasma. They identified the cancerous disease in 53 out of 54 EOC patients and the specificity was 99%. Sensitivity and specificity for ctDNA

analysis without the use of protein biomarkers were not stated in the study [46] but is expected to be reduced. Interestingly, Wang et al. used the same panel for amplicon-based targeted NGS and they detected EOC with 44% sensitivity and 100% specificity [28].

No et al. used an approach with quantitative real-time PCR to identify CNV in four genes and found no significant difference in CNV level between EOC and benign disease [47]. The last study divided cfDNA into high-, and low molecular-weight fractions followed by microsatellite analysis of loss of heterozygosity (LOH) at four loci. LOH in at least one locus could diagnose EOC with 49% sensitivity and 100% specificity [48].

The results of the studies in this section are rather inconsistent and show that it is a challenge to find biomarkers with both a high sensitivity and specificity. Based on the studies, the biggest challenge was to reach a high sensitivity. Further studies are needed to assess if ctDNA in the diagnostic setting can achieve a strong sensitivity and specificity. The aim should be that ctDNA identifies all EOC patients and to reduce, or best case eliminate, the number of healthy women undergoing surgery due to suspicion of EOC.

4.4. Monitoring of treatment response

If changes in ctDNA levels occur faster and more reliably than CA-125 levels, it could be valuable in monitoring of treatment response. When identifying a lack of treatment response, the treatment is usually changed. Faster and more reliable identification of non-responding patients, could thus potentially improve the overall survival rate and/or reduce side effects. Table 3 provides an overview of the seven studies using ctDNA to monitor treatment response.

Two studies focus on methylation status in samples during treatment. Rusan et al. used MSP to assess for methylation status in *HOXA9* during treatment of platinum-resistant EOC. After three treatment cycles, patients with undetectable methylations more often had evidence of treatment response on imaging than those with detectable methylations. The difference between the two groups was non-significant [49]. Widscwendter et al. assessed for methylations in three markers and identified a higher fraction of responders and non-responders to neoadjuvant chemotherapy than CA-125 [34]. This indicates that the tested methylation markers in ctDNA are better tools to monitor treatment response than CA-125.

The use of targeted sequencing of ctDNA to monitor treatment response was demonstrated in two studies. Han et al. used targeted NGS of plasma collected before and after surgery to assess the change in mutant allele fraction (MAF). They identified mutations in ctDNA before surgery in six out of ten patients and in these patients MAF in ctDNA was reduced after debulking surgery. Surprisingly, the four patients, with no detectable mutations in ctDNA before surgery had detectable mutations in ctDNA after surgery. It was unclear if the findings in ctDNA after surgery correlate with whether debulking surgery was optimal or suboptimal [24]. Oikkonen et al. also applied their targeted approach to monitor treatment response. They found that patients with a good clinical response to treatment had a significantly higher proportion of mutations with decreasing MAF in ctDNA than poor responding patients. An increase in *TP53*-MAF in ctDNA was detected in six patients with disease progression [29]. These results suggest that changes in MAF could be a tool to distinguish patients with a good and poor response to treatment.

Two studies used a personalized assay of *TP53* mutations for PCR of ctDNA based on Sanger sequencing of tumor DNA. Parkinson et al. found that *TP53*-MAF in ctDNA declined more rapidly during treatment than CA-125. Furthermore a stable value of *TP53-MAF* was seen during treatment in one patient and in another patient, a rise in *TP53*-MAF was detected during follow-up. In both of these patients, CA-125 was declining, but CT confirmed progressive disease [50]. Kim et al. demonstrated that the mean *TP53* mutant allele count (MAC) reduction rate before and after surgery was 93% in patients with optimal debulking surgery and 74% in patients with suboptimal debulking surgery. They found that *TP53*-MAC was significantly reduced during treatment [32], but it is unclear how the change in *TP53*-MAC corresponded to the patients' clinical response. Nonetheless, both studies indicate that changes in *TP53*-MAF or -MAC in ctDNA is useful when monitoring treatment response.

The only study demonstrating that ctDNA analysis provided no information on treatment response was done by Kuhlmann et al. They found that the detection of LOH in four loci in pre-treatment serum samples did not correlate with treatment response [48].

The studies monitoring treatment response are not comparable because they use a variation of different outcome measures and monitor response to treatment differently. Oikkonen et al. and Parkinson et al. who successfully identified poor-responding patients and patients with progressive disease presented the most promising results. Based on the described studies, the most precise approach to monitor treatment response would be by collection of ctDNA samples pre and postoperative, after 2–3 cycles of chemotherapy, and after the completion of chemotherapy. The order of sample collection should depend on whether the patient is operated upfront or treated with neoadjuvant chemotherapy.

4.5. Detection of reversion mutations

In recent years poly ADP-ribose polymerase (PARP)-inhibitors have been introduced as a maintenance treatment to newly diagnosed advanced EOC with known *BRCA1* or *2* mutations (present in approximately 20% [51]) and partial or complete response to treatment [52]. If a reversion mutation occurs in *BRCA1* or *2* it may minimize the effectiveness of the treatment. Therefore, the assessment of reversion mutations in ctDNA may help identify patients that will be unresponsive to the treatment. Reversion mutations were assessed by targeted NGS in three articles (Table 3). Christie et al. identified reversion mutations in tumor DNA in five patients but only in ctDNA from three of these five patients. The patients with detectable reversion mutations in ctDNA had a poor response to PARP-inhibitors or platinum-based treatment. It was not clear from the results whether this was different from the patients with no reversion mutations in ctDNA [53]. Since reversion mutations are identified in ctDNA in only three out of five patients with detectable reversion mutations in the tumor, it is clear that the sensitivity of the method needs improvement. Lin et al. identified *BRCA* reversion mutations in pretreatment ctDNA from 8 out of 112 patients and in 8 out of 78 patients acquired *BRCA* reversion mutations were detected during treatment with a PARP-inhibitor. Matched tumor DNA was assessed in four of the eight patients with detectable *BRCA* reversion mutations in pretreatment ctDNA. They all had at least one identical reversion mutation in ctDNA and in the tumor biopsy. Detection of acquired reversion mutations in ctDNA preceded disease progression with a median of 3.4 months in four out of eight patients. In the remaining four patients, reversion mutations were identified at the time of disease progression. Patients with reversion mutations in pretreatment ctDNA had significantly shorter progression-free survival (PFS) after PARP-inhibitor treatment than those without [25]. Thus, the detection of reversion mutations in ctDNA appears to be a valuable tool in predicting treatment response. The last study identified six putative *BRCA1* reversion mutations in ctDNA from 4/19 patients with platinum-resistant EOC [26].

Based on these studies, reversion mutations are identifiable in ctDNA, but it is either a relatively rare event or the methods need improvement to strengthen their sensitivity. It is also possible that other acquired resistance mechanism exists and ctDNA analysis might also be useful for detection of such mechanisms.

4.6. Prognosis

The capacity of a biomarker to determine the prognosis can help identify patients with a poor outcome who would benefit from either

additional or different treatment, or closer follow-up. This can potentially improve the overall survival or quality of life for these patients. The studies assessing ctDNA as a prognostic tool are presented in Table 4.

Pretreatment ctDNA as a prognostic marker was assessed in eight studies. Three of these studies evaluated the methylation status in a specific gene and found no significant correlation with overall survival (OS) and PFS, which indicates that pretreatment methylation status is not a good prognostic marker [15,16,49]. Kuhlmann et al. determined that LOH at *IGF2R* was predictive for reduced OS, but had no correlation with PFS [48]. No et al. found that a deletion in *RAB25* was an independent predictor of poor disease-free survival, and in advanced-stage disease also of poor OS [47]. Two studies focused on the association between *TP53* mutations in ctDNA and time to progression (TTP). Kim et al. found no correlation between *TP53*-MAC and TTP [32], whereas Parkinson et al. saw that TTP was significantly longer in patients with *TP53*-MAF level below the median [50]. The focus of these two studies was comparable but they did not apply the same type of PCR test or assess *TP53* mutations in a similar way. This might explain the difference in their results. In line with these studies, Swisher et al. found that OS was significantly reduced in patients with tumor-specific *TP53* mutations in ctDNA [33].

The changes in ctDNA during treatment as a prognostic tool was assessed in four studies. Two of these studies evaluated changes in *HOXA9* methylation status during treatment with either a PARP-inhibitor or tocotrienol. Both studies identified that changes in *HOXA9* methylation levels during treatment was a significant predictor of PFS and OS [49,54]. This was also the case for HOXA9 methylation status after the first treatment cycle [49]. The two studies focused on patients with platinum-resistant EOC, but the results could also be promising for other EOC patients [49,54]. Two studies, previously described, assessed changes in *TP53* mutations during treatment to predict TTP. Kim et al. observed that TTP was significantly shorter in patients with high *TP53*-MAC 3 months after completion of chemotherapy. Similarly, they also identified a significantly shorter TTP in patients where the level of *TP53*-MAC doubled from immediately after to 3 months after completion of chemotherapy [32]. Parkinson et al. assessed *TP53*-MAF during treatment and found that a decline below 60% after one cycle of chemotherapy can predict TTP within 6 months with 71% sensitivity and 88% specificity [50].

Only a minority of the studies assessing pretreatment ctDNA as a prognostic marker found a prognostic significance. On the other hand, studies monitoring changes in ctDNA during and after treatment have promising results. Most of the studies used one gene to assess prognosis, and perhaps the use of more genes would improve the results. The number of studies is rather small and more studies are needed to draw a clear conclusion.

4.7. The systematic review

This is one of the first reviews to exclusively focus on the use of ctDNA and its applications in EOC. A systematic search focusing solely on patients with EOC and ctDNA was applied, to ensure that all relevant studies were most likely identified. A limitation is the low quality of evidence available, which is limited to observational and retrospective studies. Based on our search, no randomized control trials have been performed yet. The effect measures differ substantially in the included studies, and therefore quantitative analysis including meta-analysis and comparison of the studies is impossible and irrelevant at this stage. No quality assessment of the studies was performed because of the broad focus of this review and a considerable variation in the study designs among the included studies.

Studies with no available full-text, unpublished, and grey literature were not included in the review, which means that publication bias cannot be ruled out.

4.8. Future directions

The use of ctDNA in EOC has various potential applications. The number of studies and their sample sizes are limited but results are promising. One of the clear strengths of ctDNA in EOC is that it may overcome the challenge of tumor heterogeneity. Tumors may contain subclones with different mutations and methylation patterns that are not present in a sub-sample of the tumor but may be present in the blood due to the release of tumor DNA from all subclones. In some studies, the analysis of ctDNA was based on findings in the tumor. This is highly relevant for monitoring of treatment response, recurrent disease, and as a prognostic marker, but it is irrelevant for (early) diagnosis of EOC because in this setting a tumor sample is not available. The use of a personalized assay for ctDNA analysis based on findings in the tumor is both a limitation and a strength. The limitation of this approach is that the development of a personalized assay for each patient is costly and time-consuming. On the other hand, a strength of this approach is that ctDNA analysis will be highly specific for each patient. If the number of tumor-specific mutations measured in ctDNA is sufficiently high, it could potentially be an extremely sensitive approach.

Methylations and somatic mutations including clonal hematopoiesis accumulates in noncancerous cells with increasing age [55–59]. This might be a challenge when one is trying to distinguish whether aberrations identified in the plasma are from ctDNA or released from normal aging tissue. Therefore, it is evident that the assessed aberrations need to be cancer-specific. This did however not seem to be the case for the methylations assessed in three studies on ctDNA as a diagnostic tool, where the specificity was relatively low [19,20,42]. The influence of increasing age on somatic mutations and methylations implies that it is essential that cases and controls are the same age. Thus, if controls are much younger than the cases this may overestimate the specificity. In six of the studies assessing ctDNA as a diagnostic marker, the difference in median or average age between cases and controls was 15 years or more [21,28,34,39,45,47], and in another six studies the age of the controls was not specified [15,16,18,37,44,48].

In future research it is necessary to determine whether detection of genetic alterations or methylations in ctDNA are useful biomarkers in EOC. Studies on ctDNA as a diagnostic marker are the only ones with comparable outcome measures. Among these studies, detection of methylations in ctDNA reached the highest sensitivities, but it could be premature to make a strong conclusion based on this. Firstly, almost twice as many studies assessed methylations compared to genetic alterations. Secondly, to estimate if methylations or genetic alterations are superior it would be reasonable to compare studies assessing a comparable number of genes. This is not possible in the reviewed literature because the number of assessed genes is much smaller in the methylation studies than the studies on genetic alterations. Furthermore, the type of cancer and possibly also the subtype of EOC may influence whether detection of methylations or genetic alterations is superior in daily clinical work.

The concentration of ctDNA, which is often relatively low, poses a potential challenge. Therefore, future studies should focus on how to improve the sensitivity of the methods, so that low-frequency genetic changes and methylations are identified. We identified studies focusing on genetic alterations or methylation status in one gene, and it is likely, these studies could improve the sensitivity by focusing on more than one gene. The studies using NGS, either with a targeted approach or WGS, are also likely to improve the sensitivity by deeper sequencing. In the studies with a targeted approach, sensitivity can also be enhanced by applying a broader panel. To improve NGS for ctDNA analysis the appropriate methods for bioinformatic filtering and error correction need to be identified. This would reduce the risk of identifying false positive changes in ctDNA.

5. Conclusion

In summary, this systematic review demonstrates that ctDNA is a promising biomarker for EOC in various setups. It is however clear that the best methods for the application of ctDNA in EOC need to be established in more detail before implementation in the routine clinical work. The best method will quite likely vary depending on the aim of the analysis.

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Conflict of interest statement

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Appendix A. Supplementary data

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