



# Non-invasive Technology Advances in Cancer—A Review of the Advances in the Liquid Biopsy for Endometrial and Ovarian Cancers

Mark R. Openshaw and Terri P. McVeigh\*

Cancer Genetics Unit, Royal Marsden NHS Foundation Trust, London, United Kingdom

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### \*Correspondence:

Terri P. McVeigh  
terri.mcveigh@cr.ac.uk

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Improving cancer survival rates globally requires improvements in disease detection and monitoring, with the aim of improving early diagnosis and prediction of disease relapse. Traditional means of detecting and monitoring cancers rely largely on imaging and, where possible, blood-based protein biomarkers, many of which are non-specific. Treatments are being improved by identification of inherited and acquired genomic aberrations in tumors, some of which can be targeted by newly developed therapeutic interventions. Treatment of gynecological malignancy is progressively moving toward personalized therapy, as exemplified by application of PARP-inhibition for patients with BRCA-deficient tubo-ovarian cancers, or checkpoint inhibition in patients with mismatch repair-deficient disease. However, the more recent discovery of a group of biomarkers described under the umbrella term of “liquid biopsy” promises significant improvement in our ability to detect and monitor cancers. The term “liquid biopsy” is used to describe an array of tumor-derived material found in blood plasma and other bodily fluids such as ascites, pleural fluid, saliva, and urine. It includes circulating tumor cells (CTCs), circulating nucleic acids including DNA, messenger RNA and micro RNAs, and extracellular vesicles (EVs). In this review, we discuss recent advancements in liquid biopsy for biomarker detection to help in diagnosis, prognosis, and planning of treatment of ovarian and endometrial cancer.

**Keywords:** biomarker, circulating tumor (ctDNA), microRNA, DNA, circulating tumor cell (CTC)

## INTRODUCTION

### Genomic Changes in Cancer Cells

Cancer is a ubiquitous disease, with 18 million new cases and 9.5 million cancer-related deaths reported annually worldwide (1). Cancer can be characterized by distinctive hallmarks, including abnormal growth control, escape of normal apoptotic pathways, growth factor secretion, and cell-cell interactions (2, 3). Cancer growth, invasion, and metastasis are facilitated by changes in two main classes of genes—tumor suppressor genes and proto-oncogenes. Tumor suppressor genes are a class of genes that work to inhibit cell proliferation, and loss of function of tumor suppressor genes is oncogenic. Proto-oncogenes are normal cellular genes that encode for protein products, working in concert with the products encoded by tumor suppressor genes to regulate cell growth and proliferation. Oncogenes, by comparison, are abnormal genes, the products encoded by which promote carcinogenesis, through over-activation, or over-production

of signals promoting cell growth/proliferation. Proto-oncogenes may be converted to oncogenes by a variety of mechanisms, including acquisition of gain-of-function mutations, localized copy number variation (amplification), or chromosomal translocation; which lead to constitutive over-activation or over-expression of the protein product (2). Many oncogenes, closely related to normal cellular proto-oncogenes, have been identified in common viruses, and infection with such viruses may in turn promote tumorigenesis. So-called “oncogenic viruses” include Hepatitis B and Hepatitis C (both implicated in development of hepatocellular cancer), Epstein Barr virus (Nasopharyngeal carcinoma, Hodgkin, and Burkitt’s lymphoma), Merkel cell Polyomavirus (Merkel cell cancer), HtlV-1 (adult T-cell Lymphoma), Human Herpes virus 8 (Kaposi’s sarcoma), and Human Papillomavirus (cervical cancer, ano-genital cancer, some head and neck cancers) (4).

Cells acquire genomic aberrations during each mitotic division. Uncorrected, these aberrations will then be passed on to subsequent generations. Driver mutations are those which confer a growth and/or survival advantage to the cell. The subclone bearing a driver mutation will have enhanced fitness compared to other cells and will therefore be preferentially selected for proliferation. Acquisition of 2–8 driver mutations will result in development of cancer. The molecular evolution of cancer may be mapped in parallel to morphological changes, through different phases of dysplasia, early neoplasia and late neoplasia (5). The majority of genomic changes in cancer are acquired as somatic events, but a small proportion of affected individuals carry germline variants that may be inadvertently identified by testing of tumor-derived DNA (6). Molecular aberrations in cancer provide unique targets for treatment, such biomarkers thus representing companion diagnostics (7).

Despite increasing advances in technology and improvement in our understanding of tumor biology, a number of challenges exist. These include the difficulty of obtaining tumor biopsies from which DNA of appropriate quality and quantity can be used for genomic analysis, the presence of intra-, and inter-tumoral genomic heterogeneity (8) which hinders both our understanding of tumors (9) and response to targeted agents (10), and dynamic change in tumors over time, particularly after treatment resulting development of resistance (11). Therefore, developments of circulating biomarkers that can measure this spatial and longitudinal heterogeneity are highly sought after.

The three main gynecological cancers include cervical, ovarian, and endometrial cancer. Major advances have been made in prevention of and screening for cervical cancer. Cytology-based population-level screening has existed for many decades in many developed countries, supplemented, or supplanted in more recent years with Human Papillomavirus (HPV) testing (12, 13). Vaccination against oncogenic HPV infection has resulted in significant reduction in the number of high grade cervical lesions identified in young women (14), and the World Health Organization has endorsed vaccination as one of the key strategies to try to eradicate cervical cancer as a public health problem, with a view to achieving 90% vaccine uptake in girls before age 15 (15, 16). Compared to cervical cancer, no such preventative vaccine programme exists, as yet, for

endometrial or ovarian cancer, although much research has focused on development of vaccine for Lynch syndrome-related malignancies (17–19).

Endometrial cancer is the most common cancer of the female reproductive tract, and its incidence is increasing, a fact largely attributed to increasing rates of obesity, and trends in exogenous estrogen use (20, 21). Endometrial cancer most often presents early, with irregular bleeding per vaginam. Comparatively, long heretofore known as the “silent killer,” most cases of ovarian cancer are diagnosed at late stages, as the presenting symptoms are often non-specific (22). At present, no screening test has been conclusively proven to impact mortality of ovarian/endometrial cancer in women at population-level or higher risk.

## Heritable Cancer Predisposition Syndromes

A number of heritable cancer predisposition syndromes are associated with significantly increased lifetime risks of ovarian and/or endometrial cancer (Table 1).

## Risk-Reducing Strategies for Ovarian and/or Endometrial Cancer

At present, in the absence of a proven screening test, for women with such conditions, the only proven way of minimizing their risk of endometrial and/or ovarian cancer is to undergo prophylactic surgery. In most cancer predisposition syndromes associated with increased gynecological cancer risk, the average age at diagnosis far predates that in the general population, often prior to the average age of menopause. To mitigate this risk, the age at which prophylactic surgery is recommended (35–40 years) also predates the age at which natural menopause would ordinarily occur, by many years (39–41). If premenopausal oophorectomy is undertaken, sequelae include infertility and premature menopause, with the associated risks of early cardiovascular, cognitive, or bony adverse events (42).

As it is postulated that the majority of serous epithelial ovarian cancers begin in the fallopian tube (43), early salpingectomy with delayed oophorectomy has been proposed as an alternative to standard risk-reducing bilateral salpingo-oophorectomy in certain patients with increased risks of ovarian cancer (44). It is uncertain whether such an approach has a role in women with Lynch Syndrome, given that the proportion of ovarian cancer of tubal origin in this setting is less well-established.

For women with Lynch syndrome who are also at increased risk of colorectal malignancy, requiring biannual colonoscopy, colonic surveillance following hysterectomy may be more uncomfortable, and technically more difficult (45). For women with Lynch Syndrome who wish to defer surgical prophylaxis, surveillance with transvaginal ultrasonography, endometrial pipelle biopsy, and/or hysteroscopy is not recommended, as there is no conclusive evidence to suggest this approach leads to stage-shift or improved survival in high-risk individuals (41). For other endometrial cancer predisposition syndromes (e.g., Cowden/Peutz-Jeghers syndromes), there is a paucity of evidence about the role of surveillance vs. risk-reducing surgery, and risk-management may be complicated further by a high incidence of

**TABLE 1 |** High risk cancer predisposition syndromes associated with endometrial and/or ovarian cancer, associated gene, and other cancers.

Associated gene	Ovarian cancer risk associated with germline pathogenic variant	Endometrial cancer risk associated with germline pathogenic variant	Other associated cancer types
<i>BRCA1</i> (23, 24)	43–76% <sup>a</sup>	–	Breast, Male breast, Prostate, Pancreatic
<i>BRCA2</i> (23, 24)	7.5–34% <sup>a</sup>	–	Breast, Male breast, Prostate, Pancreatic, Melanoma
<i>RAD51C</i> (25)	6–21% <sup>a</sup>	–	Breast
<i>RAD51D</i> (25)	7–23% <sup>a</sup>	–	Breast
<i>BRIP1</i> (26)	3.6–9.1% <sup>a</sup>	–	–
<i>PALB2</i> (27)	2.4–9.7% <sup>a</sup>	–	Breast, Male breast, Pancreatic
<i>STK11</i> (28)	~21% <sup>b</sup>	~9%	Colorectal, Breast, Stomach, Small bowel, Cervix (adenoma malignum), Pancreas, Testicular, Lung
<i>MLH1</i> (29)	4.8–15.4% <sup>c</sup>	33.1–52.3% <sup>c</sup>	Colorectal, Stomach, Upper gastrointestinal, Hepato–pancreatico–biliary, Brain
<i>MSH2</i> (29)	5.7–28% <sup>c</sup>	41.8–71.6% <sup>c</sup>	Colorectal, Stomach, Upper gastrointestinal, Hepato–pancreatico–biliary, Brain
<i>MSH6</i> (29)	0–31.2% <sup>c</sup>	27.3–65% <sup>c</sup>	Colorectal, Stomach, Upper gastrointestinal, Hepato–pancreatico–biliary, Brain
<i>PMS2</i> (30)	–	7–24% <sup>c</sup>	Colorectal
<i>PTEN</i> (31)	–	17–39% <sup>d</sup>	Breast, Thyroid, Colorectal, Kidney, Skin
<i>POLD1</i> (32)	–	Uncertain, moderate-high	Colorectal, Gastric, ? others

<sup>a</sup>High Grade Serous Ovarian cancer (33).

<sup>b</sup>Sex-cord stromal tumors with annular tubules (34, 35), rarely oxyphilic sertoli cell cancers (36).

<sup>c</sup>Usually non-serous ovarian cancer (37); Usually endometrioid endometrial cancer (38).

<sup>d</sup>Endometrioid common, multiple different types reported (38).

benign endometrial pathology in such conditions. For women at population-level endometrial cancer risk, the use of transvaginal sonography with endometrial biopsy as required as a screening tool is limited by a number of factors—including the intimate and invasive nature of the test, high cost, need for specialist sonographers, and the need for different cut-offs based on age and menopausal status (46). These issues have prompted discovery efforts for a non-invasive blood-based biomarker.

With respect to ovarian cancer surveillance, a multimodal strategy including transvaginal ultrasound, CA125, and considering factors such as age, menopausal status, and background predisposition has been shown to result in stage-shift at diagnosis of ovarian cancer in women at higher risk (47). However, whether such stage-shift translates to survival benefit has not yet been proven. CA125 has been included as a component of the ovarian cancer risk of malignancy index (RMI), alongside ultrasound scan result and menopausal status. An RMI score above 200 has been shown to be highly discriminate at detecting ovarian cancer (sensitivity 86%, specificity 97%) (48) in patients presenting with suspected symptoms. Major limitations of CA125 as a screening tool include observations that CA125 is raised in only 50–60% of patients with stage I and II cancers, while false positives in patients with no pathology/benign gynecological pathology are not infrequent (49). Whilst CA125 is raised in a higher proportion of advanced cases of ovarian cancer, and the measurement of CA125 combined with physical examination can detect up to 90% of relapses (50), there is evidence that commencing treatment based on elevated CA125 levels alone is not beneficial (51). Other putative blood-borne tumor biomarkers such as HE4, CHI3L1, PEBP4, and AGR2 have also been proposed, but have similar limitations when used individually, and even when multiplexed with other markers

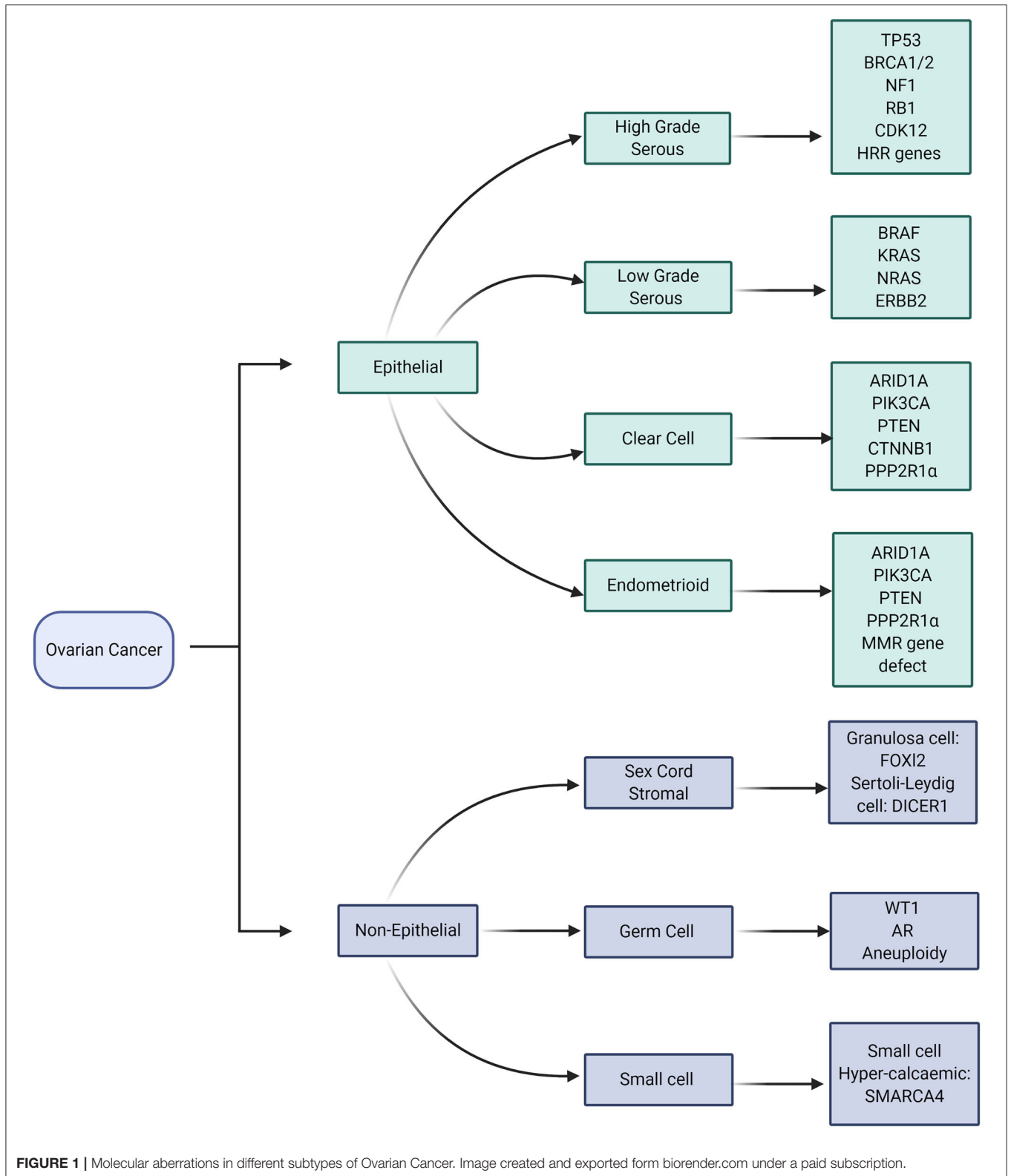
(52). The utility of such markers as part of multimodal strategies require further evaluation in large scale studies (53).

### Somatic Aberrations in Ovarian and Endometrial Cancers

The molecular characteristics of ovarian cancers are very closely aligned to the histopathological subtype (**Figure 1**). *TP53* gene mutations are almost ubiquitous in High Grade Serous Ovarian Cancer (HGSO) whilst variants in a number of other genes *NF1*, *BRCA1*, *BRCA2*, *RBI*, and *CDK12* are also commonly identified (**Figure 2**) (54). Approximately 41–50% of HGSO demonstrates Homologous Recombination Repair deficiency by virtue of somatic or germline genomic defects, which may then be exploited by therapy, with platinum-based chemotherapy and PARP inhibition (55). There are a number of ways to define HR deficiency (56), all of which currently involve a tumor biopsy; therefore non-invasive routes to defining HR deficiency would be beneficial.

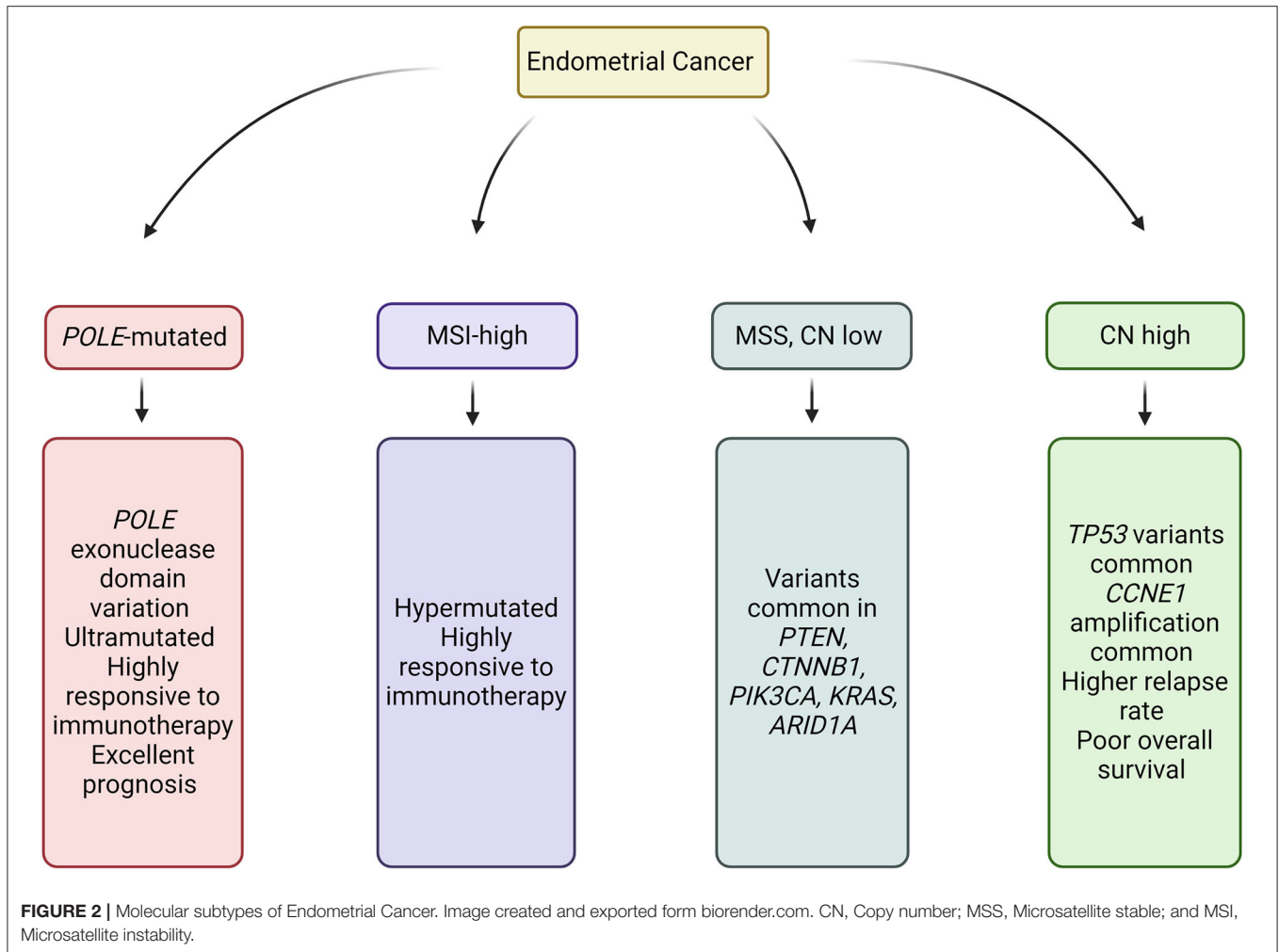
Characterization of endometrial cancer by integrated genomic, transcriptomic, and proteomic characterization has identified four sub-categories, which overlap but do not directly correlate with histopathological subtype (57) (**Figure 2**). Classification of cancer by molecular subtype rather than histological subtype is advantageous, in that it can direct therapy, provide prognostic information, and identify those patients requiring germline genetic assessment.

Mismatch Repair (MMR)-deficient (MMR-d) tumors, defined by absence of one or more MMR proteins by IHC analysis, usually demonstrate high levels of microsatellite instability (MSI-H), exhibit high expression of pro-inflammatory genes and enhanced neo-antigen expression (58). Approximately 10% of



epithelial ovarian cancers (mostly non-serous) (59, 60) and 20–40% of endometrial cancers (57, 61) demonstrate features of mismatch repair deficiency. Several studies have shown favorable

response rates and improved prognosis for patients with MMR-d tumors treated with immune checkpoint inhibitors (58, 62). A number of trials investigating the utility of checkpoint blockade



in patients with Epithelial Ovarian and Endometrial cancer are underway (63–65).

### AIM OF REVIEW

As genomic profiling becomes more cost-effective, biomarker discovery is increasingly moving away from non-specific tumor markers to highly refined “multi-omic” approaches. The aim of this review is to describe how knowledge of tumor biology and underlying molecular aberrations can be exploited for diagnostic, prognostic, and predictive biomarker design. We review emerging roles of circulating tumor cells, nucleic acids, messenger and microRNAs, and extracellular vesicles as tools for non-invasive diagnosis and surveillance in patients with or at risk of ovarian and/or endometrial cancer (Figure 3, Table 2).

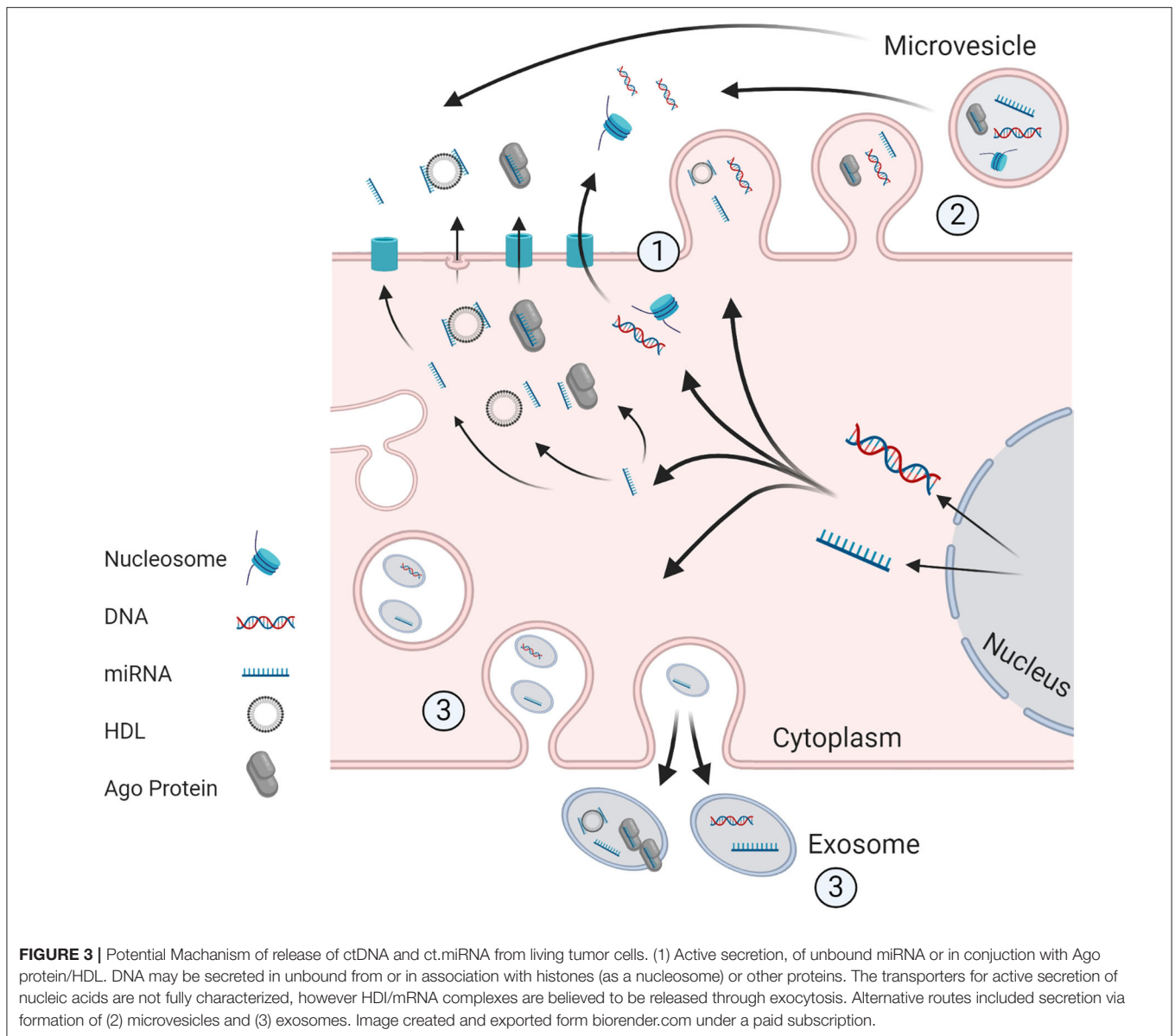
### THE “LIQUID BIOPSY”

The term “liquid biopsy” is a broad umbrella term used to describe a diverse array of tumor-derived material found in blood and other bodily fluids, including circulating tumors cells

(CTCs), extracellular vesicles (EVs), and free nucleic acids. Since these factors are derived directly from tumor cells, they carry genetic, and other biological material from the tumors from which they are derived, including (in some cases) lipids and proteins, and in the case of CTCs, living cells. As they can be obtained from non-invasive products, via repeated blood or fluid sampling, longitudinal monitoring is possible.

### Circulating Tumor Cells (CTCs)

Circulating tumors cells (CTCs) are tumor cells in the blood circulation that may exist as either single cells or cell conglomerates (clumps) (66). There is a building body of evidence to suggest that they are responsible for metastasis, although the cells must undergo a number of essential steps including intravasation into circulation, survival during dissemination, arrest at metastatic sites and then extravasation and colonization of the site before overt metastases can develop (67, 68). CTCs can be detected in low numbers even in early non-metastatic cancer indicating that dissemination of tumor cells in an early event. However, despite this early evidence of dissemination, few of these early cancers develop overt



metastases and there is evidence that later disseminating CTCs may be more suited to the development of metastases (69). The development of metastasis is a complex process with many other factors required to aid metastasis beyond the presence of CTCs (69, 70), as is clearly demonstrated by the fact that modern techniques for CTC isolation can detect many thousands of cells in the circulation of aggressive tumors, and despite this only a restricted number of metastases develop in the majority of patients (66).

Factors limiting the use of routine analysis of CTCs in clinical practice is the scarce number of cells present within the blood even in patients with metastatic disease (often <1 CTC per ml of blood) (71). Analysis of CTCs is expensive, and technically challenging. Various technologies are available which detect CTCs through recognition of cell size, or expression of cell-surface antigens (72–74). Due to their rarity in blood,

complex technology is needed to isolate and analyse CTCs, such as the epitope-dependent CellSearch<sup>®</sup> platform, and high-resolution imaging and analysis of dielectrophoretic movement of fluorochrome-labeled cells in the DEPArray<sup>™</sup> microfluidic system (71, 75, 76). Furthermore, given that tumors are heterogeneous, containing multiple cell subpopulations, analysis of single CTCs may not be representative of whole tumor biology (77).

CTCs are a valuable liquid biopsy because they are living cells derived from tumors. This allows a multitude of potential investigations including functional analysis of cultured CTCs either directly or through organoid/xenograft studies (67). Molecular characterization is also possible including but not restricted to genome, gene expression, and protein analysis, most of which can now be on a single cell basis if required (75, 76, 78). Whole genome analysis of individual CTCs has shown that CTCs

**TABLE 2** | Definitions, advantages, and disadvantages of different Components of the liquid biopsy.

Component	Abbreviation	Definition	Advantages	Disadvantages
Circulating tumor Cells	CTCs	Free floating tumor cells in the blood circulation	Protein, gene expression, and genome analysis of tumor cells CTCs maybe cultured or used as xenografts for further functional study Reflection of tumor heterogeneity	<ul style="list-style-type: none"> <li>• Rare</li> <li>• Difficult to extract and culture</li> <li>• Complexity of analysis due to Inter cellular heterogeneity</li> </ul>
Circulating tumor DNA	ctDNA	Tumor derived DNA found in bodily fluids particularly plasma	Easy to extract and store Non-invasive method to analyse tumor genomics Tumor specific genomic alterations make detection relatively easy Evidence that ctDNA is representative of body wide tumor heterogeneity	Low concentration requiring high depth NGS
Circulating MicroRNAs	c.miRNA	RNA molecules typical 18–22 nucleotides in length, found in the circulation	Non-invasive	Rarely tumor specific Analysis relies on expression levels
Extracellular Vesicles	EVs	Lipid bilayer bound cell derived vesicles, 30–2,000 nm in diameter	Contain protein, lipids and nucleic acids derived from tumor Role in intracellular communication Role in cancer development	Heterogeneity of subtypes and composition Difficulty in extraction Difficulty in characterization
mRNA		Protein encoding mRNA found in the circulation	Ability to measure gene expression Tumor specific gene variants detectable	High level of fragmentation Difficult to amplify by NGS

show a remarkable degree of variability, which gives valuable insight into the heterogeneity of the tumors from which they are derived (79). It is apparent that this degree of heterogeneity may not be captured by other liquid biopsy techniques (79). Recent developments in mRNA sequencing also allow genome-wide transcriptome analysis, allowing distinct gene expression patterns to be identified in individual CTCs (80). The analysis of CTC proteins is more difficult as there is no ability for amplification of these elements as there is for nucleic acids (via PCR), and cell staining can result in destruction of fragile cells. However, novel approaches using antibody barcode microarrays may allow protein characterization (81, 82) and the employment of miniscule nanowell chips may isolate single CTCs which can then be assayed for metabolic activity using fluorescent metabolic analogs (81) or for measurement of pH fluctuation (79).

The functional and molecular characterization of CTCs has prognostic significance and may provide the ability to monitor the development of cancer and response to anti-cancer therapy [(83, 84), #194]. The role of CTC analysis for screening, prognostication, and disease monitoring has been explored in a host of cancers, most notably breast, colorectal, prostate and lung (85–89). There have been a number of studies that have demonstrated the prognostic significance of CTCs in patients with ovarian cancer, with those patients with detectable CTCs having shorter overall survival and earlier relapse at all stages of disease (90, 91). In apparently optimally debulked patients, CTCs have been shown to be detectable in a subset of patients. These CTCs represent the presence of minimal residual disease even after R0 resection, and indicated a higher risk of death from disease (92). Presence of CTCs has also been shown to be superior than CA125 alone in the diagnosis of OC, where HE4-expression was used for CTC characterization alongside traditional CTC markers such as EpCam and cytokeratins (93). These results

suggest the future inclusion of CTCs in diagnostic screening tools maybe helpful, if the utility can be proven in larger appropriately powered studies. However, there are still significant barrier to the establishment of CTCs as a reliable biomarker for OC, in particular an agreement on the exact surface markers used to isolate the CTCs, which may account for the variability in research findings.

CTCs have likewise been identified in patients with endometrial cancer (94) and have been shown to be associated with higher risk disease (95). Molecular profiling of CTCs has revealed a suite of genes that show differential regulation that may be subject to targeted therapy during future drug development trials (68). That CTCs are generally more common in aggressive disease suggests they may play an important role in understanding the biology of aggressive disease through further molecular characterization of cultured CTCs.

## Circulating Nucleic Acids

### Circulating Tumor DNA (ctDNA)

Detection of circulating cell free DNA (cfDNA) in the plasma was first recorded in 1948 but it was not until 30 years later that levels were found to be elevated in cancer patients (96). Detection of tumor specific mutations in cfDNA was first identified in 1994 (97) and showed that the detected cfDNA was of tumor origin. This tumor derived fraction of cfDNA is termed circulating tumor DNA (ctDNA) and has been most extensively studied as a blood-borne (plasma) biomarker. However, it has also been described in a range of fluids including, urine, saliva, cerebrospinal fluid, and ascites (98).

The method by which cfDNA and, by extension, ctDNA is released into the blood and bodily fluids remains contentious. Some studies have suggested that necrosis and apoptosis are the main sources of ctDNA (99) but active secretion is also a

plausible mechanism (100, 101). It should be noted that the majority of ctDNA is not likely to be derived from CTCs, as CTCs are rare in the circulation, and ctDNA levels are often 100–1,000 times the concentration that could be generated from apoptosis of CTCs alone (98). Independent of this mechanism ctDNA has been shown to be present in the majority of patients with advanced/metastatic cancer (102), and in many cancers at an early stage (103). It is simple to extract and once extracted can be stored for prolonged periods before analysis (104). In addition the detection of tumor specific alterations can allow detection of ctDNA at very low levels using digital droplet PCR (ddPCR) (105) or NGS incorporating unique molecular barcodes (106).

In cases where high sensitivity sequencing is needed, synthetic DNA sequences corresponding to a unique “barcode” are ligated to a DNA molecule (91). This is then followed by an amplification (PCR) step to massively increase the amount of DNA available for sequencing and typically results in multiple identical DNA molecules coalescing together via a variety of methods including Bead and Flow-based techniques, to allow sequencing (107). The final sequencing step typical involves DNA amplification with detection of nucleotide addition to the end of the amplifying DNA molecule (sequencing by synthesis) via release of photons (e.g., illumina) or protons (e.g., IonTorrent), or via the binding of labeled probes (e.g., Nanostring) (108). DNA sequences are recorded, and each base called given a quality flag according to the strength of the signal for each base. Algorithms then allow this data to be improved, by checking all sequences that contain the marker of the original “barcoded” DNA molecule, and allow the determination of a consensus sequence for each original piece of DNA. Various algorithms also exist to filter out artifacts caused by the sequencing process (108, 109). The consensus DNA sequences can then be compared to the human genome sequence and variants from the standard sequence flagged. In the case of tumor DNA analysis, these flagged variants represent potential tumor specific variants. Though there is an ever increasing range of NGS techniques the majority of technologies use a variation of this description, and all nucleic acids can be detected using modifications of these sequencing techniques. The overall quality of the sequencing data produced is heavily reliant on the quality of the input DNA (110), and as such the isolation methods for each modality of the “liquid biopsy” can be very important.

Analysis of ctDNA has shown that ctDNA may be more representative of tumor heterogeneity than single tumor biopsies, as it is believed that all cells contribute to the pool of ctDNA (98). This allows assessment of body-wide tumor genomics with a single blood draw, which can be repeated at regular intervals via fresh blood sampling as required, allowing tumor monitoring (111). In addition the presence of ctDNA post operatively has been shown to predict tumor relapse indicating the detection of minimal residual disease (112–114). The presence of tumor specific mutations has significant promise in the direction of specific anti-cancer therapies (115, 116).

The main disadvantage of ctDNA is the high depth of sequencing required to detect low level ctDNA, particularly in early stage cancers, due to dilution by cfDNA derived from normal healthy cells. In addition, DNA analysis only represents a proportion of tumor biology, the remainder of which can only

be assessed by tumor biopsy (eg., histology) or the assessment of other blood borne liquid biopsy methods. Raised plasma cfDNA levels have been detected in patients with endometrial or ovarian cancers compared to unaffected individuals (117).

Given that *TP53* is the most commonly altered gene in HGSOE, the detection of *TP53* variation in plasma cfDNA has been shown using both *TP53* specific ddPCR assays (118) and NGS sequencing approaches in blood and peritoneal fluid (119). This suggests that stratification of any new drugs targeted at p53 malfunction could be selected via interrogation of cfDNA, negating the need for invasive biopsies. *TP53* variant allele fraction (VAF) in cfDNA correlates with disease burden in patients with relapsed HGSOE. In addition, a *TP53* VAF decrease of  $\leq 60\%$  in response to chemotherapy has been shown to be a strong predictor of a short time to progression in patients (<6months) (120). However, ctDNA analysis is dynamic—if malignant ascites is drained prior to blood sampling, mean *TP53* VAF may be reduced. Furthermore, other factors such as exercise or inflammation may influence ctDNA levels.

Chromosomal instability in cfDNA has been proposed as a highly specific marker of malignancy in patients with adnexal masses, with improved performance compared to traditional biomarkers CA125 and RMI (121).

In comparison to ovarian cancer, there is a scarcity of published research supporting the utility of cfDNA analysis in patients with endometrial cancer, although elevated levels of plasma cfDNA have been recorded in affected patients (117). Unlike HGSOE, in which *TP53* mutations are ubiquitous, endometrial cancer lacks a single highly mutated gene that can be analyzed in cfDNA. However, the presence of ctDNA has been demonstrated by looking at tumor specific mutations in genes such as *KRAS* (122) or tumor whole exome sequencing followed by mutation specific ddPCR in specific subgroups (high grade serous) (123). The majority of endometrioid endometrial carcinomas have at least one driver mutation in one of four genes (*CTNNB1*, *KRAS*, *PTEN*, or *PIK3CA*). Paired tumor/plasma cfDNA NGS, targeting mutation hotspots in these four genes have been shown to detect tumor specific variants in 15/48 (31%) patients with early stage endometrial cancer, with bloods taken at the time of hysterectomy (124).

Elucidation of treatment-resistance mechanisms is challenging in all cancers, in part because of a paucity of available post-treatment tumor samples for analysis. The ability of cfDNA to reflect the body-wide tumor heterogeneity is therefore particularly useful when studying resistance mechanisms. Tumors caused by *BRCA1/BRCA2* pathogenic variants demonstrate increased sensitivity to platinum-based chemotherapy agents. *BRCA*-deficient tumors have defective homologous-recombination repair (HR), which results in impaired ability to repair the double-stranded DNA breaks caused by the alkylating action of platinum-based chemotherapeutics. As a result of defective HR, *BRCA*-deficient cells are dependent upon single-stranded DNA repair mechanisms, for example that facilitated by Poly ADP-ribose Polymerase (PARP)—making them particularly sensitive to synthetic lethality induced by PARP inhibition (125). Although patients with germline pathogenic variants in *BRCA1/BRCA2*



demonstrate favorable response to both platinum-containing chemotherapy and PARP inhibitors, a large proportion of these patients go on to develop resistance to these therapies. A number of resistance mechanisms have been postulated, including the acquisition of secondary reversion mutations at or close to the initial loss-of-function variant, acting to restore functional protein production (126). These variants restore HR DNA repair, thereby removing the synthetic lethality of PARP inhibitors and allowing repair of DNA damage caused by the alkylating effect of platinum therapy.

A number of recent studies have shown that *BRCA* reversion mutations can be readily detected in the cfDNA of patients with both germline (103, 115, 127) and somatic (115) *BRCA*-mutated HGSO. Detection of these reversion events in cfDNA represents a non-invasive method of predicting resistance to platinum-based therapy, and both primary and acquired resistance to PARP inhibitors (103, 115, 127). In the largest trial, in eight patients in whom cfDNA reversion mutations were detected, concurrent, or imminent disease progression was noted, with a median time to progression of 3.4 months, where detection preceded progression (115). As such testing for cfDNA *BRCA* reversion mutations may allow a non-invasive method for selection of those patients unlikely to respond to PARP inhibitors, and by repeated sampling predict resistance to PARP inhibitors before significant disease progression has occurred. These ideas must be proved in appropriate translational clinical trials, but show that cfDNA analysis may be of significant utility in therapy stratification and prediction of treatment resistance.

## Early Detection

Early detection of cancers has been a specific goal for the development of the use of liquid biopsies. Overall the use of cfDNA analysis has been most studied as a route for detecting early cancers due to the comparative ease with which can be collected and the ability to detect at very low levels using modern sequencing (128). New technologies can be used to supplement, rather than replace, traditional biomarkers to facilitate earlier detection of cancer with improved sensitivity without decreasing specificity (129). This can be demonstrated by the CancerSEEK multi-analyte blood test, which combines the detection of a ctDNA mutation (the majority of which are tumor-specific) with presence of traditional biomarkers (Ca125, CEA, Ca19-9) and less widely used blood borne proteins, including hepatocyte growth factor (HGF), osteopontin (OPN), myeloperoxidase (MPO), and tissue inhibitor of metalloproteinases 1 (TIMP-1) to improve sensitivity (129).

Given that circulating nucleic acids and exosomes may be present in extracellular fluids, another approach to improving cancer detection has been analyse samples collected as part of the Papanicolaou (Pap) test. The role of this test in early detection of cervical cancer is well-established, but detection of ovarian/endometrial cancers by this means via histopathological analysis is not usually possible. However, diagnosis of other gynecological malignancies may be facilitated by analysis of samples collected during Pap smears for assessment of sequence variation and copy number. The PapSEEK test assesses the fluid obtained during Pap tests for 18 specific variants characteristic

of ovarian/endometrial cancers, as well as aneuploidy. The sensitivity of the PapSEEK test can be improved by collecting intrauterine samples, and with contemporaneous analysis of ctDNA (130).

## MicroRNAs

MicroRNAs (miRNAs) were first described in *C. Elegans* in 1993 (131) and our knowledge of this class of molecules has rapidly grown since this time. MicroRNAs are a group of small non-coding RNA molecules, typical 18-22 nucleotides in length that influence gene expression by binding with cis-regulatory regions in target messenger RNA, resulting in post-transcription regulation, through destabilization of the mRNA molecule (131, 132). The miRNA-mRNA network is very complex, as a single miRNA may have many hundreds of mRNA targets, and mRNA targets may be regulated by many different miRNAs (133). The human genome contains >1000 genes encoding miRNAs although the function of many of these small RNA molecules is yet to be established (132).

Cell free miRNAs (cf.miRNAs) were detected initially in 2007. Like cfDNA, cf.miRNAs can exist in plasma and various bodily fluids and their mechanism of release is not fully understood. Potential mechanisms include release from microvesicles or active secretion in association with protein complexes such as Ago2 (134) or high-density lipoproteins (135). Aberration of miRNA gene expression is seen in the majority of cancers, and miRNAs have been shown to alter the expression of a multitude of different genes. These changes in gene expression have been shown to be associated with most areas of tumor development from tumor initiation to metastasis (136).

Cell free miRNAs are relatively easy to isolate via commercially available kits and have been shown to be remarkably stable molecules despite the presence of endogenous RNase activity (137). This allows sufficient levels of miRNAs to be isolated for high quality sequencing. NGS sequencing following a reverse transcriptase step is the most commonly used analytical method (138). The reasons for their stability include their association with protein complexes or their packaging within extracellular vesicles (139). They have been detected in a number of body fluids besides plasma, including urine and saliva (140). Numerous clinical uses for cf.miRNAs have been studied in cancer including early diagnosis, monitoring of disease (140), predicting response to treatment and disease prognosis (141). Limitations in their analysis include that they are not specific to cancers, in that most miRNAs exists in both tumor and normal tissue. Analysis must rely on the relative levels of the cf.miRNA expression, which be difficult if the relative amount of cf.miRNA released from the tumors is low, or in early cancers. In addition alterations in miRNA expression are frequently shared between different tumor types, such as breast and lung cancer, as the same processes are frequently dysregulated in cancer. This ultimately reduces the specificity of cf.miRNA alteration to specific cancers.

Because of the more recent discover of cf.miRNA, research in this field is still relatively exploratory. In the quest for improved circulating biomarkers cf.miRNA has been evaluated and various labs have shown that cf.miRNA analysis can discriminate healthy from affected individuals (139). In particular single miRNAs such

as miR-205 and let7f have been shown to have high diagnostic accuracy for early stage ovarian cancer, and let7f has been shown to have prognostic value, with low levels indicating a poor prognosis (142).

Studies in endometrial cancer have also shown differential levels of cf.miRNA between healthy and affected patients. In one study miR-15b, miR-27a, and miR-223 were found to be differentially expressed between patients with endometrioid endometrial cancer and unaffected individual and therefore may have a role therefore in improving diagnosis of endometrial cancer pending further investigation (143).

Overall the work into cf.miRNAs is generating a huge number of potential new biomarkers (136, 141, 144), and significant work is needed to increase our understanding of these markers in order to improve our ability to utilize them.

## mRNA

Protein coding messenger RNA molecules (mRNA) are known to be present in plasma samples in addition to short-non-coding RNAs and other nucleic acids (138). Like cf.miRNAs, cell free mRNAs (cfmRNA) are thought to be found in association with extracellular vesicles, as well as free in the circulation (145). Analysis of cfmRNA, allows measurement of protein encoding gene expression and detection of tumor specific mutational variants, neither of which is possible using cf.miRNA. However, the latter is also possible by analysis of cfDNA which is more stable and requires less specialist extraction methods (145). Far fewer reports are available on the use of cfmRNA than cf.miRNA, because it is less stable (frequently degraded) and less abundant in the circulation, making it more difficult to detect and analyse (145).

## Extracellular Vesicles

Tumor derived extracellular vesicles (EVs) are lipid bilayer bound cell derived vesicles, 30–2,000 nm in diameter. Initially thought to be nothing more than membrane debris they have since been shown to have important roles in intracellular communication via the transfer of proteins, lipids and nucleic acids (146, 147). The three main classes are; exosomes, microvesicles and apoptotic bodies. Exosomes are derived from the endolysosomal pathway and microvesicles via budding from the plasma membrane. Apoptotic bodies are derived from the controlled death of cells via the similarly named apoptotic pathway. EVs derived from these different routes vary in size and composition, including enrichment for certain surface markers. However, the heterogeneity of different surface markers can allow for enrichment of these subtypes via marker selection.

EVs appear to have a diverse role including the regulation of immune responses, tissue repair and blood coagulation (146). Exosomes in particular play a central role in antigen presentation and immune surveillance and activation (148, 149), whilst both exosomes and microvesicles mediate genetic inter-cellular communication, by carrying nucleic acids between cells (150). Given their central role in regulating cellular processes it is not surprising that EVs may have an important role in pathogenesis of cancer and a number of other diseases. Their role in tumor development is diverse, including stimulation of growth

and angiogenesis (151), promotion of immune escape and the formation of pre-metastatic niches (70).

Given that EVs appear to play a direct role in many fundamental steps of tumor initiation and progression there is growing interest in the modulation of their effect. This includes inhibition of their formation, extracellular release or uptake and blocking specific extracellular EV components (146). EVs contain many of the other circulating components including DNA, mRNA, microRNA, and other non-coding RNAs, making analysis of any one component difficult (150). Indeed they may be one of the sources of these components on plasma analysis, although it should be noted that the composition of circulating nucleic acids differs from those found in EVs, and that the different subtypes of EV also differ in their composition (149, 152).

Difficulties with EVs are inherent in their heterogeneity and complexity. Research results based on an EV analysis from a single cell type can differ dependent on cell culture conditions, differences in purification protocols or methods used to characterize the EV (153, 154). For example ultrafugation has been the gold standard for exosome purification but concern regarding low activity of those EVs collected has led to a range of newer techniques including liquid chromatography or marker precipitation based separation (146, 155).

Tumor cells are well-known to produce large amounts of EVs. Because of their role in cellular communication, their complexity in terms of composition and their varied role in tumor growth and developments EVs contain a large amount of biological information about the tumors from which they arise. They therefore have been evaluated for varied roles in cancer including prognostic biomarkers (156). Disease monitoring is possible from EVs in blood plasma and a range of other bodily fluids such as urine and saliva (151, 157). Of interest plasma EVs maybe able to differentiate affected and unaffected patients, and may distinguish between those with early and those with late disease. They may also be enriched for immunosuppressive proteins including PD-L1, PD-1 and CTLA4, and high PD-levels have correlated with high disease activity and advanced stage of disease. Finally, monitoring of protein levels in EVs has also been shown to correlate with disease response, suggesting EVs may play a role in monitoring of response to therapy. This suggests EVs can be used as a measure of disease progression and prediction of advanced disease, and may play a role in treatment selection in the future, e.g., through stratification for the use immune checkpoint inhibitors (155, 156). There is growing evidence that due to the readily accessible nature of EVs, and the bioactive molecules that they contain, EVs may also have a role in detection of early stage disease (158).

Difficulties with the use of EVs center on methods for their extraction and purification. However, the diversity of the potential uses for EVs also means that summarizing all the current research in this review is not possible.

As EVs contain a variety of different classes of molecule significant work has concentrated on the characterization of known molecules within them. For example exosomal miRNAs could serve as potential biomarkers (159) and soluble E-cadherin bound to exosomes has been identified as both a potential biomarker and therapeutic target (160) in OC.

Herrero et al. have recently demonstrated the utility of ExoGAG technology to enrich for EVs in patients with endometrial cancer, and to facilitate detection of two known prognostic and predictive endometrial cancer biomarkers; L1 cell adhesion molecule (L1CAM) and Annexin A2 (ANXA2) in the samples (155). Increased levels of ANXA2 in EVs were found to correlate with high-risk histology, grade, stage and risk of recurrence, suggesting it may play a role in disease monitoring, recurrence and detection of early disease. Further work into the use of ANXA2 in early detection of EC is required to elucidate the best timing of samples and confirm the cut-off levels above which disease is likely to be present. This study demonstrates how known protein based biomarkers may be of enhanced value following the analysis of EVs.

The study of EVs not only reveals markers that can be monitored during therapy but also targets for therapy as EVs are known to be involved in many elements of tumorigenesis or promote the development of metastases through enhancement of the premetastatic niche. For example exosomes may promote cell invasion through transfer of surface glycoproteins in ovarian cancer (161), and exosomes released from tumor associated macrophages transfer miRNAs that contribute to an immunosuppressive microenvironment (162). Therefore, by contributing to the understanding of ovarian cancer and other cancers this may yet translate to new targets for anticancer therapy and the eventual development of new targeted drugs.

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## SUMMARY

As described the “liquid biopsy” has a wide potential for improving our understanding of cancer, including measurement of both temporal and spatial heterogeneity both of which are substantial barriers to the development of personalized anti-cancer therapies. As such, use of the liquid biopsy promises assessment of tumor heterogeneity at a greater frequency and with reduced morbidity compared to tissue biopsies. Their development therefore promises to improve detection and monitoring of tumors and identification of targeted treatment guided by precision medicine. A critical factor in the reduction of the morbidity and mortality of endometrial cancer and ovarian cancer is improvement of these very issues. Therefore, we further discuss current developments in the field of precision oncology in endometrial cancer and ovarian cancer and how improving knowledge in “liquid biopsy” research are contributing to the development of both targeted anti-cancer therapy and disease monitoring.

## AUTHOR CONTRIBUTIONS

MO and TM conceived of the review, reviewed the literature, and drafted and approved of the final manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** TM has received speaking honoraria from Roche, Novartis, Astra Zeneca, MSD, and Merck.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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