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Review

Prospects of Improving Early Ovarian Cancer Diagnosis Using Cervical Cell Swabs

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Abstract. *Ovarian cancer (OC) has the poorest prognosis and the highest mortality rate among gynecological malignancies, which is largely due to delayed diagnosis. Therefore, an effective detection strategy is a compelling need. Here, we review the potential use of cervical cell swabs (Pap specimens, liquid) for early detection of OC. It has been shown, that malignant cells exfoliate from the ovaries and may be detected in Pap specimens, routinely collected through cervical cancer screening. Using Medical Subject Headings (MeSH) for searching the PubMed database we identified eight studies reporting the use of Pap specimen in early detection of OC. Six focused on detection of gene mutations, using gene panels or analysis of TP53 variants. Two studies reported analysis of methylation profiles. Seven studies were published in 2018 or later. Additionally, we found one study without MeSH terms assigned yet, which postulated using peptide biomarkers present in Pap-test fluid. In this review we present their main findings, discuss challenges this approach presents and include ideas for improved detection.*

Ovarian cancer (OC) is the gynecological malignancy characterized by the poorest prognosis and the highest mortality rate, which is mainly due to a delay in diagnosis (1). Symptoms of OC are often discrete and non-specific, which can mislead both patients and healthcare professionals. According to the Danish Gynecological Cancer Database (DGCD) more than half of the patients are diagnosed with advanced stage cancer (stage III-IV according to International Federation of Gynecology and Obstetrics; FIGO) (2). Early diagnosis is crucial for long-term recovery/curative treatment, as the 5-year survival rate differs dramatically between early stage OC (stage I: 93%) and late stage OC (stage IV: 23%) (2). An efficient, sensitive worldwide screening program for OC could alleviate the challenges resulting from late diagnosis and improve the survival of the more than 314,000 women diagnosed with OC yearly (3). On the global scale it means 4 in 100,0000 women are diagnosed with OC every year. In Denmark this number is markedly higher than the world average, reaching approximately 15 cases per 100,000 (2).

The widely implemented screening program for cervical cancer has proven that a population-based screening can be feasible and efficient. Cytological examination of cervical smear (Papanicolaou or Pap smear) performed every 3-5 years for women aged 35-64 years has yielded an at least 80% reduction in the incidence of cervical cancer (4). However, the optimal screening strategy for OC is yet to be found; randomized controlled trials, such as the US Prostate, Lung, Colorectal and Ovarian study (PLCO) and the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), have tested screening strategies consisting of a combination of CA-125 serum levels and ultrasonography. Sadly, the screening trials did not result in significant reductions in deaths from OC (5, 6). Advances in genomic profiling and epigenetics technologies have provided new opportunities for different screening strategies. Studies focused on detection of mutations and DNA methylation profiling have explored the possibility for a screening program for OC similar to the well-established cervical cancer screening program (7, 8). This approach would ensure an accessible screening program with no additional procedures and a reasonable cost-benefit balance.

The cervical cancer screening program is widely accepted in the population, *e.g.* in Scandinavian countries the rate of

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participation ranges between 64-81% (9). This means that the clinical diagnostic framework is already in place in most high-resource countries and perhaps can be used to accommodate OC screening as well, applying material already collected, without any additional patient-related actions. Briefly, the sample collection proceeds as follows: a cytobrush used for swabbing the endocervical canal is then placed in a vial of fixative medium. In addition to the Pap smear test, performed to detect early cervical abnormalities, the liquid from the vial (hence referred to as liquid Pap specimen) can be used for diagnostics of human papillomavirus infection or, potentially, molecular testing for cancer (8, 10). The main challenge however is to identify an appropriate analysis of the cervical swab/liquid Pap specimen material that meets the requirements of high specificity and sensitivity to detect precancerous cells and/or early-stage OC. In this review, we outline the current research on the use of liquid Pap specimen for early diagnosis of OC. Studies applying analyses of mutations and DNA methylation profiling in cervical cell swabs included in this review were identified as described in Chapter 3: Search strategy. Furthermore, we assess the prerequisites of OC screening using cervical cell swabs, followed by a discussion of the existing results and the future perspectives of OC screening.

Premises for OC Screening

A prerequisite for using cervical cell swabs to detect pre-cancerous/early stage OC is the transit of OC cells to cervical canal. The cervix, uterus and fallopian tubes are part of the same anatomical unit; the ovaries are not directly connected to the fallopian tubes, but are in close proximity with the fimbriae (11). Studies have shown that in many cases OC does not originate in the ovaries but rather in the fallopian tubes (12). This precursor lesions in the tubes, named serous tubal intraepithelial carcinomas (STIC), are especially found in type II high grade serous carcinoma (HGSC), the most common type of OC (13). As the fallopian tubes communicate directly with the uterus, hence the cervix, it is reasonable to assume that cells from the tubes may reach the cervical canal. The extent of cell shedding from the ovary towards the uterus and cervical canal remains unknown. In a review, several case studies (14) have reported how abnormal cervicovaginal cytology can be indicative of OC, thus supporting the notion of cell shedding from the upper gynecological tract to the lower regions. Abnormal cytology in relation to OC encompasses the presence of atypical glandular cells and/or psammoma bodies, which are small calcifications organized concentrically (14). Psammoma bodies can be found in meningioma, thyroid cancer and gynecological cancers, but may also be an incidental finding in normal smear samples (15). A review enlisted 24 cases of OC with abnormal cervicovaginal cytology. Staging was provided for 13 cases, and 11 out of these 13 cases (85%) were staged as IIIA or higher, indicating that it is primarily advanced OC that is detected through an abnormal cytology in cervicovaginal samples (14). At earlier OC stages the quantity of material in the cervical canal originating from the ovaries or fallopian tubes may be limited, which imposes major technical requirements to new screening strategies. As the location of cancerous or precancerous cells to the cervix is a prerequisite of cervical screening for OC, it also excludes a subpopulation of women, who had tubal ligation or hysterectomy. Menon even questioned whether intrauterine device placement may impede screening using smear, which could potentially exclude a major fraction of women otherwise eligible for screening (16). Yet, the mechanistic foundation of the detection of OC using cervical smear samples is complex, as pathogenic mutations were found in Pap smear samples from an HGSC patient who already had tubal ligation (17). Another study investigating the feasibility of using cervical cell swabs for endometrial and OC screening suggested that the detection of cancer applying DNA methylation analysis might not depend on physical relocation of cells from the ovary, tubes or endometria, but could be a result of a so-called "field effect" (7). By "field effect", the authors meant that changes in DNA methylation can be found in normal tissue adjacent to tumor tissues, exemplified by a study finding changes in DNA methylation in normal cervical tissue in proximity to cancer tissue (18). Most of the studies reviewed in the subsequent sections recognize, however, that material from the ovaries and tubes needs to pass freely to the endocervix and have excluded or commented on patients with tubal ligation (17, 19, 20).

Search Strategy

The literature search was performed in MEDLINE/PubMed using the following search strategy: [Ovarian Neoplasms (MesH)] AND [Vaginal Smears(MesH) OR papanicolaou test(MesH)].

Inclusion criteria was English language and publication after 1990. The search yielded 136 unique results, herein: 1 clinical trial, 18 reviews, 37 case reports (as of August 2021).

We focused on a detailed review of studies reporting the possible use of cervical cell swabs in early diagnosis of OC. Studies (n=8) with relevance for this article are outlined in Figure 1.

Since some time may pass between publication of an article and assigning of MeSH terms, we also performed a manual search to ensure that we do not miss the most recent publications. Indeed, a manual search for terms: "Pap test/"Pap specimen" and "ovarian cancer"/"ovarian carcinoma" resulted in one additional record, namely the work by Boylan *et al.*, from January 2021, which had no MeSH terms assigned yet (21).

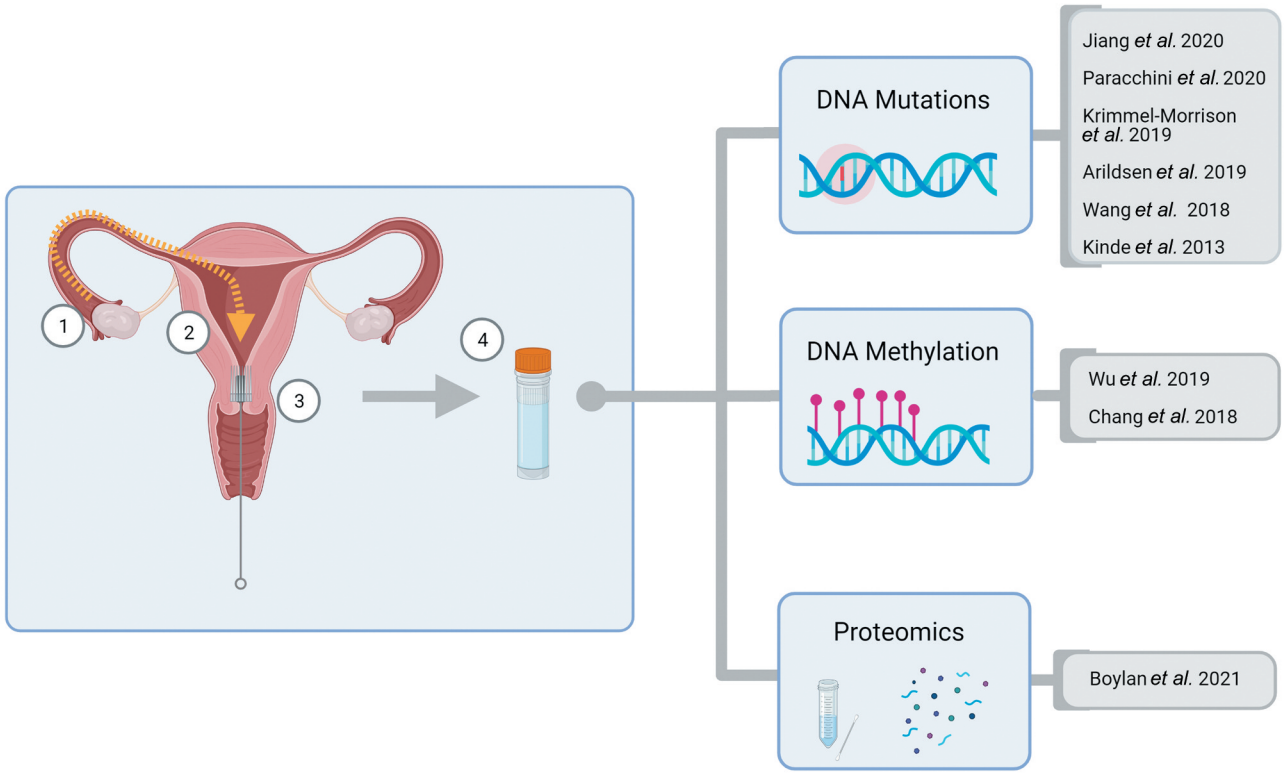

Figure 1. *Ovarian cancer screening using cervical smear/liquid Pap specimen. A premise of this screening strategy is the presence of material from a cancerous/precancerous lesion in the smear sample as follows: 1) Precancerous/cancerous cells are shed from the fallopian tube. 2) Cells reach the uterus and the cervical canal. 3) Cells can be collected from the endocervix with a Pap brush or through intrauterine sampling with a Tao brush. 4) The material may be subjected to analysis of DNA mutations / methylation patterns or protein profiles. (Figure created with the use of Biorender/licensed to RSW).*

Detection of OC Using Cervical Smears

Genetic aberrations observed in OC can be divided into sequence-dependent (mutations) and epigenetic (such as DNA methylation). Of note, sets of genes affected by either mechanism may overlap, for example both *BRCA1/2* mutations and silencing by methylation have been reported (22). Below, we review studies focused on detecting 1) mutations or 2) altered methylation profiles in OC cells, using cervical cell swabs as diagnostic material, and hence the potential use of cervical smears in early diagnostics of OC.

In Figure 1 the reviewed studies are categorized according to the primary endpoint analysis, although this division is somehow simplistic as the studies often include multiple analysis, for instance analysis of circulating tumor DNA (ctDNA) in plasma or aneuploidy detection.

DNA Mutations in OC

Malignant ovarian tumors predominantly (in over 90% cases) derive from epithelium and can be classified into two types, depending on their grade, with type I progressing relatively slowly (low grade serous tumors, mucinous, endometrioid and clear cell carcinomas) and type II, characterized by a more aggressive phenotype (high grade serous ovarian carcinoma, HGSC) (23). Between 2 to 8 driver gene mutations are needed for tumorigenesis, and genetic changes in the form of point mutations as well as structural mutations including copy number variation have been reported in OC (24, 25). Mutational profile largely depends on OC histological type (24); [an overview, see Figure 1 in (26)]. For example, in HGSC the most frequently mutated genes are *TP53* (up to 96%) and *BRCA1/2* (approximately 20%). Otherwise, these neoplasms demonstrate a high degree of genomic heterogeneity, due to frequent mutations in repair genes, including homologues recombination (HR) pathway components (24). On the other hand, clear-cell OC rarely bears *TP53* mutations, but is instead characterized by aberrations in *KRAS* (27), *ARID1A* (28) and *PIK3CA* (26). *ARID1A* (28) and *PIK3AC* are also commonly mutated in endometrioid OC, as well as *CTTNB1* (29) and *PTEN* (30). Mutations in *KRAS* is a hallmark of the mucinous subtype

(over 60%) (31, 32), whereas low grade serous OC often bears mutations in *KRAS* and *BRAF* genes (33).

Genetic (and epigenetic) changes give rise to different cell populations, contributing to tumor heterogeneity. This, on one hand, poses a therapeutic challenge, since existence of multiple tumor clones often means that therapies are effective only to a subset of them, and that the surviving cells may lead to a refractory recurrence (34). On the other hand, heterogeneity of the tumor and limitations in tumor tissue sampling may result in different mutation profiles between cervical smears and tumor samples, meaning that the smears are not necessarily representative for the lesions they shed from (25).

DNA Mutations in Cervical Cell Swabs in Early Detection of OC

We have identified six studies analyzing mutations in cervical cell swabs of OC patients (Figure 1). Three of the studies focus exclusively on *TP53* mutations, whereas others utilize gene panels. The latter typically include cohorts of both ovarian and endometrial cancer patients. In two of the studies archive Pap specimens were also analyzed.

The first study by Kinde *et al*. investigated if cervical cell swabs could be used for detection of OC (8). The purpose of their work was to verify if tumor cells shed from upper gynecological tract malignancy to the cervical canal. Authors assembled a panel of 12 genes commonly mutated in OC (*AKT1, APC, BRAF, CTNNB1, EGFR, FBXW7, KRAS, NRAS, PIK3CA, PPP2R1A, PTEN* and *TP53*), tested for mutations in tumor tissue and attempted to find the same mutations in corresponding liquid Pap smear specimens from the same patient. In 9 out of 22 patients diagnosed with OC (41%), they successfully detected the same mutations in the Pap smear samples as in the tumor tissue. However, 41% seems somewhat low given that the majority (18/22) of the OC patients presented advanced stage OC (FIGO III and IV), where shedding from the site of the cancer could be likely. This number is also much lower than for endometrial cancers in the same study, where 100% of the liquid Pap specimens bore mutations identical to those found in corresponding tumor tissues. These findings may reflect the distance between sampling site and tumor site in OC, which is why authors suggested uterine sampling for the purpose of OC screening (8).

Another work by the same group followed up on their exploratory study, attempting to increase the sensitivity of OC detection in cervical cell swabs (20). Altogether, 245 women diagnosed with OC were enrolled, along with healthy controls and endometrial cancer patients. Patient material in the form of cervical cell swabs, tumor samples and blood samples (for detection of circulating tumor DNA) were collected. For tumor detection a parameter dubbed "PapSEEK" score was applied. Samples were regarded as PapSEEK positive if they either contained aneuploidy or genetic alterations in one of 18 cancer-related genes selected for the diagnostic panel (*AKT1, APC, BRAF, CDKN2A, CTNNB1, EGFR, FBXW7, FGFR2, KRAS, MAPK1, NRAS, PIK3CA, PIK3R1, POLE, PPP2R1A, PTEN, RNF43,* and *TP53*). In 33% of OC patients genetic alterations detectable by PapSEEK in the tumor tissue were also found in cervical cell swab. This number was further increased if a so-called Tao brush was used instead of a regular Pap brush. The unquestionable strength of this study was the use of a big patient cohort, which makes its results more representative. Moreover, tumor detection was performed using an 18-gene panel, rather than only one gene (*TP53)*. As mentioned above, *TP53* is commonly mutated in HGSC, but rarely in other histological types. Therefore, limiting diagnostics only to *TP53* mutation detection may hamper detection of other OC types, which albeit less common than HGSC, are by no means less dangerous. Of note, this study also contains some new ideas (utilizing Tao brush, detection of circulating tumor DNA) to further improve sensitivity.

A more recent work by Jiang and coworkers (25) was inspired by the original study by Kinde *et al*. (8) and its continuation by Wang *et al*. (20). Like in the latter study, liquid Pap smear specimen, tumor samples and blood samples were collected from patients diagnosed with epithelial OC or endometrial cancer. First, exome sequencing of 127 cancer related genes recognized by The Cancer Genomic Atlas (TCGA) as tumor drivers was performed in OC tumor samples. Based on that, a panel of eight genes (*ARHGAP35, ARID1A, BRCA1, EGFR, LRRK2, PIK3CA, RAD21, TP53*), potentially most relevant for OC, was selected for analysis of liquid Pap smear specimens. Mutations in at least one of these eight genes were detected in 11 out of 11 liquid Pap smear specimens and showed correlation with tumor tissue. Tumor heterogeneity and limited tissue sampling may explain the fact that only in 5 out of 10 tumor samples were found mutations corresponding to variants detected in liquid Pap smears specimen.

The study by Jiang *et al*. (25) further corroborates the earlier findings and supports the claim that the DNA shed from OC cells may be present in liquid Pap smear specimens. Additionally, an interesting strategy by the authors was to use matched patient leukocytes as a self-control. Briefly, the exome sequencing of 127 selected genes was performed both in tumor tissue and in leukocytes in order to account for natural inter-individual variation.

Most studies regarding diagnosis of OC using liquid Pap smear specimens focus generally on two aspects: early detection (studies involving archival material collected when the patient was still symptom-free) and increased sensitivity. The study by Arildsen and coworkers (35) addressed both issues, analyzing somatic mutations of *TP53* in 15 patients diagnosed with HGSC. For all patients, archive liquid-based Pap specimens were available (taken 20-95 months before the diagnosis), and additionally for 9 of them diagnostic (*i.e.*,

concurrent with diagnosis) liquid Pap samples were collected. Blood samples were also collected to serve as matching self-controls, similarly to (25). In 6 out of 9 diagnostic samples, somatic *TP53* mutations were detected, giving a ratio of 66%. Interestingly, two stage II A patients had mutated *TP53* in liquid Pap samples, emphasizing the potential of the method for early detection. Moreover, mutated *TP53* was detected in one of the archive samples, taken 20 months prior to the diagnosis. Detecting OC 20 months earlier would most probably impact prognosis for the patient. However, it has been hypothesized that mutations in *TP53* may occur as early as seven years before the diagnosis. They were though absent from earlier Pap samples collected for the same patient, which may be explained by precursor lesions not shedding cells until later in the tumorigenic process (35). In the same study, the authors compared the sensitivity of two detection methods, namely regular digital droplet PCR (ddPCR; Bio-Rad) and an ultra-sensitive ddPCR-based method IBSAFE (currently: SAGAsafe®, SAGA Diagnostics, Lund, Sweden), which may improve 100- to 1,000-fold the detection limit. Indeed, IBSAFE performed better, even in samples with as little input as 0.17 ng DNA. Directly compared to regular ddPCR, it was able to detect mutated alleles in three Pap samples as opposed to two for regular ddPCR. These findings show the importance of optimizing highly sensitive and specific methods in early non-invasive diagnostics of OC (35).

A recent study by Paracchini *at el*. (36) provides further evidence that mutated *TP53* variants can be detected in Pap smears up to six years prior HGSC diagnosis. Their work focused exclusively on detection of mutated variants of *TP53*, in a rather small cohort of 17 patients. Interestingly, the authors used brush-based and dry stored Pap slides rather than liquid Pap tests. The median interval between collection of Pap test slides and the cancer diagnosis was 14.9 months, with interquartile 3.4-35.9 and maximum 68 months. For three patients more than one sample was available. In 11 out of 17 patients (65%), the same *TP53* mutation was detected in tumor samples and in the corresponding Pap smears collected within six months before diagnosis or earlier. Of note, in all but one patient mutations found in Pap smears were located in *TP53* gene hotspots. More importantly, however, mutated *TP53* variants were detected is some of the archive samples collected 25, 27, 49 and 68 months prior to diagnosis.

Krimmel-Morrison *et al*. also based their detection strategy on *TP53* alone. They compared genetic profiles of *TP53* in liquid Pap specimens with HGSC tumor tissue samples and found tumor-derived mutations only in 3 out of 8 patients (37.8%) (17). For sequencing *TP53* from Pap test, the authors utilized a highly sensitive method, namely CRISPR-DS, developed previously by the same group and designed for ultra-accurate sequencing with low DNA input (37). Therefore, the relatively low positive ratio (37.8%) should not be ascribed to technical resolution, but simply to the absence of neoplastic DNA in liquid Pap specimen. These results put into question possible implementation of Pap test in OC diagnosis. It seems, authors claim, that the sensitivity of the Pap test in the current form is most probably insufficient for detection of OC (17). Apart from correlating mutations present in tumor tissue with mutations detected in Pap samples, Krimmel and coworkers also presented an in-depth analysis of types of *TP53* mutations. First, they generated an in-silico list of all possible mutations in *TP53* coding region and determined their categories as: frequent in the cancer database, influencing protein activity, pathogenicity, if located in exons 5-8 (DNA binding domain) and/or if located in hotspots. Then, they related the distribution of various categories of mutations as predicted by the model to 1) their actual distribution, found in the Universal Mutation Database and 2) to their distribution in the cohort used in the study (30 women with or without HGSC who underwent gynecological surgery due to pelvic masses suspicious; N=9 with HGSC, N=21 with no neoplasm or benign neoplasms). This analysis revealed that indeed, likely pathogenic (affecting the protein activity) or pathogenic mutations appeared in all liquid Pap smear samples, albeit with higher frequency in liquid Pap samples from women diagnosed with HGSC. Mutations infrequent in cancer were similarly distributed in both groups. These findings suggest that, though a wide spectrum of *TP53* mutations can be seen in benign and malignant conditions alike, it may be possible to use genetic profiling to infer about pathogenicity of given mutations and, perhaps, devise a computational tool for risk assessment (17).

DNA Methylation in OC

DNA methylation is an epigenetic mechanism where gene expression is altered by methylation of the cytosine nucleotide. Several studies have documented that aberrant patterns of methylation play a role in carcinogenesis (38). One of the mechanisms is silencing of tumor suppressor genes by hypermethylation of their promoter regions (39). Activation of oncogenes by hypomethylation of oncogene promoters have also been observed, although this mechanism is less investigated compared to hypermethylation (40, 41). The TCGA Research Network found 168 genes which were epigenetically silenced due to elevated promoter methylation in HGSC as compared to normal fallopian tissue (24). Some of the most investigated sites of methylation are the promoter regions of *BRCA1, RASSF1A, OPCML and P16INK4a* (41). A study including OC tumors from 50 women staged I to IV showed hypermethylation of the promoter regions of *BRCA1* or *RASSF1A* genes in 68% of the OC cases and in none of the control cases (42). Hypermethylation of the promotor region of *P16INK4A* was also significantly associated with

OC (43). Moreover, a meta-analysis reported a significant association between promoter methylation of *OPMCL* and the risk of OC, and this association was also related to stage and poor differentiation of tumor (44).

The use of DNA methylation as a tool for OC screening/early detection was initially applied on circulating cell-free DNA in the plasma or serum of OC patients. Analyses of the methylation of promoters of different genes, among which are *BRCA1, RASSF1A,* and *OPCML* in either the serum or plasma, have shown results with specificity and sensitivity in the range of 69-90% (45-47). Although a study conducted in relation to the UKCTOCS trial (48) showed that ctDNA could be found in blood samples from up to two years prior to the diagnosis, the general assumption is that circulating DNA is mainly present in more advanced disease and not at precancerous stages (49). This could set the case for using sampled specimen in closer proximity to the site of the cancer such as cervical cell swab, thus increasing test sensitivity towards early-stage disease. DNA methylation is ideal for the purpose of screening/early diagnosis as it is present early in carcinogenesis. Furthermore, the sites are stable and tissue-specific and tests may be designed to cover a limited key targets, which is practical in large-scale implementation of test regimens (50, 51).

DNA Methylation in Cervical Cell Swabs for Early Detection of OC

The idea of using cervical cell swab and DNA methylation for OC screening is relatively new and only two studies have presented results with this approach. The first study to investigate the use of DNA methylation as a biomarker in cervical cell swabs for OC detection is the study by Chang and coworkers, where they examined hypermethylation of 14 selected genes in patients with OC, endometrial cancer and healthy controls *(ADRA1D, AJAP1, COL6A2, EDN3, EPO, HS3ST2, MAGI2, POU4F3, PTGDR, SOX8, SOX17, ST6GAL2, SYT9, and ZNF614)* (7). Using quantitative methylation specific PCR (qMSP) they identified two hypermethylated genes (*POU4F3 and MAGI2*) for validation in 30 patients with OC, 30 patients with endometrial cancer and 30 healthy controls. For OC, the test yielded a sensitivity of 61% and specificity of 62-69%. Both sensitivity and specificity were higher for the detection of endometrial cancer. The authors recognized that the study was mainly intended as a proof-of-principle due to the small sample size. Using the same target genes for endometrial cancer and OC broadens the test coverage but may reduce the specificity of the test. Despite its shortcomings, the study demonstrated the feasibility of testing for aberrant DNA methylation in cervical cell swabs and warrants further validation.

The other study on DNA methylation by Wu and coworkers (2019), focused exclusively on OC (18). Analyzing three large databases of samples from OC patients (tissue and cervical cell swabs) compared to healthy controls, Wu *et al*. identified a panel of genes on the intersection of all three databases, *i.e*., 151 genes highly differently methylated in OC compared to methylation levels in samples from healthy individuals (19). Subsequently, the authors narrowed the selection and designed a model combining three genes (*AMPD3, NRN1, TBX15*), which could predict OC incidence with sensitivity of 81% and a specificity of 84%. This model was constructed based on a so-called training (31 OC, 31 healthy patients) and testing set (21 OC and 21 healthy patients). None of these three genes has been previously reported in OC and their role remains largely unknown. The authors questioned whether these genes are indeed unique to OC or rather present a common pattern of aberrant hypermethylation found in cancers, but potentially also in benign conditions.

Wu *et al*. found no differences in methylation between various stages and grades (18). However, they observed a small, but significant difference between histological subtypes, with mucinous OC showing less methylation. A recent review on epigenetics of OC also presents similar findings, with HGSC predominantly displaying a hypomethylated phenotype while clear cell OC and endometrioid OC being associated with more hypermethylation events (49). The heterogeneity in methylation profile according to OC subtype may pose a challenge in screening and early diagnosis of OC.

The studies by Chang *et al*. (7) and Wu *et al*. (19) do not match the methylation profile of cervical scrapings with the corresponding tumor tissue from the same individual, neither they include normal tissue samples. Instead, to assess concordance in methylation profile, both studies include cervical scrapings from age-matched healthy individuals. Demonstrating that a methylation "fingerprint" from OC tumor tissue can be found in corresponding cervical cell swab would be a significant proof of concept. However, if the test is meant to be used for screening of a generally healthy population, it should be able to reliably identify women in high risk for a malignant pelvic mass. Therefore, performing validation in OC cervical scrapings *versus* samples collected from healthy individuals as done by (52) and (19), rather than in cervical scrapings *versus* matching tumor tissue seems to be well-grounded.

On the other hand, if the test is to be applied in a selected group of patients with gynecological symptoms, the ability to clearly distinguish between benign and malignant condition may be of outmost importance.

In general, the two studies further support the potential use of cervical cell swabs in the diagnosis of OC. It is yet to be determined if aberrant DNA methylation is a better predictor than the analysis of gene mutations in the cervical cell swab as described above (8, 20, 25) or it is better to combine both approaches to increase sensitivity and specificity. Additionally, one interesting aspect to pursue

would be the determination of the extent of "field effect" in OC. If there is a methylation pattern in tissue adjacent to cancerous tissue due to the so-called "field effect", as previously demonstrated in cervical cancer (18), analysis of methylation could be a promising tool for early OC detection since it may not depend exclusively on physical relocation of cancer cells to the endocervix.

Other Markers – Peptides in the Early OC Diagnosis

Interestingly, a different approach has been suggested by Boylan *et al*., who tested if OC can be detected with peptides as disease biomarkers. Using mass spectrometry (MS) the researchers compared protein profiles of three specimens: primary tumor tissue, a cervical swab and a residual cell-free fixative from a liquid-based Pap-test of one patient with late stage HGSC. Indeed, they observed a marked overlap between the profiles (2,293 out of almost 5,000 proteins analyzed were detected in all three biospecimens). Some known tumor markers (Nectin-4, UPAR, FOLR) were absent from the tumor tissue, but present in Pap test fluid and swab. This, however, can be explained by these proteins being surface markers, which can be cleaved and shed into body fluids.

Subsequently, authors compared the protein profiles of the patient's specimens with Normal Pap-test Core Proteome, a database proposed previously by the same group, consisting of 153 proteins identified in samples from healthy individuals (53), and with literature available up-to-date. Thus, they suggested several candidates, namely: CA125, mesothelin, LRG, CD44, folate receptor alpha, UPAR, Nectin-4, Kalikrein-10 and -13, as possible OC biomarkers, all of them present in Pap-test fluid.

This study is undoubtedly interesting, however, rather preliminary. First, only one patient was included, and this person was diagnosed with already advanced (metastatic) disease. Second, it must be validated to which extent the peptide profiles of OC patients are unique and to which extent they overlap with healthy controls. More extensive studies are necessary to find out if protein biomarkers can improve the early diagnosis of OC, alone or in combination with genetic testing. Notably, using proteomics in early diagnosis of OC was also postulated by Barnabas *et al*. (54) This group, however, used uterine lavage rather than liquid Pap specimens.

Early OC Diagnosis – New Strategies and Ideas for Improvement

Extending the use of cervical cell swabs to diagnosis of OC is a relatively new approach. Apart from the seminal work by Kinde and coworkers (8), which was published in 2013, eight out of nine studies reviewed by us come from the last four years (2018 and later). Though their results are encouraging, there is still room for improvement.

Most importantly, sensitivity of the detection is far too low for screening perspectives. Studies presented in the current review estimate the proportion of Pap samples where OC-related mutations can be detected to be approximately 40% (8, 17, 20). This proportion was higher (ca. 60%) in some studies focused only on detection of *TP53* alterations in HGSC (35, 36). This can be partly explained by cancer cells either not being present in the location where the sample is being collected or not present in sufficient amounts. Some of the ideas for remedying these problems are presented below.

Modifications of the Sampling Procedure

Optimization of the sampling procedure was initially suggested by Kinde *et al*. (8) and carried out by Wang and co-workers in the testing of a new screening tool (20). They showed an improvement of the sensitivity of the applied test (PapSEEK, a multiplex PCR-based test; see above) from 33% to 45% using a Tao brush instead of a conventional Pap brush. The Tao brush collects material from the endometrial cavity instead of the endocervix and may be more efficient in capturing cancerous cells shed from the ovaries or fallopian tubes. However, introducing a more invasive method collides with the intention of merely extending the cervical smear screening program.

Other modifications also aiming to collect the diagnostic material from a closer proximity to the ovaries include uterine lavage (55) or use of intravaginal tampons (56).

The uterine lavage technique [as described in (55)] enabled successful detection of *TP53* mutations in up to 80% lavage samples (24 out of 30 patients) of HGSC. Interestingly, the cohort included two FIGO stage I patients, and in both cases the mutated variants were detected. Moreover, a mutated *TP53* variant was also detected in a patient with occult OC. Briefly, the uterine lavage sample was taken prior to risk-reducing salpingo-oophorectomy (RRSO) performed due to a germline *BRCA* mutation. Histological analysis performed post RRSO revealed the presence of small lesions in the intraperitoneal cavity and on the ovaries, classifying the patient as stage IIIB. At that point, patient's CA-125 and transvaginal ultrasound remained inconspicuous, though the tumor-specific variants were present in the lavage fluid.

This method has a high diagnostic potential but is not very feasible neither comfortable for the patient and bears a theoretical risk of infection.

Another solution, potentially more convenient and less invasive than lavage, is the use of intravaginal tampons. They constitute an easily available and broadly accepted hygiene product. A small pilot study by Eriksen and coworkers (56)

presents its possible utility. From the original cohort of 33 patients, enrolled with pelvic mass suspicious for malignancy and with planned diagnostic or therapeutic surgery, only 5 fulfilled all the criteria (confirmed HGSC diagnosis, sufficient DNA yield in the tampon and the tumor sample, no tubal ligation). *TP53* mutation profiles were compared between tumor tissue samples and DNA samples obtained from tampons placed in the vagina 8-12 hours before a scheduled surgery. In 3 out of 5 (60%) participants the tumor and vaginal DNA harbored the same *TP53* mutations (56).

The use of intravaginal tampons presents an attractive way of specimen collection, as it does not require a medical professional and can be easily used for serial sampling. However, additional studies are necessary to verify the preliminary findings, especially in the context of early diagnosis.

Increasing Sensitivity of Analyses

The amount of exfoliating OC cells is small, especially in those parts of the gynecologic tract that are anatomically distant from ovaries, calling for ultra-sensitive detection methods. It is disputable to which extent cancer cells even are present in liquid Pap specimen (17) or in samples collected from the cervix or uterine cavity by one of the other aforementioned ways (Tao brush, lavage, tampons). However, no matter the sampling strategy, the right tools can improve the detection. It has been for example demonstrated by Arildsen *et al*., comparing the use of standard ddPCR with IBSAFE [see above (35)] and by Maritschnegg *et al*., who demonstrated that standard NGS often fails detecting mutated variants, that more sensitive methods (SafeSeqS, ddPCR) are more useful (55). Therefore, the progress in early OC diagnostics should also go in the direction of designing highly sensitive detection methods.

Selecting the Most Optimal Targets

Increased sensitivity of detection can potentially improve early diagnosis; however, the number of mutated variants poses a therapeutic challenge. The haploid genome contains approximately 3.1×10^9 base pairs, and, theoretically, each base can be replaced by one of the other three. Additionally, parts of the genomic sequence can be deleted or moved, further expanding the already broad spectrum of variants. Some of these alterations can result in benign mutations, while some may be pathogenic.

Screening assays in the whole population, where no tumor reference tissue is available, would require sequencing of cancer-related genes, followed by annotation to the reference genome, identifying mutations and inferring about their pathogenicity from available databases. Sequencing of *TP53* alone would allow catching a significant proportion of OC patients, since it is commonly mutated in HGSC, and HGSC is responsible for about 70% of OC-related deaths (24). Diagnosis of other histological types would most probably require sequencing of cancer-specific gene panels [such as those proposed by (20) or (25)]. Sequencing costs keep decreasing and next generation sequencing becomes more feasible and sensitive. However, limiting targets of interest would greatly facilitate the diagnostic procedure.

This can be partly addressed by implementation of methylation profile analysis along with mutation analysis. Identifying sites commonly methylated would give a lower number of variants than in the case of somatic mutations for the same sequence. In the human genome there are approximately 28.3×10^6 CpG sites (two levels of magnitude less than there are base pairs) (57), and each of them can take only one of two states (methylated or not). Identifying sites in which the methylation status commonly differs between normal and cancer tissue, between benign and malignant tumors or between histological types would greatly facilitate diagnosis. Further knowledge on methylation profiles in OC could be obtained through analysis of samples from large biobanks. That would hopefully contribute to identification of diagnostic targets in cervical cell swabs and help creating a screening strategy with an optimal coverage of most OC subtypes.

An example of a potentially promising diagnostic strategy is that presented in the study by (58). Using Infinium MethylationEPIC BeadChip array the authors identified 84 loci differently methylated in malignant and benign ovarian diseases. These exploratory results must be further validated in a larger cohort of cervical cell swabs before any strong conclusion can be drawn. These results may help in the identification of new biomarkers for differentiating between malignant and benign pelvis disease.

Optimization of diagnostic algorithms may to some extent address the issue of insufficient sensitivity of detection. That could include adding other OC-related genes to diagnostic gene panels, identifying methylation loci unique for tumor or implementing new markers, such as microRNA profiling MS-based proteomics. However, it is also plausible that the low detection rate does not result from shortcomings of the detection tools, but the very biology of OC. If it is evidenced that indeed only a proportion of OC sheds cells and/or DNA in sufficient quantities into the uterine/cervical cavity, the Pap test should most probably be supplemented with some other type of diagnostic specimen.

Other Types of Diagnostic Specimens – ctDNA and Liquid Biopsies

Detection of circulating tumor DNA (ctDNA), *i.e*., a cancer-originating component of circulating cell free DNA (ccfDNA), present in blood samples, may be considered a

potentially promising approach. Locke *et al*. postulated that it could address the problem of tumor heterogeneity and tissue sampling, which make specimens not representative for tumor, thus providing a misleading image. ctDNA may help capturing the true variation of samples and be representative for broader population of tumor cells. Moreover, using liquid biopsies and ctDNA could be a solution when collecting a biopsy is hard or impractical, like during advanced metastatic disease (50).

The downside of using ctDNA for the diagnostics may be the fact that it is present in very low amounts and does not give any information about the tumor location or its architecture. However, ctDNA can still be considered as a diagnostic tool for example in conjunction with cervical cell swabs.

Some potentially promising results have been presented by Wang and coworkers, who applied their PapSEEK gene panel to detection of OC in both Pap and serum samples. They successfully detected ctDNA in 43% of the samples from OC patients. Positive ctDNA samples, however, did not completely overlap with positive Pap samples from the same study. Collectively ctDNA and Pap identified 63% of OC cases, supporting their combined use in diagnosis (20). Detection of the mutated alleles in serum samples of OC patients have also been reported by Jiang and colleagues (25).

Just like with the enhanced sampling (tampons, cervical lavage), implementing a blood-based test would collide with the idea of simply extending the cervical smear screening program. Perhaps this approach could be reserved for women in high risk of OC.

Conclusion and Future Perspectives

Early detection of OC should be both sensitive (low false-negatives) and specific (low false-positives), though currently no satisfactory tools are available and thus further research in this direction is necessary. An ideal set-up would include a large, representative cohort of patients and a broad pool of candidate genes to catch all the histological subtypes. Of note, during the actual screening the genotype of the tumor would not be known, so the diagnostic panel must be carefully prepared considering the genetic alterations known so far. At the same time, the test should be robust enough to distinguish against benign conditions. It may be a good idea to include epigenetic markers apart from sequence mutations, since changes in the methylation profile have been reported to happen early during the tumorigenesis and since their repertoire is more limited compared to somatic mutations. Finally, it should once again be stressed that a diagnostic tool should ensure minimally invasive sample collection along with low cost to enable a broad screening of a population.

The data available to date pose the question whether applying classical liquid Pap smear specimen for early detection of OC may be sufficient, even if highly sensitive detection methods are utilized. Improvement of the diagnostic algorithms will hopefully further increase sensitivity and specificity of testing.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

EB: Conceptualization, Writing - original draft. RSW: Conceptualization; Writing - original draft. CH: Writing - review & editing. EH: EB: Conceptualization, Writing - review & editing.

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