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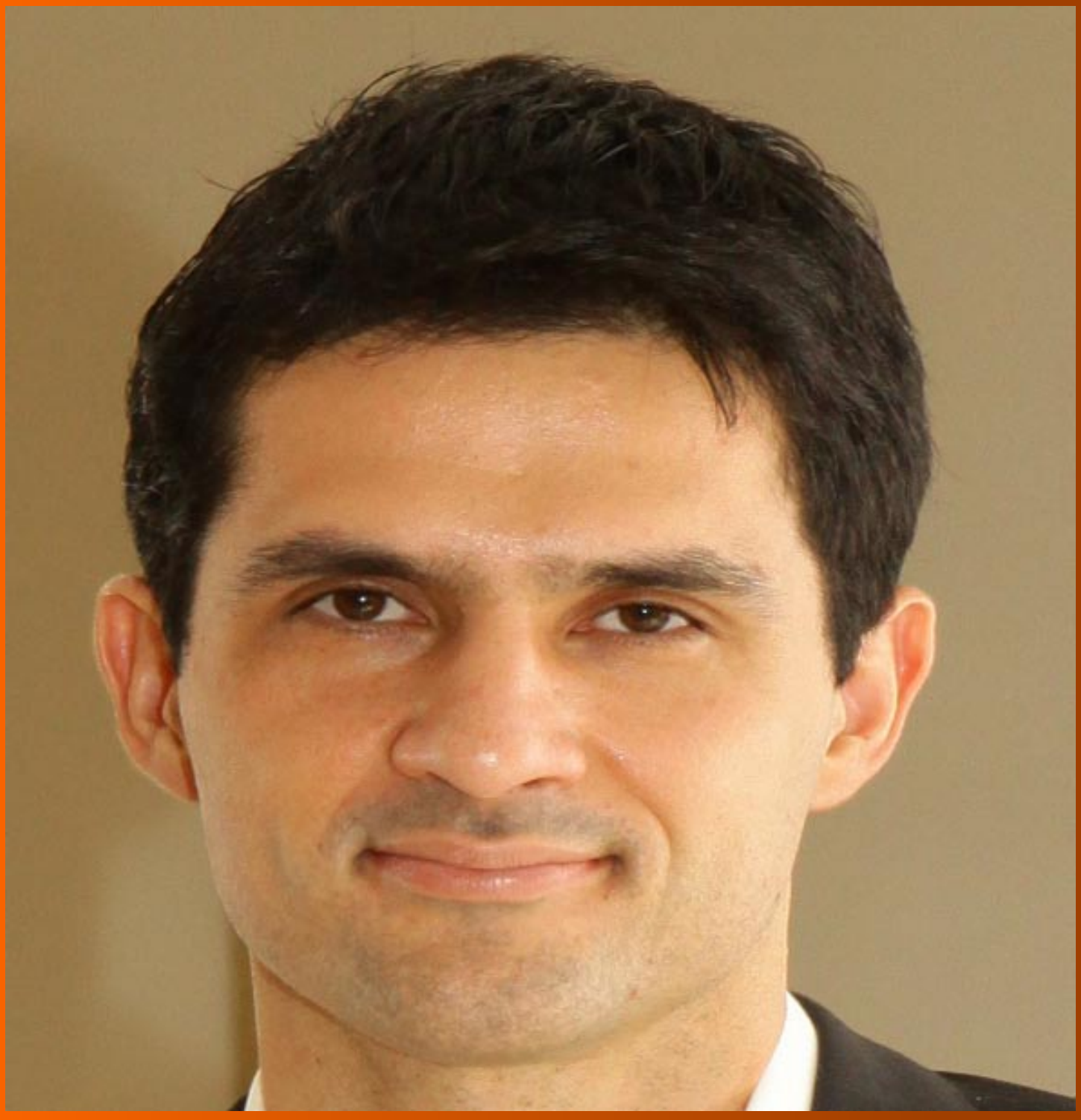

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EDITORIAL

Clinical applicability of immunotherapy of cervical intraepithelial neoplasia

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Abstract

Immunotherapy for cervical intraepithelial neoplasia (CIN) has not yet reached clinical applicability, but seems sensible and shows promising preliminary results. One of the most promising forms of immunotherapy for CIN may currently be imiquimod, because of its established role in other human papillomavirus (HPV)-induced genital conditions, its promising treatment efficacy in high-grade CIN, and its off-label availability. Although imiquimod cannot yet replace the current gold standard treatment for CIN [*i.e.*, large loop excision of the transformation zone (LLETZ)] in all patients, it may be considered in subgroups of patients; for example, young women who may wish to become pregnant in the future, or patients with recurrent CIN lesions in whom a second LLETZ is to be avoided. Immunotherapy of CIN could be extended to post-treatment vaccination, in order to prevent new HPV infections and disease recurrence.

Key words: Cervix; Cervical intraepithelial neoplasia; Large loop excision of the transformation zone; Immunotherapy; Regression; Human papillomavirus

Core tip: Immunotherapy for cervical intraepithelial neoplasia (CIN) is discussed in light of the natural history of CIN. The pros and cons of the current standard therapy (large loop excision of the transformation zone) and immunotherapy, potential side effects, and available evidence supporting the use of immunotherapy in CIN are addressed.

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IMMUNOTHERAPY OF CERVICAL INTRAEPITHELIAL NEOPLASIA

Cervical intraepithelial neoplasia (CIN) is caused by

infection with human papillomavirus (HPV). Low-grade lesions are primarily caused by low-risk HPV types and are the result of productive infections, in which viral replication takes place[1,2]. Most of these lesions are effectively cleared by the host immune response[3]. High-grade lesions are primarily caused by the more oncogenic high-risk HPV types (mainly HPV-16 and -18) and are the result of a transforming infection of cells of the squamocolumnar junction. In these infections, normal viral gene expression is deregulated: Overexpression of early viral genes in the basal cell layers leads to uncontrolled cell proliferation and cell immortalization, and makes the cell susceptible to chromosomal instability. Subsequent viral integration and epigenetic effects further enhance early viral gene expression and genomic instability, resulting in a proliferating cell population with chromosomal aberrations and leading ultimately to cervical carcinogenesis[1,2]. However, not all HPV infections lead to high-grade CIN, and not all high-grade CIN lesions lead to cervical cancer. Spontaneous regression of high-grade CIN occurs in approximately 20%-40% of high-grade lesions, while approximately 30% of high-grade CIN progresses to cervical cancer[4-7]. This suggests that the development of HPV-induced cervical pathology not only depends on the cellular changes induced by HPV infection, but is in fact determined by complex interactions among viral factors, functional cellular mechanisms, and the immune system. Clearance of HPV infection and HPV-induced lesions is mediated by the innate and adaptive immune system[3]. This immune response is a largely local process and depends on the individual characteristics of the host's immune system, but is also influenced by characteristics of the virus and the infectious process. HPV utilizes several effective immune evasion strategies, and infection leads to active downregulation of immune responses. This leads to persistent HPV infections and HPV-induced cervical lesions in a subset of patients. In patients who are unable to clear the infection, HPV resides in the host epithelium for a long time, leading to alteration of cellular processes as previously described, with neoplastic progression as a result.

Ideally, lesions that will regress spontaneously should be differentiated from those that will persist or progress into invasive disease, thus allowing for watchful waiting in the subgroup of patients in which spontaneous regression is expected. Biomarkers could be used to predict the natural history of CIN lesions. Indeed, a recent review identified several promising biomarkers in this regard, but none have yet reached clinical implementation[8]. As the natural history of high-grade CIN currently remains unpredictable, treatment of high-grade CIN is advised. Currently, the standard therapy for high-grade CIN lesions is surgical excision, which is usually done by large loop excision of the transformation zone (LLETZ). LLETZ is an effective treatment modality, but has two important disadvantages. First, residual and recurrent disease occurs frequently. Recent studies show residual and recurrent disease rates of 14%-23% after treatment for high-grade CIN or persistent low-grade CIN. Persistent or recurrent high-grade CIN occurred in 3%-10% of these patients[9-13]. Second, LLETZ is associated with an important long-term complication, namely a two-fold increase in premature birth seen in pregnancies after a LLETZ procedure, most probably as a result of cervical insufficiency[14,15]. Although some biomarkers may be promising for regression risk prediction, a considerable number of high-grade CIN lesions will not regress spontaneously, making effective treatment modalities necessary. To reduce unnecessary surgical treatment, alternative non-invasive treatment modalities for high-grade CIN are being studied. Since high-grade CIN is the result of an HPV infection that is not adequately cleared by the infected host, immunotherapy of CIN may be an effective alternative to conventional surgical treatment and/or watchful waiting.

Several forms of immunotherapy have been studied for the treatment of high-grade CIN, with varying results. These include both systemic forms of immunotherapy, such as therapeutic vaccines, interferon, and cyclooxygenase-2 inhibitors, and local forms of immunotherapy, such as topical or intralesional interferons and imiquimod[4,16-21]. Some agents show therapeutic effects, but none have yet reached clinical applicability. This is due to limited evidence, generally modest treatment results, and a high rate of side effects. We currently consider imiquimod (Aldara) to be the most promising form of immunotherapy for high-grade CIN. Imiquimod is a Toll-like receptor agonist with antiviral and anti-tumor properties. It is readily available for off-label use; its efficacy in several HPV-induced genital conditions, such as genital warts and vulvar intraepithelial neoplasia, is already established; and recent studies show promising results in the treatment of high-grade CIN. Lin *et al*[19] studied imiquimod treatment of genital HPV infections and both vaginal intraepithelial neoplasia (VAIN) and CIN lesions. They showed significantly more HPV clearance in 26 patients treated with 12 doses of imiquimod cream than in a historic control group (65% *vs* 30%). A limited number of 6 patients with high-grade VAIN or CIN lesions were treated with imiquimod, of which four (66%) showed disease remission. Treatment efficacy of imiquimod in high-grade CIN was more systematically studied by Grimm *et al*[4] in a placebo-controlled randomized controlled trial. They included 59 patients with high-grade CIN, who were treated with one to three applications of imiquimod per week for 16 wk. Significantly more histologic regression and remission was observed in the imiquimod group (73% *vs* 39%).

Although promising, it is unlikely that imiquimod will completely replace LLETZ as the standard treatment strategy for high-grade CIN. The efficacy of imiquimod and other immunotherapies has not yet reached that of LLETZ. Furthermore, imiquimod treatment is labor-intensive and time-consuming, and

is associated with frequent, albeit generally mild-to-moderate, side effects. Moreover, imiquimod and other forms of immunotherapy may treat current cervical lesions and HPV infections, but do not protect against new infections. The risk of future CIN and cervical carcinoma therefore persists. Recent new insights in the pathophysiology of high-grade CIN suggest that disease recurrence after LLETZ may in fact be the result of incomplete resection of the transformation zone in combination with persistent or new HPV infection. Herfs *et al*[22] demonstrated that high-grade CIN may originate exclusively from cells of the squamocolumnar junction. If their observations are correct, disease recurrence could be effectively be prevented by complete resection of the squamocolumnar junction. Alternatively, adjuvant vaccination may provide protection against future infections and disease recurrence. Indeed, the first trial on this topic shows a significant decrease in disease recurrence after LLETZ in combination with quadrivalent HPV vaccination[13].

In summary, immunotherapy of CIN has not yet reached clinical applicability, but seems sensible and shows promising preliminary results. One of the most promising forms of immunotherapy in CIN may currently be imiquimod, because of its established role in other HPV-induced genital conditions, its promising treatment efficacy in high-grade CIN, and its off-label availability. Although imiquimod cannot yet replace LLETZ as the current gold standard treatment for CIN in all patients, it may be considered in subgroups of patients, such as young women who may wish to become pregnant in the future, or patients with recurrent CIN lesions in whom a second LLETZ is to be avoided. Immunotherapy of CIN could be extended to post-treatment vaccination, in order to promote disease recurrence and prevent new HPV infections.

We advocate further research in the field of immunotherapy in CIN and are currently conducting a trial on the efficacy of 5% imiquimod cream in high-grade CIN [topical treatment with Imiquimod of high-grade CIN (TOPIC) trial; ClinicalTrial.gov identifier: NCT02329171]. This study also includes assessment of side effects, disease recurrence, and quality of life. Further research is also needed on the efficacy of post-treatment HPV vaccination. A combination of immunotherapy for present lesions and vaccination to prevent disease recurrence may provide a good treatment alternative to current surgical treatment for high-grade CIN in selected patients.

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REVIEW

Update on human papillomavirus vaccination: Where are we now?

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Author contributions: Chapman-Davis E and Dockery LE contributed equally to this work with literature search, text and tables; Griffith K and Stroup C contributed to literature search and tables in certain sections of the review; Chapman-Davis E and Dockery LE made several critical corrections and revisions until the submitted version was achieved.

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Abstract

Infection with human papillomavirus (HPV) is the major cause of pre-invasive and invasive lesions of the urogenital tract, resulting in morbidity and mortality worldwide. HPV-related infection is responsible for most cases of cervical cancer, a leading cause of cancer death in women worldwide. Developed countries have screening programs in place to detect precancerous lesions at early stages; in resource-limited settings however, HPV related diseases are often identified in advanced stages. This is due to limitations in the availability and roll out of effective screening programs. The relatively recent availability of the HPV vaccine has provided a new public health opportunity to decrease the incidence of HPV-related disease. The high mortality rates seen in developing countries could be reduced through effective implementation of HPV vaccination programs. Large trials have proven the efficacy of bivalent, quadrivalent vaccine and most recently 9-valent vaccine. Uptake in vaccination remains low due to multiple barriers including lack of education, lack of access, and costs. New strategies are being assessed to increase access, increase knowledge and reduce costs that may result in feasible vaccination programs worldwide. The goal of this article is to review the effectiveness and safety of the current HPV vaccines available, vaccine delivery strategies, cost effectiveness, and efforts to improve the acceptability. A literature search was conducted through PubMed using the terms "HPV vaccination, and safety, and males, and acceptability and strategies, and cost effectiveness," focusing on articles published between 2006 and 2015. The most relevant and larger scale trials were evaluated for discussion.

Key words: Human papillomavirus; Vaccines; Cervical cancer; Cancer prevention; Public health

Core tip: Human papilloma virus (HPV) represents the major cause of pre-invasive and invasive lesions of the urogenital tract. This article will review the efficacy, safety and approval of the currently available vaccines against HPV including the bivalent, quadrivalent and nine-valent vaccines. Indications for use in men, immunocompromised individuals and older cohorts will also be discussed. Additionally a summary of worldwide vaccination practices, cost effectiveness, vaccination and methods to improve vaccination uptake and acceptance will be reviewed.

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INTRODUCTION

Worldwide, cervical cancer is the fourth most frequent cancer in women affecting almost 500000 women each year and is the most common cause of cancer death among women in developing countries[1]. About 70% of the global burden falls in areas with low resources including sub-Saharan African countries[1]. Infection by certain types of human papillomavirus (HPV) is required for almost all cases of cervical cancer. HPV is the most commonly sexually transmitted infection not only in the United States but worldwide[1,2].

In the United States, 79 million people are currently infected with HPV and 14 million people are newly infected each year. Additionally, 26000 of all new oral and urogenital related cancers are attributed to HPV annually, of which approximately 17000 are in women and approximately 9000 are in men[2]. More than 4000 women die of cervical cancer each year in the United States, and as many as 93% of these cancers could be prevented by screening and HPV vaccination[3].

Infection with HPV is implicated in the development of not only cervical cancer, but also many other cancers including anal, vaginal, vulvar, penile, oropharyngeal carcinomas and oral cancers. HPVs are a family of deoxyribonucleic acid (DNA) viruses that infect skin or mucosal cells. There are over 100 types of HPV and > 40 types infect the anogenital tract. At least 13 types are considered oncogenic (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)[1,2].

Universal access to HPV vaccination, screening and treatment services are crucial in reducing the overall burden of HPV related diseases. The incidence and mortality from cervical cancer has not decreased as significantly in developing countries as it has in the United States following the introduction of the Papanicolaou smear[4]. There are many obstacles to screening, generally attributed to a lack of infrastructure and resources, as a result of technical, medical and financial constraints[4]. Lack of awareness and education among women and health care providers have also been reported to play a role[4]. Therefore a critical public health need is being addressed with the introduction of HPV vaccines as a major strategy for the prevention of not only cervical cancer but all HPV-related diseases.

The objective of this article is to review updated information regarding HPV vaccine approval, availability and safety, review the major trials of bivalent, quadrivalent and 9-valent vaccines, discuss implementation concerns including vaccine delivery strategies, cost-effectiveness of HPV vaccination and efforts to improve vaccine acceptability worldwide.

DISCUSSION

Vaccine development and rationale

Given the endemic nature of HPV infection, attempts have been made at the prevention of HPV-related sequelae such as high-grade cervical lesions and deaths due to cervical cancer with the introduction of HPV vaccines. The lifetime risk of genital infection with an oncogenic strain of HPV is thought to be greater than 80%, however in immunocompetent individuals 90% of infections become undetectable without intervention[5]. The risk of developing squamous cell carcinoma of the cervix is approximately 400 times higher following infection with HPV-16 and 250 times higher following infection with HPV-18 as compared to those not infected. HPV types 16, 18, 31, 33, 45, 52, and 58 account for approximately 90% of all HPV positive squamous cell carcinomas[1]. Wagner *et al*[6] reviewed publications investigating the genotype-specific prevalence of HPV-related cervical, vulvar and vaginal disease in women worldwide. Based on the results of these studies, HPV genotypes 16, 18, 31, 33, 45, 52, 58 are responsible for 90% of all cervical cancers providing rationale for the utilization of the 9-valent L1 VLP vaccine (9vHPV) vaccine. However, a lack of data regarding genotype-specific prevalence exists in several regions of the world with the highest age-standardized incidences rates of cervical cancer[6]. A study by Clifford *et al*[7] examined the global prevalence of HPV types in cytologically normal women. Their findings demonstrate heterogeneity in the distribution of HPV types globally. Although HPV 16 prevalence is higher in sub-Saharan Africa than Europe, these women are less likely to be infected with HPV 16 than European women[7]. Additionally, HPV 35, 45, 52, 56, and 58 (other high-risk types) and low-risk types were more prevalent in women with HPV infection in sub-Saharan Africa[7]. While this study is limited in representation of sub-Saharan countries (n = 1), it addresses the need for prevalence-specific screening and vaccination with vaccine choice (quadrivalent, bivalent, or 9-valent) tailored to regional HPV prevalence.

Much like vaccination for other communicable diseases, administration of HPV vaccines in HPV-naïve individuals attempts to provide herd-immunity for

Table 1 Phase III efficacy in women

	Future I	Future II	Patricia	CVT	Broad spectrum HPV vaccine study
Vaccine	Gardasil®	Gardasil®	Cervarix®	Cervarix®	Gardasil9®
Funding	Merck and Co., Inc.	Merck and Co., Inc.	GlaxoSmithKline	National Cancer Inst.	Merck and Co., Inc.
Number enrolled	6463	12167	18729	7466	14215
Number of countries	16	13	14	1	14
Duration of trial	4 yr	4 yr	4 yr	4 yr	4 yr
Age (yr)	16-24	15-26	15-25	18-25	
Lifetime sexual partners	< 4	< 4	< 6	No restriction	< 4
Exclusions	Pregnancy, history of abnormal Pap smear or genital warts	Pregnancy, history of abnormal Pap smear	Pregnancy, breastfeeding, history of colposcopy, autoimmune disease/ immunodeficiency, HPV 16/18-associated CIN2+ at enrollment	Pregnancy, breastfeeding, history of immunosuppression, hysterectomy, hepatitis A vaccination	Prior abnormal Pap smear, > 4 lifetime sexual partners, no prior abnormal finding on cervical biopsy
Primary endpoint	Incidence of vaccine-type HPV associated CIN1-3, AIS or cancer, combined incidence of vaccine-type HPV associated anogenital warts, VIN/VaIN1-3 or cancer	HPV 16 or 18 associated CIN2/3	Incidence of HPV 16 or 18 CIN2 or greater	HPV 16 or 18 persistent infection (12 mos.) or HPV 16 or 18 associated CIN2+	High-grade cervical, vulvar, and vaginal disease
Mean follow-up time	3 yr	3 yr	14.8 mo		
Immunogenicity	99.5% seroconversion after 3 doses	99% seroconversion to vaccine-associated HPV types	99.5% seroconversion rates in women aged 15-25 yr		Non-inferior to quadrivalent vaccine

CVT: Costa Rica HPV Vaccine Trials; HPV: Human papillomavirus; VIN/VaIN: Vulvar/vaginal intraepithelial neoplasia.

future generations and thus lower the burden of HPV-related disease. The median time from HPV infection to seroconversion is approximately 8-12 mo, however because HPV infection is restricted to the intraepithelial layer of the mucosa it does not induce a strong immune response[1]. Failure to develop a sufficient cell-mediated immune response leads to persistent infection and increased risk of progression to CIN 2 to 3[1]. The most type-specific HPV antibodies are directed against the L1 HPV viral protein providing a target for vaccine development.

Vaccine efficacy and safety

To date, two vaccines (bivalent and quadrivalent) against HPV have been approved for use in over 100 countries for the prevention of HPV-related disease.

Both vaccines are composed of non-infectious virus-like particles (VLPs) The quadrivalent vaccine (Gardasil®) targets HPV 6, 11, 16, 18, aimed at prevention of the two most oncogenic HPV types (types 16 and 18) which cause > 70% of cervical cancer worldwide, and types 6 and 11 which are responsible for approximately 90% of genital warts[8]. The bivalent vaccine (Cervarix®) contains purified viral proteins of HPV types 16 and 18, targeting only the oncogenic subtypes[1]. In the United States, the quadrivalent vaccine is administered on a 3-dose schedule (0, 2, and 6 mo), however in other countries, it is approved for a 2-dose schedule for girls and boys aged 9-13 years. The bivalent vaccine is given on a 2-dose schedule for boys and girls aged 9-14 years. Those > 15 years should receive a 3-dose schedule[1].

Efficacy: Four Phase Ⅲ efficacy trials were performed for the quadrivalent and bivalent HPV vaccines. These studies were designed to demonstrate efficacy in preventing incident vaccine-related HPV infection and preneoplastic lesions caused by incident persistent infections related to the subtypes of HPV in vaccines. The FUTURE Ⅰ and FUTURE Ⅱ trials evaluated Gardasil® while the PATRICIA and Costa Rica HPV Vaccine Trials (CVT) evaluated Cervarix®. All of the trials were large, blinded, and randomized controlled trials of young women (mean age 20). With the exception of the CVT, all studies were company-sponsored and multicenter involving multiple trial sites globally[9].

The FUTURE Ⅱ and PATRICIA trials used a precancer primary endpoint of cervical intraepithelial neoplasia (CIN) 2/3, adenocarcinoma in situ, or cervical cancer associated with HPV 16/18. FUTURE Ⅰ had an additional endpoint of HPV6/11/16/18-associated CIN1+ and external genital lesions including vulvar/vaginal intraepithelial neoplasia (VIN/VaIN). Among the trials, the median age at enrollment ranged from 15-26 years. Clinical trial details are described in Table 1 with an overall vaccine efficacy of > 99% between 14 mo and 3 years of follow up[9-13] (Table 1).

Many genotypes exist of both oncogenic (high risk) and genital wart causing (low risk) HPV. Partial cross-protection against non-vaccine oncogenic HPV types has been reported, however the clinical relevance is undetermined. While HPV types 16 and 18 are respon-

sible for 70% of global cervical cancer, oncogenic HPV types 31, 33, 45, 52, and 58 cause approximately 20% additional cervical cancer cases. A 9vHPV vaccine containing HPV types 6, 11, 16, 18, 31, 33, 45, 52, 58 has the potential to prevent up to 90% of all cervical cancers globally[14].

The Broad Spectrum HPV Vaccine Study conducted a large Phase II/III clinical trial to assess efficacy, immunogenicity, and safety of the 9vHPV vaccine. The endpoints of this trial were to prove non-inferiority of anti-HPV 6/11/16/18 antibody response, and superior efficacy in HPV 31/33/45/52/58-related clinical outcomes for the 9vHPV vaccine as compared to the quadrivalent vaccine. In addition, a non-inferiority assessment was conducted assessing the percent risk reduction for 9vHPV *vs* quadrivalent vaccine[14].

Results showed that 9vHPV vaccine was efficacious in preventing high-grade cervical, vulvar and vaginal disease related to the 5 new HPV subtypes. Additionally the 9vHPV vaccine generated a non-inferior antibody response to HPV 6/11/16/18 as compared to the quadrivalent vaccine[14,15]. The United States FDA licensed the 9vHPV vaccine for use in 2014 under the name Gardasil®9[14].

Studies have been evaluated to determine potential benefit of 9vHPV vaccine in the United States and abroad. A population-based evaluation of the subtypes of HPV in women with CIN2+ was performed in the United States. Approximately 50% of lesions were attributable to HPV 16/18, while 25% of lesions were attributable to HPV 31/33/45/52/58. Older women and racial/ethnic minorities with $CIN2^+$ diagnosed were more likely to have subtypes other than HPV 16/18[16] and would potentially benefit from the extended coverage. Serrano *et al*[17] conducted a study evaluating the potential impact of the 9vHPV vaccine on cervical cancer prevention in 4 countries (Brazil, Mexico, India and China). Based on the proportion of invasive cervical cancers attributable to HPV types 31/33/45/52/58, they estimated an increase in prevention of invasive cervical cancer by 12%-19% across the 4 countries[17]. This represents a potential target for significant decrease in HPV-related cancers worldwide if adopted into use globally.

Safety: Multiple studies[18-20] have established the safety of the 2 major HPV vaccines currently approved. A large post-licensure trial was performed evaluating the safety of the quadrivalent vaccine among females aged 9-26 years which revealed no significant increased risk of Guillan-Barré Syndrome, stroke, venous thromboembolism, appendicitis, seizures, syncope, allergic reaction or anaphylaxis[18]. A study evaluating post vaccination risk intervals in females revealed same-day syncope and skin infections at the site of vaccination as the only risks associated with recent vaccination[19]. A study performed in Australia of > 380000 doses of quadrivalent HPV vaccine given in school-aged girls identified 35 possible hypersensitivity reactions to the vaccine. Further evaluation of these 35 individuals revealed only 3 cases of true hypersensitivity reactions and most individuals tolerated subsequent doses of vaccine[20].

Vaccine approval

In the United States, Gardasil® is approved for females only between ages 9-26 while Cervarix® carries FDA approval for women ages 9-25. In countries such as Canada and Australia, the HPV vaccines are currently licensed for use in women up to age 45[21]. In Australia, the quadrivalent vaccine was added to the National Immunisation Program in 2007 and the bivalent vaccine added in 2008[22]. The VIVIANE study, a Phase 3 multinational, double-blind, randomized controlled trial is currently underway to evaluate the efficacy, safety and immunogenicity of the Cervarix® vaccine in women older than 25 years. Enrollment was stratified by age into 3 groups. The primary endpoint was vaccine efficacy against 6-mo persistent infection or CIN1+ associated with HPV 16/18. Interim analysis found significant vaccine efficacy against the primary combined endpoint overall and specifically in the 26-35 and 36-45 year age groups[21]. A study conducted in Germany and Poland assessed the immunogenicity of Cervarix® in women aged 15-55. Schwarz *et al*[23] evaluated immune responses in serum and cervicovaginal secretions 6 years after the first dose of vaccine in women ages 15-25, 26-45, and 46-55 years who received 3 doses of vaccine. After 6 years all women across all age groups were seropositive for HPV 16 and > 97% were seropositive for HPV 18 indicating a sustained immune response regardless of age at administration[23].

Interim data from the VIVIANE study establishes safety and efficacy in preventing primary acquisition of target HPV at any age[21], while Schwarz *et al*[23] demonstrate equivalent immunogenicity in all age groups; however issues addressing cost-effectiveness still need to be addressed in order to best use resources to achieve the maximum benefit to the population. The optimal target range for intervention likely still remains in women ages 9-26, however future studies using 9vHPV vaccine may prove cost effective in preventing invasive cervical cancer in the older populations with the addition of HPV types 31/33/45/52/58.

HPV vaccination of males: HPV infection is most well known for causing cervical cancer in women; however it is also responsible for other cancers, some of which are in men. In 2002, while nearly 100% of the 492800 cervical cancers were attributable to HPV, 90% of anal cancers, 40% of penile cancers, 12% oropharyngeal cancers and 3% of the mouth cancers worldwide were attributable to HPV infection[24]. HPV infection with non-oncogenic strains 6 and 11 can also cause genital warts which affect both men and women. Thus, men can also benefit from vaccination with the HPV vaccine, both to decrease their rates of cancer and genital warts, and to decrease their transmission of the virus to their male

and female partners.

Efficacy of the quadrivalent vaccine in males was established in a study by Giuliano *et al*[25]. A randomized, placebo-controlled, double-blind multi-center trial was conducted in 4055 males aged 16-26 years with the primary efficacy objective of demonstrating reduction of the incidence of external genital lesions related to HPV 6, 11, 16, or 18. An observed efficacy of 65.5% was noted in the intention-to-treat population while an efficacy of 90.4% was noted in the per-protocol group against lesions related to HPV 6, 11, 16, or 18[25]. Studies have also been conducted to measure the immune response to the vaccine in boys. A non-inferiority immunogenicity study was performed to establish the efficacy of the quadrivalent HPV vaccine in adolescent boys and girls from age 10-15. The resulting immune response in boys was found to be similar to their female counterparts[26]. These studies ultimately led to FDA approval for use of HPV vaccine in males ages 9-26 in 2010. Safety data in males has been similar to that documented in the female cohort. The most common side effects associated with vaccination include headache, pain at injection site, itching, redness, swelling and bruising[25].

A study by Bogaards *et al*[27] in the Netherlands recently looked at the benefit of including boys 12 years of age in the HPV vaccination program. The authors found that in order to prevent one additional case of anal, penile or oropharyngeal cancer among men, 795 boys would need to be vaccinated[27]. Alternatively, if vaccine coverage among girls increased from 60% to 90%, the burden of HPV related cancer in men would be reduced by 66%. This data suggests that even if vaccination increased dramatically just for girls, it would not influence the burden of anal cancer that is found primarily in men having sex with men. Therefore vaccination of boys may provide additional benefit although the cost effectiveness of this strategy comes into question[27].

In the United States, the HPV vaccine is recommended for routine vaccination at age 11 or 12 for both boys and girls. "Catch up vaccination" is also recommended for females aged 13 through 26 and males aged 11 through 21 who have not been previously vaccinated, for men who have sex with men and men who are immune-compromised through age 26[28].

Despite these recommendations, vaccination rates remain low among adolescent boys. In 2013, HPV vaccination among adolescent boys was 34.6% and in men who have sex with men aged 18-26, vaccination was significantly lower[29]. In a study of 428 gay and bisexual men aged 18-26, Reiter *et al*[30] found that only 13% of the study population had received any doses of vaccine. Another study by Meites *et al*[31] evaluated data from the 2011 National HIV behavioral surveillance system of 3221 men who have sex with men aged 18-26 and found that only 4.9% reported receiving 1 or more HPV vaccine doses. Rates of HPV vaccination also vary widely by state and region in the United States. For men, the rate of vaccine initiation and completion was 8.5% and 2.2% in the Northeast, 6.7% and 1.6% in the West and 4.9% and 1.4% in the South. For women, the rate was 58.7% and 45.6% in the Northeast, 39.0% and 24.8% in the West and 30.4% and 17.7% in the South[32].

HPV vaccination in the immunocompromised: Given the high rates of HPV infection and HPV associated cancers in HIV positive and other immunocompromised populations, HPV vaccination should be considered in these groups[33]. Several different bacterial and viral vaccines are recommended for use in solid organ transplant patients, including pneumococcal, influenza, hepatitis A virus, hepatitis B virus, diphtheria, and tetanus vaccines. These patients have diminished, but often effective, immune responses, with no major consequences[33,34]. Given that HPV VLPs represent highly immunogenic proteins as seen by the degree of humoral response in immunocompetent women, the HPV vaccine causes adequate response in even immunocompromised individuals[33]. The benefit of vaccination preventing reactivation of HPV remains unknown.

Individuals with HIV are known to be at significant risk for persistent HPV infection and the sequelae associated with persistent infection, including neoplasia and malignancy[35]. Vaccine safety and immunogenicity in at-risk populations has been studied among men, women, and children with HIV[35-39]. A randomized clinical trial found the vaccine to be safe and immunogenic in 126 HIV-infected children aged 7-12 years. By 18 mo 94%-99% had antibody to HPV 6, 11 and 16 while 76% had antibody to HPV 18[37]. Weinberg *et al*[38] examined immunogenicity of 3 *vs* 4 doses of the quadrivalent vaccine in children aged 7-12 years with HIV. Following three doses the immune response to HPV 6, 11, and 16 were sufficient, however seropositivity remained lower for HPV 18. In the cohort receiving a fourth dose of vaccine, seropositivity of HPV 18 increased to levels equivalent to HPV 6, 11, and 16[38]. Another study of 109 HIV-infected males also found the vaccine to be immunogenic and well tolerated. Responses appeared to be higher for males on antiretroviral therapy as compared to those not receiving treatment[39]. In a study by Kahn *et al*[35], the quadrivalent HPV vaccine was shown to be safe and immunogenic in HIV positive women aged 16-23 who were previously HPV seronegative[35].

In the United States, vaccination is currently recommended by the Advisory Committee on Immunization Practices through age 26 years for immunocompromised persons who have not been vaccinated previously or who have not completed the 3-dose series[34].

HPV vaccination programs and cost effectiveness

In 2014, the World Health Organization (WHO) released updated recommendations for HPV vaccination in countries where preventing cervical cancer is a public health priority and where it is both feasible and financially sustainable to introduce the vaccine. The

WHO recommended that in these countries, girls aged 9-13 be the primary target group for vaccination, prior to becoming sexually active[40]. As of June 2015, 82 countries worldwide had introduced vaccination programs. Most of the vaccination programs are directed at pre-teen and teenage children, and some specifying vaccination for females only. There are multiple countries identified by the WHO statistics that have HPV vaccination programs (Table 2). These include 12 African countries, 2 Eastern Mediterranean countries, 31 European countries, 3 South-East Asian countries, and 15 Western Pacific countries. Additionally, 7 more countries have plans to introduce vaccination programs in the coming years[41].

The WHO recommends evaluating cost effectiveness of HPV vaccination prior to implementation in countries. Studies have suggested that the most important determinant of cost effectiveness of HPV vaccination is the vaccine price, cost effectiveness threshold utilized, and whether or not screening is assumed to be in place[42]. In settings with established cervical cancer screening programs, under certain assumptions, studies have shown that HPV vaccination can reduce incidence and mortality of cervical cancer and incidence of abnormal pap tests and precancerous lesions that typically require costly follow up[42-44]. These models assume that early vaccination will lead to starting screening at later ages, and at reduced frequency, ultimately saving money in the long term.

Unfortunately in low and middle-income countries these studies have been more limited due to many different issues these countries face. There has been conflicting data within the same countries in terms of evidence of cost effectiveness[42]. This may be due to varied models used and assumptions made. In a review evaluating cost effectiveness of HPV vaccination in low and middle-income countries, introduction of HPV vaccines was found to be cost effective in 22 studies for girls aged 12 and younger[42]. Almost all these studies assumed three-dose vaccine coverage of > 70%, life long protection and did not assess delivery and program costs. Pooled results across all these studies suggest that even in countries where screening may be limited or non-existent, vaccination may be even more cost effective as long as the price for the vaccine is low[42]. Reasons for these conclusions include the competitive prices given for vaccines relative to the income level of the country, donor funding availability and high cervical cancer burden found in these countries with limited treatment options. Savings resulting from improved screening and HPV vaccination ultimately will depend on the actual costs in a given country.

HPV vaccine acceptability

Although the FDA has approved the HPV vaccine in the United States since 2006, vaccination rates have varied across countries and populations within countries[45]. Both developing and developed countries such as the United States (34% full coverage) and France (28.5% full coverage) have shown poor complete uptake rates[45]. Reasons for this include lack of knowledge and education in both adolescents and parents, cost associated with vaccine, lack of access to primary care or providers that offer vaccination and lack of provider recommendation[46-48] (Table 3).

Despite the relatively low vaccine coverage in the United States, a study did show that the prevalence of HPV subtypes 6, 11, 16, and 18 in cervicovaginal specimens in females aged 14-19 decreased from 11.5% in the pre-vaccine era to 5.1% in the vaccine era. Whereas in other age groups, the prevalence did not differ significantly between the two time periods[49]. In the United States, HPV awareness and knowledge are increasing compared to previous national surveys of HPV knowledge, with 68% of the adult population reporting knowledge of both HPV and the HPV vaccine[48]. However, this number is not consistent across populations and states. Globally, similar problems exist[46-48,50]. In the Uyghur population of China, younger women and those with a lower educational level were less likely to understand the correlation between HPV and cervical cancer[47].

Multiple studies have addressed the barriers to HPV vaccination especially as it relates to education. Barriers identified include lack of access to schooling, as well as cultural and linguistic differences[51]. Many of the adult population site obstacles to vaccination due to limited education, resulting in poor parental knowledge, holding jobs with difficult work hours, and childcare difficulties[52]. Many studies have focused on educating both patients and parents to increase awareness and vaccine acceptance; however the best method of delivery of these materials remains to be seen. Tools used in the past include educational sessions and focus groups, the media, videos, school-wide vaccination programs, and flyers[50-57] (Table 3).

School-based education has been attempted as a method to optimize uptake of the HPV vaccine. In a Korean study, fifth-grade girls and boys underwent a 2-h education session regarding the connection between HPV and cancer, as well as the effectiveness of the HPV vaccine. Awareness that "HPV vaccine can prevent cervical cancer" was significantly related to intention to obtain the HPV vaccine among both boys and girls[50].

A lower HPV vaccination rate has been seen among minority populations in the United States. Studies have shown poor vaccination rates among Black, Latina, and Asian girls in comparison to Caucasian girls[53]. Efforts have been focused on addressing individual populations to help increase acceptability. An educational video addressing HPV and vaccination was utilized as an intervention to a primarily underserved, lower income Black and Hispanic population of women. Acceptance of individual vaccination, mandatory HPV vaccination and support for school vaccination all increased significantly after the video based on survey responses[54].

In a study performed in Canada, parents were

Table 2 Worldwide vaccination protocols

	Country	Vaccine in schedule (as of December 31, 2014)	Year of introduction in entire country	Target population	Schedule
Africa	Botswana	No	2015	Girls 9-13 yr	3 doses
	Lesotho	Yes	2012	9-14 yr	3 doses
	Malawi	No	2015	Girls 9-13 yr	3 doses
	Rwanda	Yes	2011		3 doses
	Seychelles	Yes	2014	Girls 10-12 yr	
	South Africa	Yes	2014	9 yr	2 doses
Americas	Argentina	Yes	2011	11 yr	3 doses
	Barbados	Yes	2014	11 yr	2 doses
	Brazil	Yes	2014	9-14 yr	2 doses
	Canada	Yes	2009		3 doses
	Colombia	Yes	2012	9-17 yr	3 doses
	Ecuador	Yes	2014	9 yr	2 doses
	Guyana	Yes	Not available	Special groups	3 doses
	Mexico	Yes	2012	10 yr	2 doses
	Panama	Yes	2008	10 yr	3 doses
	Paraguay	Yes	2013	10 yr	3 doses
	Peru	Yes	2011	10 yr	3 doses
	Suriname	Yes	2013	9 yr	
	Trinidad and Tobago	Yes	2013	Females 11-45 yr, males 11-26 yr	3 doses
	United States	Yes	2006	11-26 females, 11-21 males (26 if high risk)	3 doses
	Uruguay	Yes	2013	12 yr	3 doses
Eastern Mediterranean	Bahrain	No			
	Libya	Yes	2013	15 yr	3 doses
Europe	Andorra	Yes	2014	12 yr	
	Austria	Yes	2008	9 yr	3 doses
	Belgium	Yes	2011	12 yr (13-14 in Wallonia)	3 doses
	Czech Republic (the)	Yes	2012	13 yr	
	Denmark	Yes	2007	12 yr	3 doses
	Finland	Yes	2013	11-12 yr	
	France	Yes	2006	Girls 11-14 yr	3 doses
	Germany	Yes	2007	Girls 12-17 yr	3 doses
	Greece	Yes	2009	11-18 yr	3 doses
	Hungary	Yes	2014	12 yr	
	Iceland	Yes	2011	12 yr	3 doses
	Ireland	Yes	2010	Girls 12-13 yr	3 doses
	Israel	Yes	2010	13 yr (or women 9-45 yr)	3 doses
	Italy	Yes	2009	Girls 12 yr	3 doses
	Latvia	Yes	2010	12 yr	3 doses
	Luxembourg	Yes	2008	12-18 yr	
	Malta	Yes	2013	12 yr	3 doses
	Monaco	Yes	2006	14 yr	3 doses
	Netherlands (the)	Yes	2010	12 yr	2 doses
	Norway	Yes	2009	Girls 12 yr	3 doses
	Portugal	Yes	2008	10-13 yr	3 doses
	San Marino	Yes	2008	11 yr	
	Slovenia	Yes	2009	11-12 yr	2 doses
	Spain	Yes	2007	12 yr	3 doses
	Sweden	Yes	2010	Girls 10-12 yr	3 doses
	Switzerland	Yes	2006	Girls 11-14 yr	3 doses
	The former Yugoslav Republic of Macedonia	Yes	2009	12 yr	3 doses
	United Kingdom	Yes	2008	12-13 yr	2 doses
	Uzbekistan	No	2015		
South-East Asia	Bhutan	Yes	2009	Girls 12 yr	3 doses
Western Pacific	Australia	Yes	2007	10-15 yr	
	Brunei Darussalam	Yes	2012	13 yr	3 doses
	Cook Islands	Yes	2011	9 yr	
	Fiji	Yes	2013	13 yr	
	Japan	Yes	2011	13 yr	3 doses
	Malaysia	Yes	2010	Girls 13 yr	3 doses
	Marshall Islands (the)	Yes	2009	11-12 yr	
	Micronesia (Federated States of)	Yes	2010	9 yr	3 doses
	New Zealand	Yes	2009	12 yr (and other eligible individuals)	3 doses
	Palau	Yes	2008	9-26 yr	3 doses
	Philippines (the)				2 doses
	Singapore	Yes	2010	Girls 9-26 yr	3 doses

Table 3 Possible interventions to increase human papillomavirus vaccine uptake

Ref.	Year	Country	Objectives	*n*	Outcome	Target population	Educational tools
Chapman *et al*[54]	2010	United States	Evaluation of a video-based educational tool to increase HPV vaccine acceptability	256	Vaccine acceptability increased following intervention	Women aged 18-60	8 min video
Kennedy *et al*[55]	2011	United States	Improvement of HPV vaccine educational materials and determination of efficacy	411	Increase in likelihood of vaccination of children and favorable view of HPV vaccine	Parents of girls 11-18 yr of age	Educational flyer
Kobetz *et al*[51]	2011	Haiti	Assessment of women's knowledge and beliefs regarding cervical cancer and HPV		Need for culturally and linguistically appropriate educational initiatives	Haitian immigrant women in Miami, FL	Focus groups
Kester *et al*[56]	2014	United States	Evaluation of the effects of a brief education session on HPV awareness	131	Higher vaccination intent among intervention group	18-26 yr old females and males	5-10 min education session
Kim[50]	2015	South Korea	Assessment of knowledge of HPV relation to cancer in children	117	HPV education at elementary school would be helpful	Fifth-grade girls and boys	2 h education session
Nodulman *et al*[57]	2015	United States	Evaluation of feasibility of increased immunization rates through middle school vaccination programs	117	Low acceptance of middle school vaccination by adolescents, parents and stakeholders	Middle school stakeholders, nurses, parents, adolescents, administrators	Middle school vaccination program

HPV: Human papillomavirus.

Table 4 Evaluation of barriers to human papillomavirus vaccination

Ref.	Year	Country	Target population	Objectives	*n*	Identified barriers to vaccination
Mortensen[46]	2010	Denmark	Women aged 16-26	Evaluation of reasons for acceptance or rejection of HPV vaccine following general vaccine availability	794	Cost, lack of information about the benefits of vaccination, and lack of knowledge about HPV
Ogilvie *et al*[58]	2010	Canada	Parents with daughters in 6th grade	Determination of parental factors associated with receipt of the HPV vaccine in a publicly funded school-based HPV vaccine program	2025	Lack of knowledge regarding the HPV vaccine
Kobetz *et al*[51]	2011	United States	Haitian immigrant women in Miami, FL	Assessment of women's knowledge and beliefs regarding cervical cancer and HPV		Lack of education
Jeudin *et al*[53]	2014	United States	Black and Latina populations	Identification of barriers to uptake of HPV vaccination among low-income and minority girls		Lack of access to primary care, lack of provider recommendation, lack of parental knowledge
Kim[50]	2015	South Korea	Fifth-grade girls and boys	Assessment of children's knowledge regarding HPV and association with cancer	117	Lack of HPV knowledge, lack of HPV education in schools
Abudukadeer *et al*[47]	2015	China	Women in Xinjiang province	Assessment of knowledge and perception of cervical cancer	5000	Lack of knowledge about cervical cancer
Blake *et al*[48]	2015	United States	National Cancer Institute's 2013 Health Information National Trends Survey Data	Assessment of population knowledge regarding HPV and the HPV vaccine as well as socioeconomic disparities	3185	Lack of HPV awareness and knowledge
Nodulman *et al*[57]	2015	United States	Middle school stakeholders, nurses, parents, adolescents, administrators	Increase of immunization rates through middle school vaccination programs	117	Lack of knowledge about HPV vaccine

HPV: Human papillomavirus.

interviewed to assess which factors were the most important barriers to vaccinating their children. The study was performed in a publicly funded, school-based HPV vaccine program, to remove the barriers of access and cost. Despite this, the main reasons for not vaccinating female children were concerns about vaccine

safety, preference to wait until the daughter is older, and not having enough information to make an informed decision[58]. Based on this study it is apparent that even when financial and health care barriers are removed, parental acceptance of vaccination remains critical in improving vaccine uptake (Table 4).

It has been shown that countries using school-based vaccination programs have the most success in uptake. Countries such as Australia, United Kingdom, and Portugal have achieved coverage rates as high as 80%[52]. Denmark has reached one of the highest vaccination rates (> 80%) through aggressive administration by general practitioners[52]. In Rwanda, government-mandated HPV vaccine coverage achieved over 90% coverage among teenage girls[59]. Programs that have achieved mass vaccination coverage rates have been able to show reduction in HPV viral prevalence in the form of high grade precancerous lesions and overall disease burden.

Vaccination strategies: Overall strategies to achieve mass vaccination continue to point towards a comprehensive approach. Continuing to raise awareness about cervical cancer and its relationship to HPV while addressing misconceptions and safety concerns to a wide range of audiences through education and health communication programs remains essential. All strategies should to be country-specific and take into account not only women, but communities, health professionals and delivery methods that provide the highest likelihood of exposure to the general public. In many developing countries, older children and adolescents are rarely routinely vaccinated or routinely evaluated by primary health care providers. New systems will need to be created including a focus on school-based immunization programs, and creating partnership programs focusing on adolescent health and sexual reproductive health programs.

CONCLUSION

Development of HPV vaccines has created opportunities to reduce cervical cancer rates and morbidity associated with other HPV related diseases. These vaccines have been found helpful in both countries with effective screening programs and those without. Financing for HPV vaccination programs will require involvement of global partners in both the private and public sector. Ongoing research regarding long term safety and efficacy of HPV vaccines will need to be evaluated in a variety of populations including those areas with high HIV prevalence. More information is needed regarding the duration of vaccine protection, long-term efficacy in males, potential need for boosters, and efficacy of two dose regimens in older girls that may reduce the overall costs of the vaccine. If uptake in vaccination increases worldwide, it may lead to increased possibilities of developing prevention and screening programs due to a subsequent decline in disease incidence.

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REVIEW

Human papilloma virus vaccination: Review article and an update

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Abstract

Human papilloma virus (HPV) is sexually transmitted and associated with uterine cervix, vaginal, and vulvar cancers in females, oropharyngeal and anal cancer in both genders, and penile cancer in males. Moreover, genital warts are benign tumors which are HPV-related and can occur in both genders. This is a review of HPV structure, HPV infection transmission, the global impact of HPV and its associated diseases, HPV vaccines and their efficacy and safety, public acceptance of HPV vaccines, the obstacles for its acceptance and strategies to address the barriers. Cervarix (a bivalent vaccine with protection against HPV types 16 and 18) and Gardasil (a quadrivalent vaccine with protection against HPV types 6, 11, 16 and 18) are 2 recommended vaccines. The longest follow up of 9.4 years has shown efficacy and protection of the vaccine against HPV types 16 and 18. The adverse effects have been minimal and the vaccine is considered safe. Numerous studies are conducted to follow the vaccinated individuals to better understand the effect of HPV vaccine on incidence of HPV-related cancers and precancerous lesions.

Key words: Human papilloma virus; Cervarix; Gardasil; Gardasil 9; Vaccine; Review

Core tip: Human papilloma virus (HPV) is sexually transmitted in both genders and it is a global issue. High risk HPVs are associated with a variety of cancers and low risk HPVs are associated with genital warts. HPV types 16 and 18 account for 70% of cervical cancer in women. Bivalent (Cervarix), and quadrivalent (Gardasil) vaccines are recommended to prevent HPV 16 and 18 related cancers with additional protective effect of Gardasil against HPV 6 and 11. Herein, HPV-related cancers and their incidences, low risk HPV related neoplasms and HPV vaccines, their efficacy and safety are reviewed. Moreover, the obstacles for global vaccination are addressed.

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INTRODUCTION

Human papilloma virus (HPV) is associated with cervical, vaginal, and vulvar cancer in females, oropharyngeal and anal cancer in both genders, and penile cancers in males[1-3]. HPV infection is underlying cause of cervical precancerous lesions including cervical intraepithelial neoplasia grade 2 (CIN Ⅱ) and 3 (CIN Ⅲ), and cervical adenocarcinoma *in situ*. HPV types 16 and 18 are the most common cause (64%) of HPV-associated cancers in both females (65%) and males (63%) in the United States[4] while HPV types 31, 33, 45, 52, and 58 account for 10% of HPV associated carcinomas in both females (14%) and males (4%)[5]. HPV 16 and 18 account for 66% and HPV types 31, 33, 45, 52, and 58 for 15% of cervical cancers[4]. Cervarix and Gardasil are two recommended vaccines for protection against HPV-related cancers. There have been barriers regarding HPV vaccination acceptance and its accessibility. This is a review of HPV, HPV vaccines, and the issues related to the vaccines.

HPV

HPVs are small, circular, double-stranded, non-enveloped icosahedral DNA viruses. HPV genome contains 6800 to 8000 base pairs coding for early (E), and late (L) functions. E1 and E2 regulate viral DNA replication, and E2 regulates viral RNA transcription. E4 regulates cytoskeleton reorganization. Finally, E5, E6, and E7 regulate cell transformation. L1 and L2 are structural component of the viral capsid. E2 is the main regulator and regulates other viral genes. This is particularly true about E6 and E7[6]. HPV life cycle requires basal layer of epidermal or mucosal epithelial cells that is still able to proliferate. This usually occurs by micro-laceration of the skin or mucosa. During infection, HPV DNA genome integrates into the host DNA, resulting in the disruption of the *E2* gene and inability of late genes to express[7]. Loss of E2 leads to up-regulation of *E6* and *E7* genes, which results in high expression of E6 and E7 proteins, genomic instability, and disruption of cell cycle. The infected cells divide and the infection spreads laterally. Following entry into the suprabasal layers, viral DNA is replicated, late viral genes are activated, and capsid proteins are formed. Viral particle formation are completed and the viral particles are released at the epithelial or mucosal surface, which may cause additional tissue infection then[6]. E5, E6, and E7 pose proliferation-stimulating activity. E5 is important at the early course of infection preventing apoptosis. E6 and E7 are oncogenes and play an important role in malignant transformation of the infected cells. E6 inhibits the tumor suppressor activity of P53 and E7 inhibits activity of RB. Only E6 and E7 of high risk HPV types are immortalized human cells[6,8,9].

HPV INFECTION TRANSMISSION

HPV has an affinity to infect either anogenital and oral mucosa (α-papillomaviruses) or skin (β- and γ-papillomaviruses)[10]. Infection is commonly transmitted by sexual contact including vaginal, rectal sex and initially the pathologic changes in squamous epithelial cells are inconspicuous[11]. The age at first sexual intercourse, number of sexual partners, smoking, the age of the woman and her partner, and male circumcision are important risk factors for HPV transmission[12]. Open mouthed kissing and oral sex are associated with oral HPV infection[13-15]. Most of the infections are cleared by 6 to 12 mo after appearance, which is probably due to immunologic response to infection.

HPV AND ITS GLOBAL IMPACT

HPV, by far, is one of the most common viral sexually transmitted diseases. At least 50% of adult population is infected with this virus during their lifetime[16-19]. The prevalence of HPV in cervical cancer has been estimated 85%-99% worldwide[20]. Cervical cancer is reported as the third most common cancer in females, and overall it is the seventh most common cancer[21]. Screening for cervical cancer has significantly decreased the incidence of cervical cancer in developed countries while the results in developing countries have been only marginal. According to International Agency for Research on Cancer, developing countries account for greater than 85% of the global burden, where 13% of all female cancers occur[16,21]. The highest rates of cervical cancer are seen in: (1) Western; (2) Eastern Africa (age standardized rates greater than 30 per 100000); (3) Southern Africa (26.8 per 100000); (4) South-Central Asia (24.6 per 100000); (5) South America; and (6) Middle Africa (23.9 and 23.0 per 100000 respectively). The lowest rates of cervical cancer are seen in North America, Western Asia, and Australia/New Zealand (less than 6 per 100000). In South-Central Asia, Melanesia, and Eastern Africa, cervical cancer is still the most common cancer in women[16,21].

INCIDENCE OF HPV IN HUMAN IMMUNODEFICIENCY VIRUS PATIENTS

HPV-related neoplasms tend to occur at a younger age in human immunodeficiency virus (HIV)-infected patients and exhibit a more aggressive and advanced stage compared to those in HIV-negative patients[22,23]. In fact, infection with HIV was listed as an important risk factor for cervical cancer[24,25]. Low $CD4^+$ counts ($\leqslant$ 200 cells/μL) are the most significant independent predictor for infection with both high risk and low risk HPV genotypes[26]. Moreover, HIV-infected patients with genital warts show more resistance to standard treatment for genital warts and even relapse is more likely in HIV-positive women treated for cervical neoplasia compared to the healthy HIV-negative population[26-28]. Large cohorts from the United States and Europe demonstrated standardized incidence

ratios ranging from 5-10 for cervical cancer for HIV-infected women compared with the healthy general population[29-32]. Several studies from sub-Saharan Africa with limited generalizability have reported similar proportion of HPV 16 and 18 related cervical cancers in HIV-infected women *vs* general female population[33,34].

HPV ASSOCIATED DISEASES

HPV is the underlying cause of a large number of benign, precancerous, and malignant conditions in both females and males. HPV shows a predilection to the skin and mucosa. Harald Zur Hausen made the most significant contribution in this field. He described the strong association between HPV infection and cervical cancer for first time. Using cloning and sequencing methods, he identified HPV types 16 and 18 from cervical cancer[35].

HPV-associated cancers

HPV infection is associated with greater than 50% of infection-related cancers in females and 5% in males worldwide[36]. The most well-known HPV-associated cancer is cervical cancer. In anogenital area, HPV contributes to more than 99% of cervical squamous cell carcinoma and adenocarcinoma[1,37], 40% of vulvar cancers, 60% of vaginal carcinoma[38] and 97% of anal cancers[39]. Men having sex with men (MSM) and immunocompromised patients (HIV-infected patients and organ transplant recipients) are in greater risk for anal carcinoma[40]. HPV is associated with 45% of penile cancer[41]. In head and neck, HPV infection accounts for 47% of oropharyngeal cancers and 11% of oral and tongue cancers[42].

HPV-associated precancerous lesions

HPV infection is associated with high grade squamous intraepithelial lesions (SILs) of cervix (CIN Ⅱ, CIN Ⅲ)[43], vagina (VAIN Ⅱ, VAIN Ⅲ), vulva (VIN Ⅱ, VIN Ⅲ), and anus (AIN Ⅱ, AIN Ⅲ).

Oral lichen planus (23%), leukoplakia (63%), epithelial dysplasia[37,44], and erythroplakia (50%)[45] are considered precancerous disorders in oral cavity attributed to HPV infection. Common warts and flat warts of skin are also attributed to HPV infection[46]. Epidermodysplasia verruciformis is a complex disease which is associated with HPV infection[47].

HPV-associated benign lesions

Anogenital warts or condyloma acuminate, atypical squamous cell of uncertain significance, and low grade SIL of cervix are benign lesions associated with HPV in anogenital area. Oral squamous papilloma, verruca vulgaris (common wart)[46], recurrent respiratory papillomatosis[48], and focal epithelial hyperplasia (Heck disease) are benign HPV-associated lesions in head and neck area.

HPV SUBTYPES AND THEIR ASSOCIATED DISEASES

The HPV are classified into low-risk and high-risk HPV types according to their oncogenic potential[49]. HPVs 16 and 18 are the most common high-risk HPV types causing anogenital and oropharyngeal cancers in both females and males. The most common low-risk HPVs are types 6 and 11, causing genital warts and laryngeal papilloma[50]. Worldwide, the overall HPV prevalence in cervical cancer is greater than 99%[1]. Cervical cancer is associated with several types of HPV. The most common type is HPV 16 (61%), followed by HPV 18 (10%), HPV 45 (6%), HPV 31 (4%), HPV 33 (4%), HPV 52 (3%), HPV 35 (2%), and HPV 58 (2%)[1,20]. HPV 16 and 18 are also the most common HPV types detected in cervical cancer biopsies in HIV-infected patients[51].

HPV 16 and HPV 18 are the most common HPV types associated with vulvar (HPV 16, 32% and HPV 18, 4%)[38], vaginal (HPV 16, 54%, HPV 18, 8%)[38] anal cancer (HPV 16, 75%, HPV 18, 3%)[39]. HPV types 16 (60%), and 18 (13%) are the most common HPV types associated with penile cancer in males followed by HPV-6/11 (8.13%), HPV-31 (1.16%), and HPV-45 (1.16%)[41].

HPV 16 (45%), HPV 31 (9%), HPV 33 (7%), HPV 18 (7%), HPV 58 (7%), HPV 52 (5%), and HPV 35 (4%) are the most common HPV types detected in cervical high grade SILs[43].

HPV 16 (9%), HPV 6/11 (5%), HPV 31 (4%), HPV 33 (2%), and HPV 18 (2%) are the most common HPV types detected in low-grade SILs of the cervix[37,52].

Among benign anogenial lesions, HPV 6 (89%) and HPV 11 (11%) have been associated with condyloma acuminate[53,54].

In head and neck, HPV 16 is strongly associated with oropharyngeal squamous cell carcinoma and oral cavity carcinoma accounting for 90% and 96% of cases, respectively[42]. HPV types 6 and 11 are associated with recurrent respiratory papillomatosis[48], and oral squamous cell papilloma[55].

HPV vaccines

Targeting L1 and L2 proteins of HPV, major and minor capsid proteins, respectively, is the current strategy for the development of vaccines that are safe and effective. The expression of recombinant L1 in various hosts such as insect[56,57], yeast[58], bacteria[59] and even mammalian cells[60,61] generate virus-like particles (VLPs), which are similar to native virions both morphologically and immunologically. Studies have shown that L1 VLPs induces high titers of neutralizing serum antibodies, particularly immunoglobulin G (IgG). In fact, L1 VLPs are immunogenic and protective against HPV infecting skin or mucosa[57,62-64]. A conducted clinical trial by Koutsky *et al*[65] showed 100% protection from the natural acquisition of persistent HPV16

infection in individuals vaccinated with HPV 16 L1 VLPs, formulated in the adjuvant alum, over an average of 17.4 mo. The adjuvant is incorporated into a vaccine in order to enhance or direct the immune response of the vaccine[66]. Other clinical studies confirmed the high rate of protection and efficacy of L1 VLP vaccines against persistent infection of the same HPV type infection[67-73]. HPV vaccine is not recommended during pregnancy.

Cervarix

Cervarix is a L1 VPL manufactured by GlaxoSmithKline Biologicals, based in the United Kingdom. It is a bivalent recombinant vaccine to prevent cervical cancer, high grade cervical intraepithelial neoplasia (CIN) grade 2 or worse, cervical adenocarcinoma *in situ*, and CIN grade 1 caused by HPV types 16 and 18. It was approved by the United States Food and Drug Administration (FDA) in 2009 for use in females 9 to 25 years of age. HPV types 16 and 18 account for 70% of all cervical cancers worldwide[74]. Immunization with Cervarix consists of 3 doses of intramuscular injection, 0.5-mL each, on 0, 1, and 6 mo[75]. Cervarix is produced and formulated in the proprietary Adjuvant System 04 (ASO4) using insect cells infected with recombinant baculovirus. ASO4 consists of aluminum hydroxide and TLR4 agonist monophosphoryl lipid A (3-O-desacyl-4′-monophosphoryl lipid A) (MPL). Cervarix is the first vaccine with MPL adjuvant, which is licensed by the FDA.

Syncope may occur immediately after receiving Cervarix. Therefore, it is recommended to observe the vaccinees for 15 min after administration of the vaccine. A tonic-clonic movements and other seizure-like activity might be associated with syncope which is usually transient. A supine or Trendelenburg position restores cerebral perfusion. Redness, swelling, and pain at the injection site are the most common local adverse reactions and are seen in greater than 20% of subjects. Fatigue, headache, myalgia, gastrointestinal symptoms, and arthralgia are reported as the most common general adverse effects. Severe allergic reactions, such as anaphylaxis, to any component of Cervarix is a contraindication[75].

Gardasil

Gardasil is a quadrivalent L1 VLPs recombinant vaccine manufactured by Merck and Co., Whitehouse Station, New Jersey, United States. It protects against cervical, vaginal, and vulvar cancers and precancerous lesions, and genital wart associated with HPV types 6, 11, 16, and 18[76]. HPV types 16 and 18 have demonstrated the strongest association between infection and cervical cancers and high grade SILs (CIN Ⅱ and 3), and they are responsible for 70% of all cervical cancers and CIN Ⅱ, and CIN Ⅲ[77]. HPV types 6 and 11 are associated with 10% of low grade SILs[78], and more than 90% of all genital warts[53,79].

Gardasil consists of four types of L1 VLPs produced in saccharomyces cerevisiae using recombinant DNA technology. The DNA-free VLPs are purified and adsorbed on a proprietary amorphous aluminum hydroxyphosphate sulfate adjuvant (225 mg per dose)[76]. Each dose is 0.5 mL containing 20, 40, 40, and 20 μg of VLPs for HPV types 6, 11, 16, and 18, respectively. The vaccine is administered by intramuscular injection as a three-dose regimen, at months 0, 2, and 6[76].

Gardasil 9

Gardasil 9 is a nonavalent recombinant human papillomavirus vaccine (by Merck and company, Kenilworth, New Jersey, United States) to prevent approximately 90% of cervical, vaginal, vulvar, and anal cancers caused by HPV types of 16, 18, 31, 33, 45, 52, and 58 and to prevent genital warts associated with HPV types 6 or 11. Compared to Gardasil, Gardasil 9 covers five additional HPV types including 31, 33, 45, 52 and 58, which account for approximately 20% of cervical cancers. The vaccine was approved by FDA for use in females ages 9-26 years and males ages 9-15 years in December 2014[80,81]. Gardasil 9 is administered as intramuscular injection in three doses. The combined follow up studies on females vaccinated by Cervarix, or Gardasil, and or Gardasil 9 confirm high efficacy of HPV vaccination in prevention of cervical precancerous lesions[81].

HPV VACCINATION IN HIV-INFECTED PATIENTS

The American Council of Immunization practices recommends HPV vaccination for HIV-infected patients ages 11-26 years. This recommendation is based on a few studies that evaluated the immunogenicity of the quadrivalent HPV vaccine in perinatally HIV-infected patients[82] and HIV-infected men[83]. A longitudinal, prospective, non-randomized, controlled, open-label clinical study on 46 HIV-infected adolescents and young adults ages of 13 to 26 years and 46 HIV-negative controls ages of 14 to 27 years was conducted to evaluate the long-term immunogenicity effect of Gardasil administered intramuscularly in three doses (0, 8 and 24 wk). Naive ($CCR7^+/CD45RA^+$) and central memory (CM) ($CCR7^+/CD45RA^-$) $CD4^+$ and $CD8^+$ T lymphocytes were diminished at 28 wk, whereas effector memory (EM) ($CCR7^-/CD45RA^-$) $CD4^+$ and $CD8^+$ T lymphocytes were increased. No differences were noted between HIV-infected patients and HIV-negative healthy controls[84].

Safety of HPV vaccination

A Danish study reported post-vaccination symptoms in 35 females following vaccination with Gardasil. The patients were older than general target population (23.3 ± 7.1 years), with a high (71%) to moderate (29%) level of physical activity prior to vaccination, and BMI of 22.1 ± 4.7 kg/m^2. The window period between vaccination

and onset of symptoms was 9.3 d. The symptoms included orthostatic intolerance (100%), postural orthostatic tachycardia syndrome (60%), nausea (94%), chronic headache (82%), fatigue (82%), palpitations (77%), reduced cognitive function (77%), skin changes for instance aggravation of acne (76%), intermittent tremor/myoclonic twitches (72%), neuropathic pain such as "burning", "a deep stabbing", or "jolts of electricity" starting distally, often in one limb, and then progressing proximally and often spreading to the contralateral side (68%), sleep disturbances described as new-onset insomnia and non-refreshing sleep (61%), and muscular weakness (61%). Symptoms were reported to appear after the first vaccination in 24%, after the second vaccination in 51%, and after the third vaccination in 25%[85]. Another study from the same center in Denmark reports more symptoms in addition to what they reported earlier in 53 females. Only symptoms that were experienced in more than 25% of the patients were presented. The mean age at symptom onset was 21.0 ± 7.4 years. Mean time between vaccination and onset of symptoms was 11.1 ± 12.5 d and symptoms were reported to appear after the first vaccination (40%), after the second vaccination (36%), and after the third vaccination (25%)[85].

The additional symptoms included visual symptoms such as new-onset hypersensitivity to bright colors and light (70%), and intermittent blurring of vision (83%), gastrointestinal symptoms including feeling bloated (77%); abdominal pain of varying character, intensity and location (70%); and changes in bowel habits (55%), dyspnea (66%), voiding dysfunction with respect to frequency, urgency, nocturia and incomplete bladder emptying (59%), limb weakness, mostly experienced as muscle weakness and confined to lower extremities (57%), vascular abnormalities described as intermittent changes in skin color to blue, red, pale or blotchy in the lower parts of the legs and in fingers and toes - the color changes were often accompanied by painful swelling of the involved limbs (51%), irregular periods in females who did not take oral contraceptive pills reported as hypermenorrhea and worsening of menstrual discomfort and pain (48%), SICCA symptoms including new-onset dry mouth (40%), and dry eyes (28%), and hyperventilation in 18%[86]. An earlier study in 2006[76] reported injection-site reaction (pain, swelling, erythema, and pruritus) and fever as the most common Gardasil-related adverse effects.

The most commonly reported side effects of Gardasil 9 in 13000 females and males were swelling, redness, and pain at the injection site, and headaches[80]. HPV vaccines are considered safe even for immunocompromised individuals including HIV-infected women because they do not contain any live pathogens[34,83,87].

Efficacy of HPV vaccines

Phase III trials were conducted to evaluate efficacy of HPV vaccines for both bivalent and quadrivalent HPV vaccines in young women[88]. All of the trials were relatively large, blinded, randomized, and controlled with at least 4 years of follow-up. The trials included 5500 to 18500 young women vaccinees with age range of 15-26 years (mean = 20 years). In the PATRICIA trial, the cohort-naïve analysis of the vaccinated young women showed 100% efficacy to protect against high grade CIN 3 related to HPV types 16 and 18[72,89]. Cervarix is reported a vaccine with a long term immunogenicity and efficacy in an 8.4-year follow-up after the first dose of vaccine[90]. Gardasil showed a high efficacy and protection against CIN 3 associated with HPV types 16 and 18 in the final intention-to-treat (ITT)-naïve analyses. Efficacy of quadrivalent HPV vaccine was greater than 95% against HPV 16 and 18 -related high grade vulvar intraepithelial neoplasia (VIN 2/3) or vaginal intraepithelial neoplasia (VAIN 2/3) and greater than 75% against genital warts in the ITT-naïve and ITT cohorts[91,92].

Immunogenicity of HPV vaccines

The immunogenicity of Cervarix and Gardasil are expected to vary due to their different adjuvants. ELISA (Enzyme-linked immunosorbent assay) is used to measure immune response for the bivalent HPV vaccine and competitive Luminex immunoassay (cLIA) for the quadrivalent HPV vaccine. The PBNA (pseudovirion-based neutralization assay) detects the neutralizing ability of the induced antibodies with highest accuracy[88]. The humoral response to Gardasil has been defined by total IgG and cLIA as a different method measuring immunogenity of the vaccine[90,93]. The cLIA is a sensitive assay and it measures specific IgG level corresponding to a specific neutralizing epitope on each of the four HPV types of Gardasil, while the total IgG assay is less sensitive and measures a non-specific and broad response to HPV vaccine. Applying the cLIA assay, 98.5%, 64.8%, 90.2%, and 95.5%, of vaccinated women remained seropositive to HPV 16, HPV 18, HPV 6, and HPV 11, respectively, at month 48 while the total IgG cLIA assay showed 100%, 96.7%, 100%, and 100% of vaccinated women remained seropositive to HPV 16, and HPV 18, HPV 6, and HPV 11, respectively, at the same time. These results probably explain the possible differences among results of HPV-vaccine studies due to using different serologic assays[88,93]. Both the humoral and cellular immunity are responsible for viral clearance and long-term protection by HPV vaccine[94]. Moreover, a study showed similar efficacy and immunogenicity of quadrivalent HPV-vaccine in HIV-infected young adults and adolescent and in HIV-negative controls[95].

Cost-effectiveness of HPV vaccines

Multiple studies were conducted in the Europe and the North America indicating cost-effectiveness of HPV vaccination. However, the comparison among these studies might be challenging due to different adopted models[96-104].

Long-term protection

An HPV-16 vaccine (monovalent) trial was conducted by Merck and a long-term follow-up showed that up to 86% of young women aged 16-23 years remained seropositive for anti-HPV 16 antibodies, for an average of 8.5 years and there were no breakthrough cervical disease cases[105].

Bivalent HPV vaccine protection was noted in women after 9.4 years follow-up. The efficacy of Cervarix was estimated 95.1% over the 9.4-year follow-up. All vaccinated women remained seropositive for both HPV types 16 and 18 and the serum antibody titers were several-times higher than naturally acquired levels. The safety of the vaccine was clinically acceptable[90,106].

A study in boys 10-18 years received 3 doses of bivalent HPV vaccine showed a protection against HPV 16/18 after 24 mo of follow-up. Importantly, post-vaccination antibody titers against both HPV 16 and 18 were up to three times higher in boys than in young women and antibody levels against HPV 16 and 18 were four- and two- fold higher at month 2. A previous study reported a higher levels of antibodies detected against HPV 16 and 18 in 10-18 or 10-14 year old boys compared with 15-25 years old women or 10-14 years old girls[107].

Women vaccinated with quadrivalent vaccine (Gardasil) showed protection against all four types of HPV 6, 11, 16, and 18 after a nine-year follow-up[71]. Using a total IgG Luminex immunoassay, seropositivity rates were 100%, 91%, 98%, and 96% respectively for HPV types 16, 18, 6, and 11 were at nine years[88,91].

The efficacy of quadrivalent HPV vaccine to prevent HPV-related infection and genital disease in males has been assessed in 16-26 years old healthy boys and young men and followed up for three years. The quadrivalent vaccine was effectively prevented HPV types 6 and 11 related genital warts in 89.4% of cases compared with placebo[108]. The efficacy of Gardasil to prevent HPV types 6, 11, 16, and or 18 related anal intraepithelial neoplasia (AIN) was assessed in healthy HIV-negative males who have sex with males over 36 mo. The overall efficacy of Gardasil in preventing HPV types 6, 11, 16, and or 18 related AIN (anal intraepithelial neoplasia) was 77.5% (95%CI: 39.6%-93.3%) in the vaccinated study population[109].

ISSUES REGARDING HPV VACCINES

HPV vaccines provide type-specific immunity against HPV infection and do not prevent cancer caused by other types of HPVs. The bivalent HPV vaccine has developed to protect against infection and anogenital diseases related to HPV types 16 and 18 and the quadrivalent HPV vaccine protects against infection and anogenital disease associated with HPV types 6, 11, 16 and 18, respectively. Therefore, Cervarix and Gardasil together can prevent approximately 70% of all cervical cancer cases. Studies support that the overall protection against cervical cancer with these two vaccines is probably close to 80% due to cross-reaction and close relation of HPV 16 and HPV 31 and HPV 18 and HPV 45[66,69,110].

The 15 most common types of HPVs causing cervical cancer worldwide, in order of high to low frequency, are 16, 18, 45, 31, 33, 52, 58, 35, 59, 56, 39, 51, 73, 68 and 66. HPV type distribution in cervical cancer varies depends upon geographic region. The most common types of HPV detected in cervical cancer in Europe and North America are HPV types 16 and 18. North Africa shows higher frequency of HPV 16 than average, South Asia of HPV 18, sub-Saharan Africa of type 45, and Central and South America of HPV 31. Although Cervarix and Gardasil together could presumably prevent 70% of cervical cancers worldwide, their preventive impact would be potentially higher in Europe, North America, and Asia with regard to the variable geographic distribution of HPV. It was postulated that a vaccine with protection against the seven most common HPV types would prevent approximately 87% of cervical cancers worldwide, with minimal geographic variation[111].

Overcoming these two issue, Gardasil 9, a non-avalent vaccine was developed to protect against infection with seven most common types of high-risk HPVs including 16, 18, 31, 33, 45, 52, and 58 and two types of low-risk HPVs including 6 and 11 to enhance protection against cervical cancer worldwide[78].

An alternative approach is the application of cross-protective antigen, for instance minor capsid protein L2 to induce cross-neutralizing antibodies[112-114]. Clinical trials are designed to assess the efficacy and safety of HPV L2 vaccines.

Currently, HPV vaccine is recommended for girls in most countries. Only the United States, Canada, Austria, and Australia offer HPV vaccine to both males and females. The debate regarding cost-effectiveness and universal vaccination remains open. Studies in boys who received HPV vaccines have shown higher titers of antibodies against HPV 16/18 in comparison to those in females. It can be argued that vaccinating boys might be more effective in defeating cervical cancer since the virus is sexually transmitted. The HPV infection may persist through MSM, even if all the girls are vaccinated. In addition, the incidence of HPV-related head and neck squamous cell carcinoma is on the rise, which might be another consideration for universal HPV immunization[115]. HPV-related carcinoma is a global burden that affects both genders, female in particular.

OBSTACLES OF ACCEPTANCE OF HPV VACCINE

Ethnicity, race, and income are considered important factors for acceptance and use of HPV vaccine in the United States[116]. Both incidence and mortality rate of cervical cancer are much higher among blacks, 25% and 95% respectively, and Latinos, 53% and 41% respectively, compared with whites[117]. Studies

show that there are significant differences among Blacks, Hispanic, and Whites in their age of first sexual experience, number of their sexual intercourses, and number of their lifetime partners. Having the first sexual experience before age 13 is reported to be more common in African-Americans (14%) and in Hispanics (7.1%), compared with Whites (3.9%)[118]. It is reported that Black teens (60%) have more often sexual intercourse compared with Latino (48.6%) and White (44.3%) teens. In addition, having four or more lifetime partners is more common in Black teens (24.8%), compared with Hispanics (14.8%) and Whites (13.1%)[118]. Therefore, the recommended age for HPV vaccination is 11-12 years in the United States since 6.2% of adolescent initiate their sexual activity prior to age 13 nationwide[118]. Despite the above facts, only 53% of the American female adolescents initiate the HPV vaccine and among them only 35% complete the HPV vaccine series[119]. Black mothers have expressed an interest in preventing cervical cancer and protecting their children after education about HPV vaccine and cervical cancer. They were concerned about: (1) lack of robust information; (2) long-term side effects; (3) vaccine being experimental; (4) challenges to completion of all three doses of HPV vaccine; (5) lack of health insurance, especially among the poor in Southern United States, and (6) language barriers and inaccurate information among foreign-born subgroups[120]. Concerns and issues for Hispanics were: (1) language barriers; (2) safety concerns; (3) not knowing where to get the vaccine; and (4) lack of health insurance, especially among the poor and undocumented immigrants. Asians' issues and concern were: (1) language barrier; (2) limited knowledge about HPV vaccine; (3) concerns about sexual activity, and finally (4) lack of health insurance, especially among the poor. The issues addressed above are mainly due to lack of mandatory school-based HPV vaccination programs in the United States. In general, vaccination in the United States is largely dependent on individual decision. Therefore, the overall HPV vaccination rate may be greatly influenced by factors such as personal awareness of HPV, health insurance status, race, and access to healthcare.

Vaccine cost is labeled as major obstacle for HPV vaccine acceptance in the Caribbean and Latin America, followed by public knowledge about cervical cancer as a health issue, political will, competition of HPV vaccine with other lifesaving vaccines, access to vaccine, and finally acceptance of the HPV vaccine among the high risk populations[121].

It is estimated that cervical cancer affects the lives of 37500 women in Europe every year[21]. Moreover, genital warts are considered one of the most common sexually transmitted diseases in Europe; and its incidence is reported even more than 10% in women from the Nordic countries including Denmark, Finland, Iceland, Norway, and Sweden[122]. The HPV vaccine cost and a lack of knowledge about advantages of vaccination were major barriers for vaccine acceptance. The main reason for vaccine acceptance was prevention of cervical cancer, followed by parental endorsement, providing financial support, personal experience of knowing someone with cancer and HPV vaccine recommendation by health-care professionals[123].

In Africa, knowledge gaps regarding HPV and cervical cancer, lack of access to HPV vaccine, and its cost were among major obstacles for receiving HPV vaccine[124,125]. Over 50% of cervical cancer patients worldwide are from the Asia Oceania region[21]. The obstacles for receiving vaccines in these countries are its cost, followed by access to vaccines, and acceptance of HPV vaccination by the public[126].

STRATEGIES FOR THE FUTURE

Vaccine cost is considered as a major barrier worldwide. Finding ways to lower the cost of vaccine production can overcome this main obstacle. Public awareness of HPV and its association with cervical cancer and other related cancers and genital warts is the second most important step to enhance HPV vaccine acceptance. Willingness by local governments to support HPV vaccination and to facilitate public access to vaccine will increase the vaccine acceptance globally.

CONCLUSION

HPV-related cancers, cervical cancer in particular, is a global health concern with a high burden on developing countries. Migration, changes in sexual behavior, discovery of HPV in a subset of head and neck cancers and of high percentage of anal cancers, increase in travel rate and having more global life are other reasons to act globally for HPV vaccination in both boys and girls. Meanwhile, more studies should be conducted toward producing low-cost vaccines with long time efficacy and minimal or no adverse effects. Public education will enhance vaccine acceptance.

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REVIEW

MicroRNAs: New players in endometriosis

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Abstract

Endometriosis is an estrogen-dependent inflammatory disorder that limits the quality of life of affected women. This pathology affects 10% of reproductive-age women, although the prevalence in those patients experiencing pain, infertility or both is as high as 35%-50%. Endometriosis is characterized by endometrial-like tissue outside the uterus, primarily on the pelvic peritoneum, ovaries and the pouch of Douglas. Despite extensive research endeavours, a unifying theory regarding the exact etiopathogenic mechanism of this high prevalent and incapacitating condition is still lacking, although it has been suggested that epigenetics could be involved. MicroRNAs (miRNAs), one of the epigenetic players, are small non-coding RNAs that can act as post-transcriptional regulators of gene expression, reducing the expression of their target mRNAs either inhibiting its translation or promoting its degradation. MiRNA expression profiles are specific of tissue and cell type. Abnormal miRNA expression has been described in different pathological conditions, such as a myriad of oncological, cardiovascular and inflammatory diseases and gynecological pathologies. In endometriosis, miRNA expression patterns of eutopic endometrium from patients and control women and from different endometriotic lesions have been described. These small non-coding molecules have become attractive candidates as novel biomarkers for an early non-invasive diagnosis of the disease, which could suppose a valuable benefit to the patients in terms of improvement of prognosis and reduction of the ratio of recurrence. In this systematic review we will focus on the role of miRNAs in the pathophisiology of endometriosis.

Key words: MicroRNAs; Endometriosis; Epigenetics;

Angiogenesis; Biomarkers

Core tip: Endometriosis is an estrogen-dependent inflammatory disorder that limits the quality of life of affected women. Nowadays, a unifying theory regarding its exact etiopathogenic mechanism has not been achieved yet. Our objective is to review the current literature to better understand the role of microRNAs, one of the epigenetic players, in the pathophisiology of endometriosis and their potential as novel diagnostic biomarkers to guide therapeutic interventions in endometriosis.

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INTRODUCTION

Endometriosis is an estrogen-dependent inflammatory disorder that limits the quality of life of affected women[1-3]. This pathology affects 10% of reproductive-age women, although the prevalence in those patients experiencing pain, infertility or both is as high as 35%-50%[4]. The prevalence of this condition is estimated around 176 million worldwide, with an average diagnostic delay of 7 years[5], being the mean age at diagnose 32.5-36.4 years, depending of the study population[5,6].

Endometriosis is characterized by endometrial-like tissue outside the uterus, primarily on the pelvic peritoneum, ovaries and the pouch of Douglas. These extrauterine lesions are responsible for the main symptoms, pelvic pain and infertility[1].

Despite extensive research endeavours, a unifying theory regarding the exact etiopathogenic mechanism of this high prevalent and incapacitating condition is still lacking. Several authors have reported a hormonal, immunity and a genetic base for this gynecological disorder. However, a growing body of evidence suggests that epigenetics could also be involved[7], with an exponential increase of papers published on this issue in recent years.

Epigenetics refers to the study of mechanisms that control gene expression in a potentially heritable way without affecting DNA sequence. MicroRNAs (miRNAs), DNA methylation and modifications of the chromatin structure represent the different types of the known epigenetic modifications, exerting their regulatory effect additively[8]. In this review we will focus on the role of miRNAs in the pathophisiology of endometriosis.

MiRNAs are small (19-22nt) non-coding RNAs that can act as post-transcriptional regulators of gene expression, reducing the expression of their target mRNAs either inhibiting its translation or promoting its degradation. MiRNAs usually regulate gene expression by binding to the 3' UTR (Untranslated Region) of their target mRNA. Importantly, several miRNAs can target a given mRNA and a single miRNA can target several mRNA, increasing the complexity of the regulatory mechanism mediated by these molecules[9-13]. In malignancies, miRNAs can act as oncogenes or tumor suppressors, depending on their targets[14-16]. It is important to highlight that the miRNA expression profiles are specific of tissue and cell type[9]. To date, more than 1881 miRNA precursors, coding for more than 2500 mature miRNAs have been described in humans[17].

MiRNAs were first described in 1993 by Lee *et al*[10] in the worm *Caenorhabditis elegans*. Since then, studies about biogenesis, functions, roles and characterisation of the mechanism of action of miRNAs have grown considerably and nowadays they are considered as excellent biomarkers of some diseases such as coronary artery disease[18-20], cancer[21,22], and several gynecological pathologies, including endometriosis[23,24].

PERITONEAL FACTORS AND ENDOMETRIOSIS

Endometriosis is a multifactorial disease in which endometrial and peritoneal factors such as those related to angiogenesis and proteolysis may be involved[25-27]. Peritoneal fluid (PF) is a complex suspension containing large amount of macrophages as well as endometrial and red blood cells, small molecules diffused from plasma through the mesothelial wall and other components dependent on ovarian contribution and local secretion such as steroid hormones and growth factors, respectively[28]. Because ectopic lesions located in the pelvic peritoneum are completely submerged in this fluid, their components have emerged as an important field of study[28-31].

It is well documented that endometriosis is characterized by an important inflammatory process[32-34] with and increased production of reactive oxygen species (ROS)[35-37]. Berkes *et al*[38] and Santulli *et al*[39] have identified significantly increased levels of protein oxidative stress markers in the PF from women with deep infiltrating endometriosis when compared with endometriosis-free controls. On the other hand, NETosis describes the mechanisms by which activated neutrophils expel their entire chromatin, serving as catch and kill scaffold against microorganisms, a structure designated as neutrophil extracellular traps (NETs). Furthermore, it is known that ROS are the major activator of NETosis. The involvement of NETosis in endometriosis was studied Berkes *et al*[38], who observed the presence of NET formation in virtually half of the patients with endometriosis, primarily in the

stage Ⅰ and Ⅱ group and rarely in controls, suggesting that NETosis is implicated in the initiation of the disease.

The contribution of immune system disorders to endometriosis has been proposed by several authors[2,40-42]. In this context, macrophage migration inhibitory factor (MIF) is arousing growing interest. MIF is a major pro-inflammatory factor found elevated in PF from women with endometriosis. Apart from its effect on activating and inhibiting macrophage mobility, it is also considered a critical upstream activator of innate immunity. MIF may be required for ectopic endometrial tissue growth and progression of endometriosis lesions *in vivo*[43]. Interestingly, miR-451 has been postulated to target MIF[44]. By using a murine model, Nothnick and coworkers[45] concluded that disruption of miR-451 expression in endometrial tissue impairs the ability of this tissue to establish ectopically. These authors also found elevated expression levels of miR-451 and diminished of MIF in ectopic endometriotic lesions (mainly peritoneal lesions) when compared with matched eutopic tissue. In addition, *in vitro* luciferase assays corroborated MIF as a target of miR-451 and forced expression of miR-451 reduced MIF and cell survival. Consequently, the aforementioned authors hypothesized that miR-451 over-expresses in ectopic lesions in an attempt to curtail endometriotic lesion/cell survival[46].

MIRNAS IN ENDOMETRIOSIS

Abnormal miRNA expression has been described in different gynecological pathologies, including malignancies[47-49], bening conditions as leiomyoma[50], adenomyosis[51], and endometriosis[11,52-54]. Among gynecological tumors, ovarian cancer represents the second most prevalent and the most lethal malignancy in developed countries[55,56], what could be explained by the difficulty of its diagnosis at early stages and the lack of effective treatments[49]. Recently reviewed by Davidson *et al*[57], miRNAs could be an invaluable tool to overcome the above mentioned limitations, regarding their potential role in diagnosis and progression of ovarian carcinoma as well as prediction of response to chemotherapy. For instance, miRNAs of the miR-200 family, the miR-199/14 cluster and the let-7 paralogs have emerged as potential therapeutic targets in ovarian cancer[49]. In addition, Lee *et al*[58] found that higher expression of miR-181d, miR-30c, miR-30d, and miR-30e-3p was associated with significantly better disease-free or overall survival in this condition. These both miR-30 and miR-200 families have also been associated with endometrial cancer, the most frequent gynecological malignancy[56,59]. In a recent work, Kong *et al*[59] reported miR-30c to be a tumor suppressor *via* the miR-30c-MTA-1 signaling pathway, with a decreased expression of this miRNA in tumor cells.

Regarding endometriosis, miRNA expression patterns of eutopic endometrium from control women and patients[53,60] and ectopic lesions from patients have also been described[11,53,61]. Although endometriosis is a benign condition, it shares common mechanisms with tumors (*e.g.,* tissue invasion, inflammation, reduced apoptosis and aberrant angiogenesis)[55]. In this context, the relationship between endometriosis and ovarian cancer, specially endometrioid and clear cell ovarian carcinoma, has been long reviewed[62-66], but recent literature on this issue points that existing data is not enough to establish a doubtless causality[55].

Among the pioneering studies addressing the miRNA expression patterns in endometrial and endometriotic tissues was the work published by Burney *et al*[67]. Four endometrial samples from women with endometriosis and three from endometriosis-free women in the early secretore phase of the menstrual cycle were assessed for miRNA expression by means of microarray analysis. After real time quantitative polymerase chain reaction (qRT-PCR) validation, the authors reported a downregulation of four miRNAs (miR-34c-5p, miR-34b*, miR-9 and miR-9*) belonging to two miRNA families (miR-34 and miR-9, respectively) in the eutopic endometrium of women with endometriosis compared to endometrium from control women. Notably, members of the miR-34 family mediate the p53-dependent suppression of proliferation[68]

Furthermore, Laudanski *et al*[60] conducted a study enrolling 25 endometriosis-free women and 21 patients with ovarian endometriosis in the proliferative phase in which the expression of 667 human miRNAs was examined. Validation of array results led to the corroboration that miR-483-5p, a regulator of IGF2, and miR-629-3p, involved in inflammation, were downregulated in the eutopic endometrium of patients in comparison to controls. The authors pointed to the idea that dysregulation of these genes could contribute to the overgrowth of endometrial tissue outside the uterus.

Human endometrium is a unique tissue that undergoes complex molecular, cellular, and functional changes on a cyclic basis under ovarian hormone regulation[69,70]. These changes are essential for uterine receptivity and can be grouped in three distinct phases: Proliferative, secretory and menstrual[71]. Thus, some authors hypothesized that miRNA expression could vary across the menstrual cycle[11,72]. For instance, Kuokkanen *et al*[72] showed that miRNA expression profiles of human endometrial epithelium were under hormonal regulation and, therefore, varied across the physiological phases of the menstrual cycle. Particularly, miRNAs targeting several cell cycle regulators were over-expressed in the midsecretory phase. Conversely, others have identified no effect on menstrual cycle phase on endometrial miRNA expression[11,53]. These discrepancies could be explained by the cell-type specificity in the response to sex steroid hormones of the human endometrium[72] and the different type of cellular populations studied in each study.

Filigheddu *et al*[61] described a set of miRNAs dif-

ferentially expressed in ovarian endometriomas in comparison to paired eutopic endometrium. By means of microarray technology, 84 significant differently expressed miRNAs were identified. In addition, the use of bioinformatic tools allowed researchers to identify the predicted targets of these dysregulated miRNAs, as well as the molecular networks and the biological function they affected. Interestingly, one of the most significantly up-regulated miRNAs was found to be miR-202-3p. In a recent report[53], our research group corroborated these results, with a 200-fold over-expression of miR-202-3p in ovarian endometriomas in comparison to paired eutopic endometrium. With regards to miR-202-3p, it has been reported[73] that this miRNA targets the glioma-associated oncogene homolog 1 (GLI1) transcription factor, a strong positive activator of downstream target genes involved in proliferation, migration, invasion and angiogenesis, such as BCL-2, CD24, metalloproteinase-2 (MMP-2) and MMP-9[74]. GLI1 also regulates the transcription of vascular endothelial growth factor A (VEGF-A), which has been postulated as the main regulator of angiogenesis[74-77]. Thus, the over-expression of BCL-2 in the eutopic endometrium of patients with endometriosis[4,67] could be a consequence of the GLI1 regulation by miR-202-3p.

Using a Next Generation Sequencing approach, Hawkins *et al*[78] found 10 miRNAs up-regulated (miR-100, -193a-3p, -193a-5p, -202, -29c, -485-3p, -509-3-5p, -574-3p, -708) and 12 miRNAs down-regulated (miR-10a, -34c-5p, -141, -200b, -200c, -200a, -203, -375, -429, -449b, -504, -873) in ovarian endometriomas in comparison to control endometrium, suggesting that miRNAs could also play a significant role in these ovarian lesions. Interestingly, one of the most dysregulated miRNAs in ovarian endometrioma was miR-29c, in agreement with our own data[53].

Different miRNA profiles have been described in peritoneal lesions compared to paired eutopic endometrial tissues[11]. Through miRNA microarray analyses and *in silico* studies, the authors identified 22 differently expressed miRNAs that putatively regulated the expression of 673 differently expressed mRNA targets. Of them, 14 were up-regulated in peritoneal lesions (miR-1, -29c, -99a, -99b, -100, -125b, -125a, -126, -143, -145, -150, -194, -223, -365) and 8 were down-regulated (miR-20a, -34c, -142-3p, -141, -196b, -200a, -200b, -424) compared to paired eutopic endometrial tissue. Interestingly, the mRNAs targets of these miRNAs had been previously related to endometriosis-associated molecular pathways, including cell death, cell proliferation and angiogenesis[11].

More recently, Saare *et al*[79] identified five over-expressed miRNAs (miR-34c, -449a, -200a, -200b, -141) in peritoneal endometriotic lesions in comparison to eutopic endometria using a high-throughput miRNA sequencing approach. This set of miRNAs allowed the discrimination of peritoneal lesions from the healthy surrounding tissue. Finally, they concluded providing a note for caution when evaluating peritoneal lesions, due that analyses carried out in biopsies also containing healthy surrounding tissues could mask aberrant miRNA expression intrinsic of peritoneal endometriotic tissues.

Although efforts have been made in the identification of the role of miRNAs in the pathogenesis of endometriosis, we are aware that future research will provide new regulatory functions for known miRNAs and that new identified miRNAs will expand our knowledge of this condition. Hence, several authors are focusing on the discovery of new miRNAs associated with human female reproductive tract disorders. For instance, Creighton *et al*[80] performed a next generation sequencing of over 100 tissues or cell lines derived from human female reproductive organs in both healthy and pathological states. As a result, 7 confirmed and 51 highly confident predicted novel miRNAs were identified.

Even though the involvement of miRNAs in the pathophysiology of endometriosis requires further investigation, nowadays these small non-coding molecules are considered as putative biomarkers for an early non-invasive diagnosis of the disease, which could suppose a valuable benefit to the patients in terms of improvement of prognosis and reduction of the ratio of recurrence, as recently demonstrated in other miRNA regulated diseases[81-83].

ANGIOGENESIS-RELATED MIRNAS IN ENDOMETRIOSIS

The involvement of angiogenesis in the physiopathology of endometrisis has been long discussed, as the endometrial tissue migrated to the peritoneum requires a blood supply in order to survive, proliferate, invade the extracellular matrix and establish the endometriotic lesion[84]. VEGF represents one of the most potent angiogenic factors. Several studies have reported an increase in VEGF-A levels in endometriosis and it has been suggested that VEGF-A plays an important role in the progression of the disease[84,85]. Regarding angiogenesis inhibitors, alterations of thrombospondin-1 (TSP-1) expression has been reported to be involved in endometriosis, in which vascularisation is mandatory for the survival of migrated tissues[85].

In previous publications, our research group has found and up-regulation of the expression of angiogenic and proteolytic factors in endometrial tissue from patients with endometriosis[85,86] and we have suggested that this increase might contribute to the invasive potential of endometrial cells.

The miRNA regulation of angiogenesis has been long reported in several pathologies, including endometriosis[12,53,54]. The miR-17-92 cluster, also known as oncomir-1, encodes six mature miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a)[87] and has been reported to play an important role in the tumor neovascularisation[27]. Two miRNAs encoded in this cluster, miR-17-5p and miR-20a, have been found

to be down-regulated in ovarian endometriotic cysts in comparison to eutopic endometrium[11,61]. As miR-17-5p targets TSP-1, a decrease of the miR-17-5p levels in ovarian cysts could repress the down-regulation of TSP-1 expression and provide an explanation for the clinically observed low invasion grade of these endometriotic lesions to the surrounding ovarian tissue.

Extracellular matrix remodelling is a crucial process in the regulation of angiogenesis[88] and plays a critically important role in the establishment of the endometriotic lesion[89]. mRNA levels of key components of the metalloproteinase systems have been reportedly observed to be deregulated in eutopic and ectopic endometria of patients with endometiosis[85,86,89,90]. In a recently published paper[53], we observed that miR-29c-3p was up-regulated in several endometriosis tissues (ovarian endometrioma, peritoneal lesions and rectovaginal nodule). Provided that miR-29c-3p regulates different genes of the extracellular matrix, our results are in agreement with previously published studies[11,78] accounting for the coordinated role of several miRNAs in the remodelling process necessary for the implantation of migrated endometria in ectopic locations and the establishment of endometriotic lesions.

Taking into consideration the importance of angiogenesis in the pathophisiology of endometriosis, several therapies targeting VEGF as blockers or inhibitors have been proposed aiming to decrease the number of lesions, inhibit growth and reduce vascular density. In this context, soluble truncated VEGF receptors (Flt-1), antibodies to human VEGF and bevacizumab[91], among others, have been tested in murine models of endometriosis. Although results from these studies are promising, it should be taken into account that the use of an animal model that neither menstruate nor develop spontaneous endometriosis is a major limitation. Furthermore, the use of molecules that might block the expression or mimic functions of angiogenesis-related miRNAs could represent new therapeutic approaches in the treatment of endometriosis as recently demonstrated in other miRNA-regulated diseases[92].

CLINICAL UTILITY OF MIRNAS AS BIOMARKERS OF ENDOMETRIOSIS

Despite the fact that endometriosis is one of the most common benign gynecological diseases, there is a lack of non-invasive or semi-invasive diagnostic test that overcomes the need for the current surgical diagnosis[53,67]. Laparoscopy with histological confirmation, the gold standard for diagnosis, is a minimally invasive procedure. However, patients usually undergo general anesthesia and a certain degree of expertise from clinicians is necessary and it is a costly procedure. Additional concerns are related to the delay in the diagnosis of the disease, which has been estimated of around 7 years[32,93]. This may be due to multiple reasons including non-specific symptoms of the disease (pelvic pain and infertility), which leads to multiple tests for differential diagnosis[45]. As a consequence, patients are diagnosed at advanced stages of the disease, which impairs the prognosis and increases the risk of recurrence. For all these reasons, there is a great interest among researchers to find a non-invasive or semi-invasive test for the diagnosis of endometriosis that would ideally diagnose patients in initial steps of the disease and overcome the need for an invasive procedure.

Since they were first described to be present in blood[82], circulating miRNAs have become interesting biomarkers in different conditions[16,23,24,94,95]. The presence of miRNAs in different biofluids, including blood[96], could be explained by different mechanisms: (1) passive release of miRNAs from broken cells and tissues following tissue injury, chronic inflammation, cell apoptosis or necrosis, or from cells with a short half-life, such as platelets; (2) active secretion *via* cell-derived microvesicles (including exosomes and shedding vesicles); and (3) active secretion by cells as RNA-binding-protein conjugated complexes. Mechanisms (2) and (3) also offer an explanation for their highly elevated stability in plasma, despite the presence of elevated amounts of RNAses[97]. Although so far the biological functions of circulating miRNAs remain to be completely defined, some authors have proposed a role into cell-to-cell communication for these short nucleic acids[97-100]. In any case, it is clear that their presence in plasma/serum and the distinct advantages that they offer over other biomarkers (for instance and unlike mRNAs, miRNAs show high stability in blood, can be both amplified and detected with high sensitivity and specificity[101] and are highly resistant to storage handling[97,101]) offers an opportunity to use them as biomarkers.

In the field of gynecological pathologies, several authors have explored this possibility[94,102,103]. In ovarian cancer, miRNA expression profiles have been analyzed in whole blood and sera from patients, either as free-circulating miRNAs or encapsulated in exosomes. An example of the last is the study conducted by Taylor *et al*[95] in serum exosomes from patients with serous papillary adenocarcinoma of the ovary. Eight miRNAs (miR-21, -141, -200a, -200b, -200c, -203, -205, -214) were found to be up-regulated in tumor-derived exosomes compared with serum from benign ovarian disease patients. Interestingly, these 8 miRNAs showed a high correlation between their cellular and exosomal levels. In another study, published by Resnick and coworkers[104], 8 miRNAs were found to be deregulated (miR-21, miR-29a, miR-92, miR-93 and miR-126 up-regulated and miR-99b, miR-127 and miR-155 down-regulated) in serum obtained from 19 patients with epithelial ovarian carcinoma (serous, clear cell, endometrioid and mucinous) in comparison to miRNAs analysed in sera from 11 controls. Interestingly, three out of the five up-regulated miRNAs (miR-21, miR-92

Table 1 Current studies assessing the clinical utility of circulating miRNAs as biomarkers of endometriosis

Sample	Anticoagulant	Main results	Participants (patients/controls)	Ref.
Serum	-	↓ let-7b and miR-135[a] let-7d and let-7f showed a tendency towards down-regulation[a]	$n = 24/n = 24$[b]	[102]
Plasma	EDTA	↓ miR-17-5p, miR-20a and miR-22	$n = 23/n = 23$[c]	[103]
Serum	-	↑ miR-122 and miR-199a ↓ miR-9*, miR-141*, miR-145* and miR-542-3p[a]	$n = 60/n = 25$[d]	[106]
Plasma	EDTA	↓ miR-200a-3p, miR-200b-3p and miR-141-3p[a]	$n = 61/n = 65$[e]	[110]

Down-regulated (↓) and (↑) up-regulated miRNAs in samples from patients in comparison to control women. [a]Combination of miRNAs in bold yielded the best diagnostic value; [b]Control group presented dermoid cysts ($n = 10$), serours cystadenoma ($n = 5$), mucinous cystadenoma ($n = 3$), simple ovarian cysts ($n = 3$) and paratubal cysts ($n = 1$); [c]Control group presented uterine leiomyoma ($n = 14$), mature teratoma ($n = 4$), simple cysts ($n = 3$) and unexplained infertility ($n = 2$); [d]Main diagnosis: Infertility due to tubal factors; [e]Thirty-five endometriosis-free women with primary ($n = 10$) or secondary ($n = 15$) infertility, suspicion of endometriosis ($n = 5$), polycystic ovaries ($n = 3$) and pelvic pain ($n = 2$) and 30 self-reported healthy women. EDTA: Ethylenediaminetetraacetic acid.

and miR-93) were overexpressed in 3 patients with normal CA-125 levels. This finding could be explained by the high sensitivity and accurancy of the RT-PCR quantification, suggesting that miRNAs could provide an advantage as biomarkers in terms of sensitivity in comparison to those in current clinical use.

Häusler *et al*[105] analysed the miRNA expression profile in whole blood from 24 patients with epithelial carcinoma (mainly serous histotype) and from 15 healthy donors. As a result, the expression of miR-30c1* was found to be up-regulated and the expression of miR-181a*, miR-342-3p and miR-450b-5p down-regulated in patients in comparison to controls, enabling a discrimination between populations.

Regarding endometriosis, an interesting recent review from Fassbender *et al*[23] pointed to the possibility of developing a semi-invasive test for endometriosis from PF obtained *via* transvaginal ultrasound-guided aspiration. Although this is an interesting approach, current research is mainly focused on developing serum/plasma biomarkers as a noninvasive diagnotic tool. Jia *et al*[103] explored this possibility, conduct a study that enrolled 23 women with histologically proven endometriosis and 23 endometriosis-free controls. RNA from plasma was extracted to perform a miRNA microarray profiling. Three out of the six miRNAs selected for qRT-PCR (miR-17-5p, miR-20a and miR-22) were proven to be significantly down-regulated in patients and useful to discriminate women with endometriosis from patients. Wang *et al*[106] performed a circulating miRNA profiling with a different approach. For miRNA profiling, 2 pools of sera from 10 endometriosis patients and 10 control women, respectively, were prepared. Results from array were validated by qRT-PCR in sera from 60 patients and 25 control women, finding that miR-199a and miR-122 levels were up-regulated and miR-145*, miR-141*, miR-542-3p and miR-9* down-regulated in samples from patients in comparison to control women and could therefore serve as biomarkers of the disease. In a very recent study, Cho *et al*[102] quantified the levels of miR-135a,b and let-7a-f in sera of 24 endometriosis patients and 24 disease-free women. The selection of these miRNAs was based on their previous association with endometriosis[107,108]. Employing a logistic regression approach, researchers found that a combination of let-7b, let-7d and let-7f during the proliferative phase yielded the highest area under the curve value in discriminating patients with endometriosis from control women. Of note, several miRNAs were found to be differently expressed depending on the phase of the menstrual cycle in patients but not in controls, in agreement with previous reports[109]. Finally, Rekker *et al*[110] performed the last published study regarding circulating miRNAs as biomarkers of endometriosis. Based on previous literature, authors selected 3 miRNAs from the miR-200 family (miR-200a-3p, miR-200b-3p and miR-141-3p) whose expression was assessed in plasma samples from 61 patients and 65 control women. The expression of all 3 miRNAs was down-regulated in patients and miR-200a-3p and miR-141-3p showed the highest potential as noninvasive biomarkers for this benign condition. Remarkably, authors also analyzed variations of the levels of the three miRNAs of interest with time of sampling (morning/evening) finding lower levels in evening samples, perhaps due to circadian fluctuations in their expression. This is an interesting approach and points to the time of sampling as an important factor to be taken into account when performing circulating miRNAs studies. All these studies on the role of circulating miRNAs as biomarkers of endometriosis are summarized in Table 1.

Importantly, it should be noted that the circulating miRNA pool is not a mirror of tissue miRNAs content[83,111] and that changes in tissue miRNA will not be reflected in the same extent in the circulating miRNA profile[101]. Therefore, the aforementioned differences in endometrial miRNA expression profiles found in endometriosis should be considered in the context of a semi-invasive diagnosis of endometriosis by means of endometrial biopsy, because of the low probability of finding such differences in serum or plasma from the same patients.

CONCLUSION

MiRNAs, one of the epigenetic players, are small non-coding RNAs that can act as post-transcriptional regulators of gene expression reducing the expression of their target mRNA. The involvement of miRNAs in

different pathological conditions has been well established and miRNA expression profiles have been performed in biopsies from different conditions, including gynaecological pathologies as endometriosis. Despite being a benign gynaecological pathology, endometriosis deeply impairs the quality of life of affected women in terms of pain and infertility. The prevalence of endometriosis in reproductive-age women is estimated around 1 out of 10 and raises to 5 out of 10 in patients experiencing both pain and infertility. Research endeavours are being conducted in order to find a non-invasive or semi-invasive biomarker of the disease that ideally diagnosis the disease at initial stages and overcomes the need for the current laparoscopy gold standard diagnosis. In this area, circulating miRNAs have emerged as attractive molecules to be considered as biomarkers. Up to date, only few studies have been performed in order to obtain a circulating miRNA-based diagnostic tool. However, differences in experimental design among them make it difficult to compare results. From our point of view, there is a need for standardization of clinical data annotation, sample collection and handling among research projects that takes into account several aspects: (1) surgical and non-surgical data; (2) type of sample (serum/plasma) and processing protocols. In the case of plasma, the choice of anticoagulant is not a minor feature in experimental design and must be carefully addressed; (3) time of sampling is also an important factor and a decision has to be made between morning fasting samples or evening samples, as demonstrated by Rekker *et al*[110]; and (4) number of participants in circulating miRNAs as biomarkers of endometriosis studies is scarce and usually control population is heterogeneous, including self-reported endometriosis-free women, patients with different benign gynaecological conditions and infertile women due to tubal factors. For all these reasons, we encourage researchers in the field to follow recommendations from the World Endometriosis Research Foundation[112-115] in order to solve the observed heterogeneity in experimental designs and improve reproducibility between studies. In addition, validation of experimental algorithms in different cohorts is needed so as to improve quality of research and reach the ultimate goal, benefit patients with an earlier diagnose of endometriosis and avoiding unnecessary assisted reproductive techniques in those women whose fertility is not affected by the disease. To achieve this ambitious objective, we do encourage researchers to collaborate and synergistically add efforts to be able to recruit larger cohorts of patients and endometriosis-free women for circulating miRNAs studies, adopt standardized protocols and improve research outcomes.

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REVIEW

Pathobiological role of MUC16 mucin (CA125) in ovarian cancer: Much more than a tumor biomarker

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Abstract

MUC16 (CA125) has remained the mainstay for ovarian cancer assessment and management since the early 1980's. With the exception of HE4, it is the only reliable serum biomarker for ovarian cancer. MUC16 belongs to a family of high-molecular weight glycoproteins known as mucins. The mucin family is comprised of large secreted transmembrane proteins that includes MUC1, MUC4 and MUC16. These mucins are often overexpressed in a variety of malignancies. MUC1 and MUC4 have been shown to contribute to breast and pancreatic tumorigenesis. Recent studies have uncovered unique biological functions for MUC16 that go beyond its role as a biomarker for ovarian cancer. Here, we provide an overview of the literature to highlight the importance of MUC16 in ovarian cancer tumorigenesis. We focus on the growing literature describing the role of MUC16 in proliferation, migration, metastasis, tumorigenesis and drug resistance. Accumulating experimental evidence suggest that the C-terminal domain of MUC16 is critical to mediate theses effects. The importance of MUC16 in the pathogenesis of ovarian cancer emphasizes the need to fully understand the signaling capabilities of MUC16 C-terminal domain to develop more efficient strategies for the successful treatment of ovarian cancer.

Key words: MUC16; CA125; Mucin; Ovarian cancer; Tumorigenesis; Biomarker

Core tip: MUC16/CA125 has been a mainstay biomarker for ovarian cancer but its pathobiological role has remained mostly unknown. Recent literature has shown that MUC16 is much more than a biomarker. MUC16 has oncogenic properties and plays an important role in tumorigenesis. Here, we will review the current knowledge regarding the oncogenic role of MUC16.

Piché A. Pathobiological role of MUC16 mucin (CA125) in ovarian cancer: Much more than a tumor biomarker. *World J Obstet Gynecol* 2016; 5(1): 39-49 Available from: URL: http://www.wjgnet.com/2218-6220/full/v5/i1/39.htm DOI: http://dx.doi.org/10.5317/wjog.v5.i1.39

INTRODUCTION

Cancer antigen 125 (CA125) is the most widely used and the best characterized biomarker for the management of epithelial ovarian cancer (EOC)[1-16]. CA125 was initially identified in 1981 as an antigen present in the serum of patients with EOC[17,18]. However, it was not until 2001 that the gene was cloned and identified as MUC16[19-22]. It was later realized that the CA125 antibody recognizes a repeating epitope located in the extracellular domain of MUC16[5,19,20]. For the purpose of this review, the term CA125 will be used to designate the cleaved extracellular domain that can be found in serum of EOC and the term MUC16 will designate the full length mucin glycoprotein. For CA125 to be detected in the serum of EOC patients, the MUC16 glycoprotein undergoes a proteolytic cleavage resulting in the release of most of the extracellular domain from the cell surface. Serum levels of CA125 are routinely used in clinic to monitor patients with EOC[2,7,9,10,12,13,15,18]. There is indeed a strong correlation between rising and falling levels of serum CA125 with the progression and regression of the disease. Although the MUC16 mucin may be expressed in other cancers, its use in clinic has been mostly limited to patients with EOC.

Despite the fact that serum CA125 has been the mainstay of EOC assessment and management since the early 1980's, there are considerable gaps in our knowledge regarding the biological functions of MUC16 in normal physiology and particularly in oncogenesis. Not surprisingly, most of the early studies (before 2001) have focused on the clinical applicability of CA125 as a biomarker. However, the cloning of *MUC16* gene and the characterization of its structure, which revealed that MUC16 belong to membrane-bound mucins, has encouraged studies aiming to elucidate its physiological normal function as well as its role in malignant conditions. Because other membrane-bound mucins, such as MUC1 and MUC4, have oncogenic properties, it has been hypothesized that MUC16 may possess oncogenic capabilities as well. However, the very large size of the molecule (MUC16 is the biggest mucin identified to date) has hampered the characterization of MUC16 functional domains leading to an incomplete understanding of the physiological and pathological roles of MUC16. Nonetheless, in the last few years, a number of studies have begun to unravel the biological functions of MUC16. In particular, these studies have demonstrated that MUC16 possesses oncogenic properties. Here, we will review the current knowledge regarding the oncologic role of MUC16 in ovarian cancer. Our discussion will focus on cancer cells signaling, invasion and metastasis, and on regulation of drug-induced apoptosis. This review will also highlight gaps in our knowledge to provide a framework for future research studies.

CA125 AS A BIOMARKER

CA125 is the most widely studied serum biomarker for EOC. CA125 has been recognized as a tumor-associated antigen based on the characterization of the OC125 monoclonal antibody raised against the human ovarian cancer cell line OVCA433 in 1981[17,18]. This antibody detected a molecule that was named CA125 in the serum from patients with EOC[17]. It was later realized that CA125 is an epitope located on a repeated extracellular domain of MUC16 mucin[5,19,20]. Measurement of serum CA125 is an important part of the clinical management for EOC patients[2,10]. Its clinical utility has been evaluated as a screening test for the early detection of EOC, to distinguish benign diseases from malignant conditions, and to monitor EOC progression and regression following treatment.

The early detection of EOC remains a clinical challenge and minimal progress has been achieved to detect early diseases, which are often clinically asymptomatic, at a more curable stage. The use of serum CA125, as a single biomarker, for the early detection of EOC is tempered by several factors. First, MUC16 glycoprotein may be expressed in various malignant and non-malignant conditions. MUC16 is expressed at low levels in the normal airway epithelium and levels can increase in some chronic conditions such as cystic fibrosis[23-25] or vary with dexamethasone treatment[26]. MUC16 is expressed at the apical surface of the ocular and conjonctival epithelium where it is part of the glycocalyx protecting corneal cells from bacterial infections and dryness[27-30]. Immunohistochemistry of human tissues using the OC125 antibody detected MUC16 expression in fetal coelomic epithelia and its derivatives such as Müllerian duct, fallopian tube, endometrium, endocervix[8,14]. MUC16 is expressed by mesothelial cells of the peritoneum, the pleura and the pericardium[8,14]. Furthermore, CA125 serum levels can be elevated in various benign diseases including menstruation, first trimester pregnancy, endometriosis, adenomyosis, salpingitis, uterine fibroids, chronic renal failure or in inflammation of the pleura, peritoneum or pericardium[31-34]. However, MUC16 expression is not found in normal adult colon, rectum, cervix, small intestine, liver, pancreatic ducts, spleen, kidney, skin and ovaries[35]. Secondly, the expression of MUC16 in EOC tissues varies according to the histotype. MUC16 is expressed by 56% to 85% of serous, 65% of endometrioid, 40% of clear cell and 36% of undifferentiated adenocarcinomas of the ovary, but by only 12% of mucinous ovarian cancers[6,7,35]. Therefore, elevated levels of CA125 are most strongly associated with serous EOC subtypes and its expression is more limited in other subtypes. Consequently, the detection of serum CA125 is notably less sensitive for subtypes other than serous EOC. MUC16 is also detected in normal airways as well as in a small percentage of invasive breast carcinomas, lung carcinomas and pancreatic carcinomas[23,36-39]. Therefore, measurements of serum CA125 levels, as a single modality, have a limited utility for screening of EOC because of its lack of sensitivity and specificity.

The clinical utility of monitoring of serum CA125, as a screening test, has been evaluated by the ovarian component of The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial[40]. Results from this study showed that annual screening with CA125 and transvaginal ultrasound had no mortality benefit. Furthermore, the majority of the screen detected cancers presented in advanced stages. Another large ovarian cancer screening trial is currently ongoing, the UK Collaborative Trial of Ovarian Cancer Screening. The multimodal arm of this study utilizes the Risk of Ovarian Cancer Algorithm (ROCA) to predict the probability of EOC as a first-line screen[41]. The ROCA is based on the slope of serial serum MUC16 measurements. According to ROCA, patients are classified as low, intermediate and high risk of developing EOC. When the risk is intermediate or high, patients undergo a transvaginal ultrasound. Results of this trial are expected soon and it thus remains to be seen whether CA125 serum levels will be useful for screening of ovarian cancer in the general population.

Another potential application of CA125 serum monitoring, is its ability to distinguish benign gynecological conditions from EOC. However, as mentioned previously, the expression of MUC16 in a number of benign gynecological conditions limits the utilization of serum CA125 as a single biomarker. To overcome this limitation, a number of risk prediction scores have been developed to identify women that have high risk of EOC. Examples of risk prediction models include: (1) the Risk of Malignancy Index combines serum CA125 levels with ultrasound and menopausal status to identify women at high risk of having EOC[42]; (2) the Risk of Ovarian Malignancy Algorithm (ROMA), which uses menopausal status with CA125 and HE4 serum values[43-45]; and (3) OVA1, which uses CA125, β2-microglobulin, apolipoprotein A1, transthyretin and transferrin[46]. The use of ROMA added more accuracy for differentiating the benign and malignant conditions compared to CA125 alone. Other algorithms are being investigated and there is an intense effort to search for new potential biomarkers for EOC. Recently, the combination of serum CA125 with IL-6 ascites levels was evaluated for its ability to discriminate between benign conditions and EOC[47]. Despite encouraging data, further evaluation is needed to determine the clinical utility of these panels of markers.

The clinical utility of serum CA125 has been best validated for monitoring of therapy as well as detection of recurrence. Indeed, CA125 is the only biomarker currently recommended for the monitoring of therapy and for the detection of recurrent diseases. Elevated levels of serum CA125 are common in patients with advanced disease of serous histiotype (approximately 90%). It was shown by several groups that rising and falling levels of serum CA125 correlate with progression and regression of the disease and this formed the basis for monitoring CA125 serum levels for patient follow-up[14,15,18]. However, up to 20% of patients with advanced EOC have normal serum level of CA125. CA125 nadir serum values were associated with a significantly longer progression-free survival (PFS) and overall survival[9,13]. Pretreatment CA125 serum level is an independent predictor of PFS in patients with advanced EOC who received a standard chemotherapy regimen[16].

CLONING OF *MUC16* GENE

The cloning of the *MUC16* gene in 2001 revealed that MUC16 is a tethered mucin[19-22]. The deduced amino acid sequence of MUC16 demonstrated that it shares common features with other membrane-bounded mucins with high serine, threonine and proline contents. MUC16 therefore belongs to the family of membrane-bound mucins. Other membrane-associated mucins include MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17 and MUC20[48]. With a molecular weight of > 2 MDa, MUC16 is the largest membrane-bounded mucin known to date[21,22]. This glycoprotein is composed of a heavily glycosylated N-terminal domain, a large multiple repeat domain (up to 60 tandem repeats of 156 amino acids each), an ectodomain proximal to the putative cleavage site, a transmembrane domain (TM) and a small cytoplasmic domain of 31 amino acids[21] (Figure 1). MUC16 extracellular domain possesses much longer repeats (156 amino acids) as compared to MUC4 (16 amino acids) and MUC1 (20 amino acids). The N-terminal domain (12068 amino acids) and the repeat domain are heavily glycosylated with both O- and N-linked oligosaccharides[22]. In contrast to other membrane-bound mucins such as MUC1, which harbor a single sea urchin sperm protein, enterokinase and agrin (SEA) domain, MUC16 contains approximately 56 SEA domains (Figure 1)[49,50]. SEA domains consist of about 120 amino acids. Sequence analysis of MUC16 SEA modules showed that they display some sequence variability[50,51]. The second MUC16 SEA domain, proximal to the TM, however is relatively conserved and most closely resembles the SEA domain found in other mucins. Thus, MUC16 was speculated to contain a putative proteolytic cleavage site located in a SEA domain proximal to the membrane which could be involved in releasing the ectodomain[21]. Direct experimental validation has been lacking. However, a recent study has challenged this model. The investigators suggest that MUC16 cleavage occurs outside the proximal SEA domains and within the 12 extracellular amino acids proximal to the TM domain[52,53]. According to these authors, MUC16 cleavage takes place in the acidic pH of Golgi/post-Golgi compartments[54]. Notably, these authors did not found any evidence of intramembrane proteolysis with their constructs. The ectodomain of MUC16 has been reported to be released by metalloproteases [matrix metalloproteinase 7 (MMP-7), MMP-9, ZmpC] and neutrophil elastase[28,55]. ZmpC, a zinc metalloproteinase secreted by *S. pneumoniae*, selectively cleaves the ectodomain of

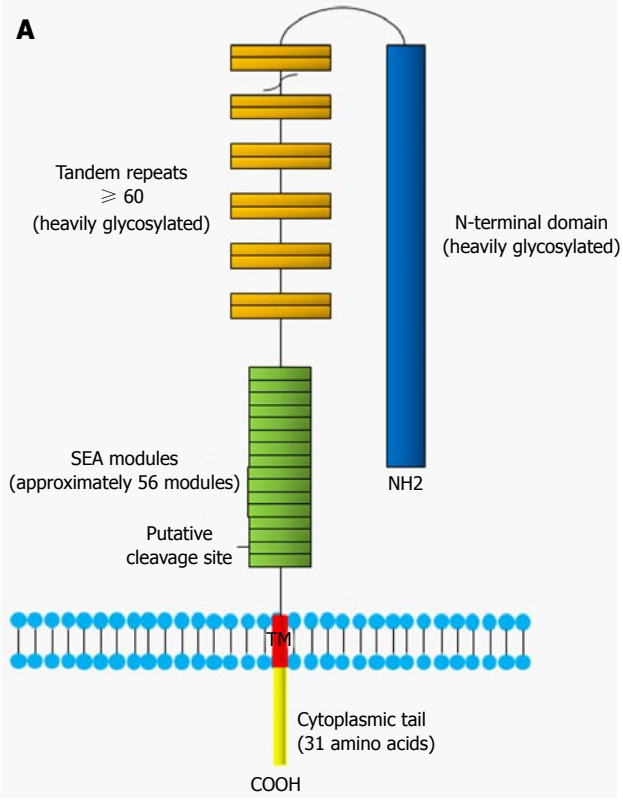

Figure 1 Schematic structure of MUC16 mucin. A: The major domains of MUC16 include the N-terminal domain, the tandem repeat domain and the C-terminal domain. The SEA modules contain a putative proteolytic cleavage site which divides MUC16 in two subunits. The extracellular larger subunit consists of the N-terminal (> 12000 a.a.) and tandem repeat domains (156 a.a. each), and are heavily glycosylated. The smaller subunit contains SEA domains, a transmembrane domain (TM) and the cytoplasmic tail (31 a.a.); B: MUC16 is usually expressed at the apical surface of normal epithelial cells. In EOC cells, this pattern of expression is lost and MUC16 is expressed through the entire surface of the tumor cells. The micrograph represents OVCAR3 cells probed with M11 antibody. SEA: Sea-urchin sperm protein, enterokinase and agrin; a.a: Amino acids.

MUC16 mucin from conjunctival and corneal epithelial cells[55]. The SEA domain is presumably the cleavage site for these proteases[28]. These findings contrast with that of Das *et al*[54] which showed that MUC16 cleavage was independent of neutrophil elastase and MMP-7. Post-translation modifications such as glycosylation can potentially regulate the release of MUC16 ectodomain and could explain these different findings. Intriguingly, mutation of Ser106Ala or Thr84/85Ala in the cytoplasmic tail did not affect the cleavage of MUC16[54]. According to these results, it would mean that there is no requirement for cytoplasmic cues for MUC16 extracellular cleavage. This is unexpected because previous studies showed that the phosphorylation of MUC16 cytoplasmic tail (Ser/Thr phosphorylation) has been associated with its secretion[56]. The secretion of MUC16 was also reported to be stimulated by epidermal growth factor (EGF) or tyrosine phosphatases[57]. Its shedding is decreased by glucocorticoids[26]. One limitation of the study by Das *et al*[54] is that, because of the lack of specific antibodies to MUC16 CT, they were not able to demonstrate cleavage of endogenous MUC16 in ovarian cancer cells to validate their observations, particularly in the context where most of the data were generated in non-ovarian cancer cell lines. Therefore, although MUC16 cleavage may transit in the Golgi before it reaches the cell surface, it is possible that its cleavage may also be triggered once the full length MUC16 is anchored in the cell membrane. The findings of Das *et al*[54], if confirmed, have important implications from a clinical standpoint. Since the cleavage of MUC16 appears to occur before it reaches the plasma membrane, antibodies raised against CA125 (extracellular portion of MUC16) may not be the most effective means to assess expression of MUC16 and could have been one of the reasons for the limited success in clinical studies of CA125 monoclonal antibodies[58-62].

Unlike MUC1 and MUC4, MUC16 lacks an EGF-like domain. Through their EGF-like motif located at C-terminal domain (extracellular portion), MUC1 and MUC4 bind to growth factor receptor tyrosine kinases (RTKs) such as erbB family and fibroblast growth factor receptor 3[63-66]. The formation of heterodimer with RTKs causes cross-phosphorylation of their respective cytoplasmic domain leading to the activation of various signaling pathways[67]. Because MUC16 lacks an RTK binding motif in its C-terminal domain, it is not clear whether MUC16-induced signaling is affected by RTKs although MUC16 extracellular domain shedding from the cell surface is stimulated by EGF. Consistent with the lack of an RTK binding motif, the intracellular interaction between MUC16 and β-catenin was not affected by EGF[68].

MUC16 31 amino acids long cytoplasmic tail (CT) contains serine/threonine/tyrosine residues that serve as potential phosphorylation sites. MUC16 CT also contains a polybasic sequence of amino acids (RRRKK), which is predicted to bind to the ezrin/radixin/moesin (ERM) family of proteins (Figure 2). This motif is not found in MUC1 and MUC4. The ERM proteins can interact with numerous membrane-associated proteins and the actin cytoskeleton. In line with this, MUC16 was recently shown to interact with E-cadherin and β-catenin,

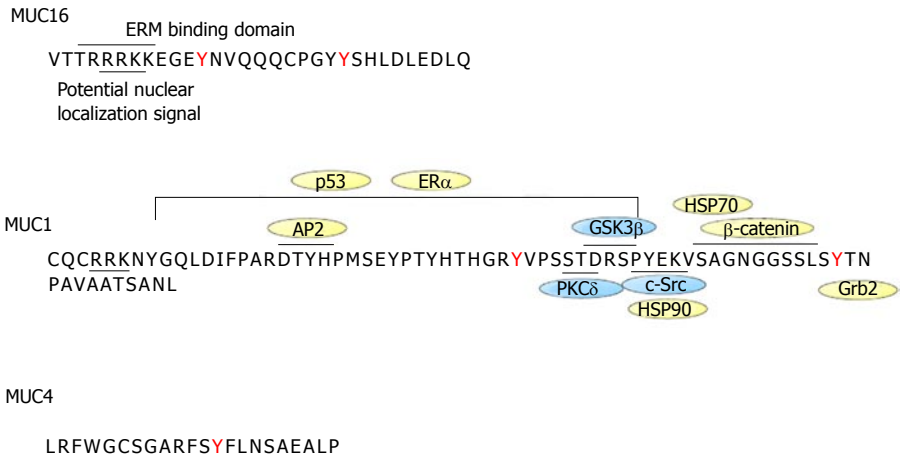

Figure 2 Sequence of MUC1, MUC4 and MUC16 cytoplasmic tails. The intracellular sequence of the different mucins is shown along with protein interaction sites. MUC1 is the best characterized mucin. MUC1 cytoplasmic tail interacts with c-Src, GSK3β, PCKδ, β-catenin, p53, ERα, HSP70/90, Grb2, AP-2. Proteins with kinase activity are in blue whereas those without kinase activity are in yellow. HSP70 binds to MUC1 cytoplasmic tail in the same region as β-catenin. HSP90 binding to MUC1 depends on c-Src-induced Y-46 phosphorylation. MUC16 cytoplasmic tail has an ERM motif for potential interaction with the cytoskeleton. Both MUC1 and MUC16 contain a potential nuclear localization signal motif. MUC4 has no known interaction binding partners. EMR: Ezrin/radixin/moesin.

causing alterations in the actin cytoskeleton[68]. It remains unclear however whether MUC16/β-catenin and MUC16/E-cadherin interactions are mediated through the ERM motif of the MUC16 CT. Janus kinases (JAKs), which are non-receptor tyrosine kinases, contain an ERM domain. Previous studies showed that MUC16 co-immunoprecipitates with JAK2 in breast cancer cells resulting in the activation of STAT3, which may be involved in breast cancer development[69].

MUC1 cytoplasmic tail has been shown to bind to β-catenin and a serine-rich SXXXXXSSL motif in MUC1 CT is responsible for this interaction *in vitro*[70-72]. This motif is notably absent in MUC16. Interestingly however, the binding of MUC1 to β-catenin in cells was independent of the serine-rich motif[72]. These observations suggest that MUC16 interaction with β-catenin is mediated by an indirect mechanism, probably through another protein.

The positively charged R-K rich region of MUC16 CT also constitutes a putative nuclear localization motif[67]. Up until recently, evidence were lacking to support nuclear localization of MUC16 CT. However, a recent study demonstrated that MUC16 CT is cleaved from the cell surface and co-localized with JAK2 in the nucleus[52]. MUC16 CT-mediated increased nuclear JAK2 leads to up regulation of LMO2 and NANOG, two proteins shown to induce stem cell-like properties emphasizing the signaling capabilities of MUC16's CT domain[52].

MUC16 AND TUMORIGENICITY OF CANCER CELLS

MUC16 is overexpressed by most serous EOC, by fallopian tube cancers and by cancers of the uterus[6,11,32,38]. Consistent with the role of MUC16 in tumorigenicity, overexpression of *MUC16 mRNA* and amplification of genomic regions encoding *MUC16* DNA have been observed in The Cancer Genome Atlas (TCGA) ovarian cancer project that included 316 cases of serous EOC[73]. However, MUC16 expression was not correlated with overall survival or resistance. MUC16 is also aberrantly expressed by several other malignancies, including cancer of the lung, pancreas and breast. Along with MUC16, overexpression of other membrane-bound mucins, such as MUC1 and MUC4, is common in breast and pancreatic cancers[74]. Interestingly, both MUC1 and MUC4 mucins have been shown to possess oncogenic properties and promoted proliferation, invasion and metastasis in pancreatic cancer models[75-77]. These observations suggested that MUC16 could play a role in ovarian cancer progression as well as in other cancers.

Indeed, there are now several studies demonstrating that MUC16 C-terminal domain enhanced proliferation, migration, invasion and tumorigenicity of ovarian, breast and pancreatic cancer cell lines. The first study demonstrating the tumorigenic-enhancing properties of MUC16 was published by Thériault *et al*[78] in 2011. In this study, the knockdown of MUC16 in MUC16 overexpressing EOC cell line OVCAR3 decreased tumorigenicity as shown by the decreased number of colonies in soft agar and the reduction of *in vivo* tumor growth. In the same study, the enforced expression of MUC16 C-terminal domain (last 284 C-terminal residues) enhanced soft agar colony formation and tumor growth in nude mice. Consistently, intraperitoneal injection of MUC16 C-terminal domain-expressing tumor cells significantly reduced the survival of SCID mice compared to those injected with vector-expressing controls[78]. Subsequently, Lakshmanan *et al*[69] showed that MUC16 knockdown in overexpressing breast cancer cell lines decreased cell proliferation and *in vivo* tumor growth. Similarly, Reinartz *et al*[79] demonstrated that MUC16 knockdown in ovarian and breast cancer cells was associated with decreased migration and invasion. In line with these observations, more recent studies showed that ectopic expression of MUC16 C-terminal

domain (last 114 C-terminal residues) enhanced proliferation, migration and *in vivo* tumor growth in pancreatic cancer cells whereas MUC16 knockdown reduced migration and invasion in these cancer cells[52,80]. Collectively, these studies clearly support a critical role for MUC16 in tumor growth and metastasis. Most importantly, expression of the C-terminal domain appears to be sufficient to mediate these effects.

The mechanism by which MUC16 enhances tumorigenicity of cancer cells remains poorly defined however. Although MUC16, as for MUC1 and MUC4, enhances tumorigenicity in cancer cells, the cytoplasmic tails of these three mucins are poorly conserved[50]. Size and amino acid sequence considerably vary suggesting a variety of functions in cell signaling. The ectopic expression of MUC16 C-terminal domain induces an epithelial to mesenchymal transition which has been associated with tumorigenesis and tumor progression[78]. Constitutive MUC16 C-terminal expression was associated with reduced E-cadherin expression which may enhance metastasis[81]. In addition, both Akt and ERK pathways may be activated by constitutive expression of MUC16 C-terminal domain[69,82]. The precise role these pathways was not however further investigated and therefore it is not clear whether their activation is essential to mediate MUC16 effects on tumor progression. However, Akt and ERK activation is frequently observed in serous OC and have been previously shown to be important for tumor progression[73,83,84].

Ectopic expression of MUC16 C-terminal domain in cancer cells has been associated with altered gene expression profile with increased expression of genes coding for proteins involved in invasion such as MMP2, MMP9, CXCL12 and CDH1[85]. MMPs are major proteolytic enzymes that are involved in cancer cell migration, invasion, and metastasis[86]. The CXCL12/CXCR7 axis plays an important role in ovarian cancer as it enhances cancer cell invasion by up-regulating MMP9 expression through the MAPK pathway[87]. MUC16 knockdown in breast cancer cells was associated with decreased expression of cyclin B1 and D1, both involved in regulation of cell cycle control[69]. The cytoplasmic domain of transmembrane mucins is usually embedded in the apical membrane of normal epithelial cells. However, upon transformation, the cytoplasmic tail of mucins such as MUC1 accumulates in the cytoplasm[88-90]. As mentioned above, there is evidence that MUC16 cytoplasmic tail gets cleaved and translocates in the nucleus[52]. However, whether this cleavage is required for MUC16 to enhance tumorigenicity remains to be determined.

MUC16 AND TRANSFORMATION

Membrane-bound mucins such as MUC1 and MUC4 possess transforming properties[91,92]. For example, when the C-terminal portion of MUC1 was stably transfected into 3Y1 fibroblast cells, soft agar colonies and subcutaneous tumors in nude mice were readily obtained[91]. Interestingly, MUC16 is not expressed by normal ovarian surface epithelial (OSE) cell but its expression is induced in transformed OSE cells[93]. These observations suggest that MUC16 may play an important role at early stages in the development of EOC. Two recent studies indeed confirmed the transforming properties of MUC16. Giannakouros *et al*[82] stably expressed MUC16 C-terminal domain (last 284 C-terminal residues) and a similar construct lacking most of the cytoplasmic domain in immortalized NIH3T3 mouse fibroblast cells using lentiviral vectors. It was observed that MUC16 C-terminal domain-expressing cells displayed enhanced anchorage-dependent and -independent growth and enhanced the formation of sub-cutaneous tumor nodules in athymic nude mice[82]. Notably, these effects were abolished by the deletion of the cytoplasmic tail. In line with these observations, Rao *et al*[85], using constructs containing either the last 114 C-terminal amino acids or last 344 C-terminal amino acids, showed that both constructs significantly enhanced the number of soft agar colonies compared to vector only in NIH3T3 cells. In addition, both constructs formed larger sub-cutaneous tumors relative to control NIH3T3 cells with no significant differences suggesting that the proximal extracellular part of the molecule has little effect on the oncogenic effects of MUC16. These studies establish that MUC16 is an oncogenic glycoprotein much like MUC1 and MUC4.

There are, however, conflicting data regarding the minimal functional domain of the C-terminal domain required for transformation. Although, the SEA domain appears to be dispensable for tumorigenicity, the respective role of the ectodomain and the cytoplasmic tail are more controversial. Rao *et al*[85] showed that ectopic expression in NIH3T3 cells of a construct containing the ectodomain (58 amino acids), the TM (22 amino acids) and the first 6 amino acids of the CT had transforming effects mostly similar to a construct carrying the full length CT. In contrast, Thériault *et al*[78] and Giannakouros *et al*[82] showed that deletion of the CT in a construct consisting of the last 284 amino acids completely abolished the tumorigenic and transforming effects of MUC16 C-terminal domain. The reasons for this discrepancy are unclear but may reflect differences in integration of extracellular and intracellular signals with the different constructs that were used in these experiments.

MUC16 AND DRUG RESISTANCE

Silencing MUC16 using single-chain antibodies increased the sensitivity of MUC16 overexpressing OVCAR3 cells to genotoxic agents such as cisplatin and doxorubicin but not to microtubule assembly inhibitors such paclitaxel and vinorelbin[94]. Conversely, expression of MUC16 C-terminal domain conferred increased resistance to cisplatin[94]. Consistent with the role of MUC16 in drug resistance, MUC1 has been shown to modulate the

sensitivity of cancer cells to genotoxic agents. Indeed, expression of MUC1 C-terminal domain conferred resistance to cisplatin and etoposide in colon cancer cells[89,95]. The downregulation of MUC16 in OVCAR3 cells activates the PI3K/Akt pathway[68]. The authors also reported that MUC16 knockdown in these cells decreased FOXO3a nuclear localization. FOXO3a function is controlled in part by activation of the Akt pathway. Akt phosphorylates FOXO3a, resulting in binding of FOXO3a to 14-3-3 proteins and retention of FOXO3a in the cytoplasm. In contrast, dephosphorylation of FOXO3a induces its nuclear localization where it transactivates gene expression[96]. FOXO3a modulates the expression of several genes that regulate the cellular response to stress at the G2-M checkpoint. The growth arrest and DNA damage response gene Gadd54a is a target of FOXO3a that mediates part of FOXO3a's effects on DNA repair[97]. Thus, preventing the nuclear localization of FOXO3a contributes to the apoptotic response to genotoxic drugs. It is therefore possible MUC16 knockdown sensitizes tumor cells to genotoxic drugs by activating Akt which in turn prevents FOXO3a nuclear localization. Although MUC16 expression may be associated with cisplatin resistance *in vitro*, no correlation was observed between MUC16 expression and resistance to chemotherapy in the TCGA ovarian cancer project[73]. Interestingly, however, a recent study suggested that the combination of serum CA125 and ascites leptin levels have a high discriminating potential to distinguish clinically resistant patients (intrinsic resistance) to first-line therapy from patients that responded to first-line chemotherapy[47].

Death receptor ligands such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) trigger rapid apoptosis in various tumor cell types[98-102]. TRAIL binds to death receptors, TRAIL-R1 (DR4) and -R2 (DR5), whose cytoplasmic death domain (DD) signals downstream caspase activation to mediate TRAIL-induced apoptosis[103]. Upon receptor activation, FADD and pro-caspase-8 are recruited to form a death-inducing signaling complex (DISC)[104]. When recruited to the DISC, pro-caspase-8 becomes activated and subsequently activates downstream effectors caspases-3, -6 and -7, leading to apoptosis. The cellular FLICE inhibitory protein (cFLIP) regulates both recruitment and processing of pro-caspase-8 within the DISC[105]. MUC16 ectopic expression, like MUC1, was shown to attenuate TRAIL-induced caspase-8 and mitochondria activation, resulting in decreased apoptosis[106]. Notably, MUC16 C-terminal expression was sufficient to attenuate TRAIL-induced apoptosis and signaling. MUC16-induced resistance to TRAIL was related to decreased TRAIL-R2 expression and recruitment at the DISC, and by increased cFLIP expression. MUC1-blockade of TRAIL-induced apoptosis has been attributed to localization of MUC1 C-terminal domain to the mitochondria or to the ability of MUC1 to directly bind caspase-8 and FADD, thereby inhibiting recruitment of caspase-8 at the DISC[107]. Thus, although both MUC1 and MUC16 can inhibit TRAIL-induced apoptosis, the mechanisms by which these mucins inhibit TRAIL signaling differ. This could be related to the amino acids composition of their cytoplasmic tail.

CONCLUSIONS AND FUTURE DIRECTIONS

Since its discovery in the late 1970s, MUC16 glycoprotein has been recognized as a useful clinical biomarker in advance diseases. However, evidence now suggests that MUC16 is much more than a biomarker for disease progression. Recent studies have established that MUC16 can act as an oncogene. MUC16 expression leads to the transformation normal fibroblastic cells and it contributes to the pathogenesis and progression of EOC. These phenotypic effects are shared by other membrane-bounded mucins such as MUC1 and MUC4. However, the mechanisms by which these mucins exert their effects differ. The complex biochemical structure of MUC16 is a constant challenge to define functional domains. Although progress has been made toward understanding the role of MUC16 in tumorigenesis, many question remains. Further studies are required to gain a better understanding of the oncogenic role of MUC16. An area of critical importance is deciphering the mechanism by which MUC16 C-terminal domain enhances tumorigenesis and the downstream signaling associated with this effect. A better understanding of the contribution of MUC16 C-terminal domain to tumorigenesis may yield specific intracellular or extracellular signaling targets. Exploiting these targets could provide novel treatment for ovarian cancer which are urgently needed.

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MINIREVIEWS

Implications of multigene testing for hereditary breast cancer in primary care

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Abstract

Approximately 1 in 8 women will develop breast cancer during their lifetime and the risk factors include age, family history, and reproductive factors. In women with a family history of breast cancer, there is a proportion in which a gene mutation can be the cause of the predisposition for breast cancer. A careful assessment of family and clinical history should be performed in these women in order to determine if a genetic counseling referral is indicated. In cases of hereditary breast cancer, genetic testing with a multigene panel can identify specific genetic mutations in over 100 genes. The most common genes mutated in hereditary breast cancer are the high-penetrance *BRCA1* and *BRCA2* genes. In addition, other mutations in high-penetrance genes in familial cancer syndromes and mutations in DNA repair genes can cause hereditary breast cancer. Mutations in low-penetrance genes and variants of uncertain significance may play a role in breast cancer development, but the magnitude and scope of risk in these cases remain unclear, thus the clinical utility of testing for these mutations is uncertain. In women with high-penetrance genetic mutations or lifetime risk of breast cancer > 20%, risk-reducing interventions, such as intensive screening, surgery, and chemoprevention, can decrease the incidence and mortality of breast cancer.

Key words: Genetic testing; *BRCA1*; *BRCA2*; Hereditary breast cancer; Multigene testing

Core tip: Multigene testing for hereditary breast cancer is readily available and some panels can identify over 100 gene mutations. Risk-reducing strategies are available for women with mutations in high-penetrance genes, whereas strategies for managing women with mutations in low-moderate penetrance genes is less

clear. Appropriately identifying women who should undergo genetic counseling for hereditary breast cancer and implementing recommended guidelines in those who are found to be high risk can reduce the incidence and mortality of breast cancer.

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INTRODUCTION

Approximately 1 in 8 women will develop breast cancer during their lifetime[1]. There are many risk factors for the development of breast cancer, including increasing age, reproductive factors, and family history of breast cancer. Thirteen percent of women diagnosed with breast cancer have at least one first-degree relative with breast cancer and the risk of developing breast cancer increases with increasing numbers of affected first-degree relatives when compared to women with no affected relatives[2]. A proportion of these women develop breast cancer due to inheriting a mutated gene. This is classified as a hereditary breast cancer. Hereditary breast and ovarian cancer (HBOC) syndrome is secondary to mutations in the *BRCA1* and *BRCA2* genes and accounts for 20%-25% of breast and ovarian cancers in families with multiple affected family members[3,4]. There are several other defined syndromes associated with hereditary breast cancer, including Cowden (*PTEN* mutation), Li-Fraumeni (*TP53* mutation), Peutz-Jeghers (*STK11* mutation), and hereditary diffuse gastric cancer (*CDH1* mutations) syndromes[5]. Additionally, there are other low and moderate penetrance genes, including *PALB2*, *CHEK2*, and *ATM*, that can cause clustering of breast cancer in affected families.

Genetic testing for these gene mutations can allow for identification of these patients prior to the development of cancer. In the case of some mutations, there are interventions that can reduce breast cancer incidence and mortality in mutation carriers. Recent advances in technology allow for rapid and low cost identification of inherited mutations. While individuals can be tested for only *BRCA1/2* mutations specifically, there is also multigene testing that utilizes next generation sequencing (NGS), which can identify mutations in over 100 genes in one test[6]. Though multigene testing is readily available in the clinics and through direct-to-consumer testing[7], the clinical utility of evaluating the large number of genes remains uncertain.

This review article will summarize the indications for genetic assessment for hereditary breast cancer, the evidence on interpretation of multigene testing results, and breast cancer risk management options for women who are found to be carriers of mutations.

INDICATIONS FOR GENETIC ASSESSMENT IN HEREDITARY BREAST CANCER

Several professional organizations, including the National Comprehensive Cancer Network (NCCN)[8], the United States Preventive Services Task Force (USPSTF)[9], the American Society of Clinical Oncology (ASCO)[7,10], the National Society of Genetic Counselors (NSGC)[5,11], the American College of Medical Genetics (ACMG)[11], and the American College of Obstetricians and Gynecologists (ACOG)[12] have published guidelines regarding genetic assessment for hereditary breast cancer in cancer-free women. As part of a genetic assessment, these guidelines all emphasize the importance of genetic counseling prior to and after genetic testing by a health care provider knowledgeable in genetic testing. While the specific criteria for referral to genetic assessment vary among different organizations, the criteria are based on the clinical features and history that increase the likelihood of a hereditary breast cancer. Table 1 shows the guidelines for hereditary breast cancer genetic assessment in a woman without a cancer diagnosis as published by the NCCN, USPSTF, ASCO, NSGC, ACMG, and ACOG.

There are also specific guidelines for genetic assessment in patients who have received a diagnosis of cancer and also for men; however, these guidelines will not be discussed in this review.

MULTIGENE TESTING FOR HEREDITARY BREAST CANCER

There are several commercially available multigene panels for hereditary breast cancer, such as BreastNext by Ambry Genetics, OncoGeneDx by GeneDx, and myRisk by Myriad Genetics, that are capable of sequencing a range of 6 to more than 100 cancer-associated genes depending on the test[6,13]. The specific genes tested in each multigene panel vary depending on the laboratory offering the testing. Some of the genes included in these panels are genes that are known to be associated with cancer syndromes with breast cancer component (*i.e.*, *BRCA1/2* for HBOC syndrome, *PTEN* for Cowden syndrome, *TP53* for Li-Fraumeni syndrome), genes shown to have a moderate risk association with breast cancer, or genes that function in DNA repair pathways with or similarly to *BRCA1/2*. However, there are concerns that other genes included in multigene panels have breast cancer risk associations that are not well established[6,13,14]. This complicates the testing of these less-established genes on three levels of uncertainty: (1) the magnitude of cancer risk associated with the gene; (2) the clinical scope of cancer risk; and (3) the clinical relevance of variants of the genes[13].

Table 1 Guidelines for hereditary breast cancer genetic assessment in a woman without a cancer diagnosis

Organization	Indications for genetic assessment referral
National Comprehensive Cancer Network[8]	Family history of any of the following: A known mutation in a cancer susceptibility gene within the family ⩾ 2 breast cancer primaries in a single individual ⩾ 2 individuals with breast cancer primaries on the same side of family ⩾ 1 individual with invasive ovarian cancer primary First- or second-degree relative with breast cancer at age ⩽ 45 yr Three or more of the following (especially if early onset): Pancreatic cancer, prostate cancer (Gleason score ⩾ 7), sarcoma, adrenocortical carcinoma, brain tumors, endometrial cancer, thyroid cancer, kidney cancer, dermatologic manifestations and/or macrocephaly, hamartomatous polyps of gastrointestinal tract, diffuse gastric cancer (can include multiple primary cancers in same individual) Male breast cancer
United States Preventive Services Task Force[9]	Family history of any of the following: Breast cancer diagnosis before age 50 yr Bilateral breast cancer Breast and ovarian cancer Breast cancer in ⩾ 1 male family member Multiple cases of breast cancer in the family ⩾ 1 family member with 2 primary types of BRCA-related cancer Ashkenazi Jewish ethnicity OR use of a familial risk stratification tool, such as the Ontario Family History Assessment Tool, Manchester scoring system, Referral Screening Tool, Pedigree Assessment Tool, and Family History Screen 7, to determine need for genetic counseling
American Society of Clinical Oncology[7,10]	When all 3 criteria are met: The individual being tested has a personal or family history suggestive of genetic cancer susceptibility The genetic test can be adequately interpreted The test results have accepted clinical utility
National Society of Genetic Counselors[5,11] and American College of Medical Genetics and Genomics[11]	If patient or any of their first-degree relatives meet one of the following criteria: Breast cancer diagnosis at age ⩽ 50 yr Triple-negative breast cancer diagnosis at age ⩽ 60 yr ⩾ 2 primary breast cancers in the same person Ashkenazi Jewish ancestry and breast cancer at any age ⩾ 3 cases of breast, ovarian, pancreatic, and/or aggressive prostate cancer in close relatives, including the patient Breast cancer and one additional Li-Fraumeni Syndrome tumor in the same person or in two relatives, one diagnosed at age ⩽ 45 yr Breast cancer and ⩾ 1 Peutz-Jeghers polyp in the same person Lobular breast cancer and diffuse gastric cancer in the same person Lobular breast cancer in one relative and diffuse gastric cancer in another, one diagnosed at age < 50 yr Breast cancer and 2 additional Cowden syndrome criteria in the same person
American College of Obstetricians and Gynecologists[12]	Women with greater than an approximately 20%-25% chance of having an inherited predisposition to breast and ovarian cancer are recommended for genetic counseling referral, including: Women with a close relative with known *BRCA1* or *BRCA2* mutations In women with greater than an approximate 5%-10% chance of having an inherited predisposition to breast and ovarian cancer, genetic counseling referral may be helpful, including those with a close relative that has: Breast cancer at age ⩽ 40 yr Ovarian cancer, primary peritoneal cancer, or fallopian tube cancer of high grade, serous histology at any age Bilateral breast cancer (particularly if the first case of breast cancer was diagnosed at age ⩽ 50 yr) Breast cancer at age ⩽ 50 yr and a close relative with breast cancer at age ⩽ 50 yr Ashkenazi Jewish ancestry with breast cancer at age ⩽ 50 yr Breast cancer at any age and 2 or more close relatives with breast cancer at any age (particularly if at least one case of breast cancer was diagnosed at age ⩽ 50 yr)

Additionally, NGS has the potential to discover variants of uncertain significance (VUS) at high rates[15]. In a study of 198 women who met NCCN guidelines for *BRCA1/2* mutation testing, multigene testing with a panel of 42 genes was performed and participants were found to have an average of 2.1 VUS among 42 genes[16]. The high rate of VUS poses a risk of causing unnecessary anxiety and potentially interventions that are without evidence base[13].

In families that do not have *BRCA1* or *BRCA2* mutations, it is likely that other high-penetrance genes or a number of moderate- or low-penetrant genes account for familial breast cancer[3]. In a study in the United States, among women who met NCCN guidelines for *BRCA1/2* testing and had negative genetic testing, 11.4% had pathogenic mutations in 1 of 40 other genes[16]. A study in Germany performed multigene testing with a panel of 10 genes on 620 patients who met criteria for HBOC genetic testing and found almost 33% more mutations could be discovered with the addition of 8 genes to *BRCA1* and *BRCA2* testing[17]. The use of multigene testing could help identify mutations

that may cause the predisposition to breast cancer of the approximately 75%-80% of familial breast cancers that are not associated with *BRCA1/2* mutations.

While mutations in genes that have a well-established risk association with breast cancer, such as *BRCA1/2*, have clear clinical implications, there is uncertainty with how to interpret and communicate the results of mutations in genes with less robust evidence, as well as gene *VUS*. Genes that have evidence of an association with breast cancer are described below.

BRCA1/BRCA2 (HBOC)

BRCA1/2 genes play a role in DNA repair and mutations in these genes are of high-penetrance. Women with a *BRCA1/2* mutation have elevated lifetime risks of breast and ovarian cancer of 40%-60% and 20%-40%, respectively[18-21]. There is also an increased risk of other cancers, such as pancreas cancer. While the prevalence of the genetic mutation is less than 1% (1 in 400) in the general population, the prevalence of a founder mutation in the *BRCA1* (5382insC or 185delAG) or *BRCA2* (6174delT) genes is up to 2.5% (1 in 40) among individuals of Ashkenazi (Central and Eastern European) Jewish descent[21,22]. There are over 2000 different known mutations in the *BRCA1/2* genes[23]. There is also evidence to suggest that there are genetic modifiers of breast cancer risk for carriers of *BRCA* mutations and that the type and location of *BRCA* mutation affects breast cancer risk[24,25]. Genetic test results are reported as positive, VUS, uninformative-negative, or true negative[9]. The difference between uninformative-negative and true negative is that a true negative result is when no *BRCA* mutation is found in the setting of a known *BRCA* mutation in the family[26]. In a large cross-sectional study of non-Ashkenazi women who underwent *BRCA* mutation testing, 6.2% were found to have VUS. Populations that are genetically distinct and/or under-tested, such as racial/ethnic minorities, will have higher rates of VUS when compared to the white/European reference population; however, with increased volume of testing and reclassification of VUS, the rates of VUS reporting have declined over time[27].

Genes responsible for tumor syndromes

There are several well characterized high-penetrance hereditary tumor syndromes in which breast cancer is one manifestation of the syndrome. While the association of these syndromes with an increase in breast cancer risk is known, the exact increase in risk is difficult to estimate due to ascertainment bias[6]. Li-Fraumeni syndrome is due to a germline mutation in the *TP53* gene, a tumor suppressor gene, and is characterized by an increased risk for childhood sarcomas, brain tumors, adrenocortical carcinoma, childhood leukemia, and other cancers, in addition to breast cancer[28]. In a study assessing the cancer incidence in 56 *TP53* germline mutation carriers and 3201 non-carriers, there was a significantly higher risk of breast cancer in female *TP53* mutation carriers, with a standardized incidence ratio of 105.1 (95%CI: 55.9-179.8)[29]. Cowden syndrome, or multiple hamartoma syndrome, is due to a germline mutation in the *PTEN* gene, a tumor suppressor gene[30]. The clinical phenotype has a wide array of abnormalities, including behavioral disorders, macrocephaly, gastrointestinal hamartomas, thyroid cancer, and endometrial cancer, in addition to breast cancer[31]. The estimated lifetime risk of breast cancer in a female *PTEN* mutation carrier is 85.2%, with a standardized incidence ratio of breast cancer of 25.4 (95%CI: 19.8-32.0)[32]. Germline mutations in the *STK11* gene, another tumor suppressor gene, cause Peutz-Jeghers syndrome. This syndrome is characterized by mucocutaneous pigmentation and gastrointestinal polyposis as well as an increase in gastrointestinal, breast, ovary, uterus, lung, and testis cancers[33]. The risk of developing breast cancer in a female *STK11* mutation carrier is 45% by age 70 years, a 6-fold increase when compared to the general population[33]. Hereditary diffuse gastric cancer is due to a germline mutation in the *CDH1* gene, which encodes for the E-cadherin protein. This mutation results in an increased risk of diffuse gastric cancer, colorectal cancer, and breast cancer, specifically lobular breast cancer. In female mutation carriers, the cumulative risk of breast cancer by age 80 is 39%, with a relative risk of developing breast cancer of 6.6 when compared to the general population[34]. Finally, neurofibromatosis type 1 (NF1) is caused by a germline mutation in the *NF1* gene, which encodes for neurofibromin, and is also a tumor suppressor gene. This syndrome has a phenotype of dermatologic manifestations, vascular disease, bone deformities, cognitive difficulties, and an increased risk of neoplasms, including breast cancer[35]. Cohort studies have shown a higher than expected number of breast cancer cases in women with NF1 with an estimated relative risk of 2.6 (95%CI: 2.1-3.2)[6,35,36].

Genes involved in DNA repair

Mutations in other genes that are involved in DNA repair, such as *PALB2, CHEK2, ATM,* and *NBN*, have also been shown to be associated with an increased risk of breast cancer and are characterized as moderate-penetrance variants. The *PALB2* gene encodes a protein that interacts with both BRCA1 and BRCA2 in DNA repair[37]. Several studies have shown that mutations in *PALB2* are associated with increased risk of breast cancer[38-41]. A meta-analysis found the combined relative risk to be 5.2 (95%CI: 3.0-9.4)[6]. The *CHEK2* gene encodes a kinase that responds to DNA damage. In 2 large case-control studies, a specific variant of *CHEK2*, c.1100delC, was found to increase the risk of breast cancer by an estimated relative risk of 3.0 (95%CI: 2.6-3.5)[6,42,43]. The *ATM* gene encodes a protein kinase that functions in monitoring and repairing double-strand DNA breaks. The disease ataxia-telangiectasia is caused by biallelic mutations in the *ATM* gene. A case-control study of 964 patients found that the relative risk of breast cancer associated with *ATM* mutation is 2.37 (95%CI: 1.51-3.78)[44]. The *NBN* gene encodes a protein that,

like *BRCA1* and *BRCA2*, plays a role in the homologous recombination repair pathway. In a meta-analysis of 10 case-control studies on the association of *NBN* 657del5 variants and breast cancer risk, which included 25365 subjects, the pooled odds ratio was found to be 2.66 (95%CI: 1.82-3.90)[45].

INTERVENTIONS FOR HIGH RISK PATIENTS

BRCA mutation carriers

Risk management options for women found to have *BRCA1/2* mutations have been well studied and the NCCN has published expert-opinion based guidelines for the management of these patients. The risk management options for mutation carriers include intensive breast cancer screening with clinical breast exam, mammography, and breast magnetic resonance imaging (MRI), risk-reducing surgeries such as prophylactic mastectomy and bilateral salpingo-oophorectomy, and chemoprevention.

NCCN guidelines recommend clinical breast exam every 6-12 mo starting at age 25 years, annual breast MRI or mammogram (if MRI unavailable) from age 25-29 years, and annual breast MRI and mammogram from age 30-75 years[8]. Screening with MRI should begin earlier than 25 years if there is a family history of a breast cancer diagnosis prior to age 25 years[8]. In a meta-analysis of 11 prospective studies that screened women at high risk for breast cancer with mammography and MRI, the sensitivity of MRI was greater than mammography (77% *vs* 39%), but specificity of mammography greater than MRI (94.7% *vs* 86.3%). When mammography was combined with MRI, sensitivity was 94% and specificity was 77.2%[46]. In *BRCA1/2* mutation carriers, annual surveillance with MRI is associated with a significant reduction in advanced-stage breast cancer[47].

With regards to risk reducing surgery, NCCN guidelines recommend counseling *BRCA* mutation carriers on risk-reducing mastectomy (RRM) and risk-reducing salpingo-oopherectomy (RRSO)[8]. In a prospective cohort study of 1619 *BRCA1/2* mutation carriers, of the 247 women who underwent RRM, none developed breast cancer, while there were 98 cases of breast cancer in the 1372 women who did not have surgery[48]. NCCN guidelines recommend RRSO between the ages of 35-40 and upon completion of childbearing[8]. RRSO resulted in 72%-86% reduction in risk of ovarian cancer, 37% reduction in risk of breast cancer in *BRCA1* carriers, and a 64% reduction in risk of breast cancer in *BRCA2* carriers[48]. Mutation carriers who underwent RRSO compared to those who did not had a 79% reduction in ovarian cancer-specific mortality, 56% reduction in breast cancer-specific mortality[48], and a 60%-77% reduction in all-cause mortality[48,49].

The use of tamoxifen, raloxifene, and aromatase inhibitors has been studied as chemoprevention in women at high risk for breast cancer, though data in *BRCA1/2* mutation carriers is limited. The Breast Cancer Prevention Trial was a randomized placebo-controlled trial investigating whether tamoxifen reduces the incidence of breast cancer in high-risk women and found a 49% reduction in the incidence of breast cancer with the use of tamoxifen[50,51]. Of the 288 breast cancer cases in the trial, 19 were *BRCA* mutation carriers. Analysis showed that tamoxifen reduced breast cancer incidence among *BRCA2* carriers by 62%, but had no effect on breast cancer incidence in *BRCA1* carriers[52].

Negative BRCA1/2 testing

Despite negative *BRCA1/2* testing, families with a significant family history of breast cancer still have an approximately 4-fold increased risk of breast cancer[26]. In these women, there are still interventions available that can decrease the risk of developing breast cancer. In women with a greater that 20% lifetime risk of breast cancer, either as calculated by a risk model or due to the presence of a high- or moderate-penetrance mutation (*i.e.*, *ATM*, *CDH1*, *CHEK2*, *PALB2*, *PTEN*, *STK11*, or *TP53*), NCCN guidelines recommend intensive screening with MRI[8]. Additionally, for women with *CDH1, PTEN,* or *TP53* mutations, option of RRM should also be discussed[8].

In women who have a 5-year breast cancer risk $\geqslant$ 1.67% or lifetime breast cancer risk $\geqslant$ 20%, chemoprevention is also an option. There is evidence that in this high risk population, tamoxifen, raloxifene, and aromatase inhibitors can decrease the incidence of breast cancer by approximately 50%, 40%, and 65%, respectively[51,53-55].

CONCLUSION

Women with a family history of breast or ovarian cancer should be screened for referral to genetic assessment for hereditary breast cancer. Advances in NGS have made multigene testing for hereditary breast cancer readily available; however, there remain questions about the clinical utility of such testing. For high- and moderate-penetrance mutations, there is greater clinical utility as there are established guidelines on risk management interventions that can be performed to reduce incidence and mortality from breast cancer.

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MINIREVIEWS

Amniocentesis: A contemporary review

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Abstract

Amniocentesis is an essential tool in obstetrics. Invasive testing remains the only modality for diagnostic genetic testing and the only way to provide comprehensive testing for chromosomal abnormalities. Despite increasing use of cell free fetal deoxyribonucleic acid (DNA) testing, amniocentesis should still be offered to all women who desire more complete and accurate genetic testing. Counseling patients on the limitations of screening tests is of the upmost importance and amniocentesis should continue to be recommended to confirm positive results from cell free fetal DNA testing or in the case of failed cell free fetal DNA test. As cell free fetal DNA screening has not adequately been studied in multiple gestations, its use is not recommended in this population and invasive testing should be offered. Amniocentesis is also very useful in providing additional information in settings other than genetic testing the second and third trimester. If intraamniotic infection is suspected, but the clinical findings are not enough to guide management, amniocentesis can provide testing that can both immediately clarify the picture (interleukin-6, gram stain, glucose levels) and finally confirm the presence of infection (culture). It can also be used to detect the presence of intrauterine viral infections. Additionally, amniocentesis may be used to test for markers of fetal lung maturity. The American Congress of Obstetricians and Gynecologists recommends that amniocentesis for this indication not be used in cases where late preterm delivery is indicated. It may be useful in guiding decision-making, however, when late preterm delivery is indicated, but when exact timing is unclear. Regardless of the indication, amniocentesis appears to be a relatively low risk procedure with minimal risk to the patient. Additional randomized controlled trials are not likely, as they are not feasible to due extremely high number of participants that would be needed to detect a difference in loss rates. Based on current literature, however, the risk of pregnancy loss from second trimester amniocentesis is low in both singleton and twin gestations. We counsel patients that technique has changed since the original studies in the 1970s and feel comfortable quoting a loss rate of 1/500-1/1000 based on contemporary data.

Key words: Amniocentesis; Prenatal diagnosis; Invasive genetic testing; Procedure related loss rates; Cell free fetal DNA testing; Fetal lung maturity; Intraamniotic

infection

Core tip: Invasive testing remains the only way to provide comprehensive testing for chromosomal abnormalities. Despite availability of cell free fetal DNA testing, amniocentesis should still be offered to all women who desire complete genetic testing. Amniocentesis is also useful if intraamniotic infection is suspected, but the clinical picture is unclear. Additionally, when late preterm delivery is indicated, amniocentesis need not be used. There are, however, some instances when delivery timing is unclear and amniocentesis for fetal lung maturity may provide information to guide delivery timing. Amniocentesis is a relatively safe procedure. We counsel patients that technique has changed since the original studies in the 1970s and feel comfortable quoting a loss rate of 1/500-1/1000 based on contemporary data.

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HISTORICAL CONSIDERATIONS

Amniocentesis has been widely used since it was first performed in 1956[1]. Originally reported as a method of determining fetal sex *in utero*, Fuchs and Riis then hypothesized that it could be possible to diagnose chromosomal abnormalities *in utero via* this technique. By 1963, it had been confirmed that the karyotype of fetal and amniotic cells are identical[2]. Steele and Brag then showed in 1966 that amniotic cells can be sampled and cultured in sufficient quantity to be karyotyped, showing that *in utero* chromosome analysis was possible[3]. Shortly thereafter, the first prenatal diagnosis of an abnormal karyotype was reported[4]. Since that time, its utility has only continued to expand in both the second and third trimesters.

In 1979, the National Institutes of Health (NIH) suggested that amniocentesis be routinely offered to women 35 years or older, based on the balance between the risk of procedure-related pregnancy loss and the incidence of aneuploidy based on age[5]. Its use for genetic testing has only continued to expand over the years and is now the most commonly used invasive genetic test in obstetrics[6]. With the recent advent of cell free fetal DNA screening, understanding the continued need for and risks of amniocentesis has become increasingly important.

TECHNIQUE

Amniocentesis is generally performed after 15 wk gestation. The amniotic fluid volume at this gestational age is approximately 150 cc[5]. Prior to performing procedure, an ultrasound should be performed to evaluate the placental and fetal location and to confirm gestational age and fetal viability. After adequately prepping the maternal abdomen with antiseptic solution, the procedure should be performed under continuous ultrasound guidance to decrease the number of insertions and bloody taps[7]. If possible, transplacental passage of the needle should be avoided. If this is not possible due to an anterior placenta, care should be taken to avoid large vessels and echolucencies seen with ultrasound guidance. In the hands of experienced providers, there does not appear to be an increased loss rate with transplacental amniocentesis compared to procedures during which the needle does not pass through the placenta[8].

The effect of operator experience itself on outcomes of amniocentesis has independently been studied. In a retrospective review, Margioula-Siarkou *et al*[9], examined loss rates of a single operator over a 13 year period (n = 5913) and found the loss rates in the first 10% of amniocenteses performed to be significantly higher than the last 10%, suggesting there is benefit to experience. In a review of 6332 amniocentesis specimens that revealed male karyotype (46 XY), maternal cell contamination was seen more frequently in samples obtained by physicians who perform < 50 amniocenteses annually (0.67% *vs* 0.19%, P = 0.0021)[10]. There has never been a study defining an exact number of procedures after which a provider becomes "experienced," though reports of single institutions' experiences show that fetal loss rates are related to operator experience[11].

Needle size is another technical aspect of the procedure that has been postulated to have effect on loss rates; however studies on this issue are extremely limited. Athanasiadis *et al*[12], showed that while a larger caliber needle may facilitate a faster collection of fluid, it may also be associated with increased fluid leakage rates. From a review of the literature, it appears that 20 or 22 gauge needles are most commonly used today. When these two sizes were compared in a randomized trial with 200 participants, it was shown that procedure time was statistically significantly lower when a 20 gauge needle was used (9.6 s *vs* 26 s, P < 0.0001). There was no difference in intrauterine bleeding at the insertion site, patient discomfort 30 min post-procedure, or complication rates within 2 wk of the procedure between the two groups[12]. This decrease in procedure time is not clinically significant and we use a 22 gauge needle.

INDICATIONS

Amniocentesis allows for fetal DNA in the amniotic fluid to be analyzed for chromosomal abnormalities. This can be done in response to abnormal serum genetic screening, an abnormal ultrasound finding, or in order

to specifically test for a genetic condition for which a patient or partner is a carrier, including autosomal recessive, autosomal dominant, X-linked conditions or microdeletion/microduplication syndromes. This analysis of the fetal DNA is only possible with diagnostic testing via amniocentesis or chorionic villus sampling (CVS). One advantage of amniocentesis over CVS, however, is the ability to directly analyze fetal DNA. This avoids the potential issue of confined placental mosaicism that may be encountered in CVS samples. In 1%-3% of CVS samples, chromosomal mosaicism is seen[13]. This mosaicism is usually confined to the placenta, however is also present in the fetus in 10% of cases. In all cases of mosaicism on CVS, amniocentesis is recommended in order to determine whether it is confined to the placenta or is seen in the fetus as well.

Another important application for amniocentesis that deserves its own attention is in twin gestations. It has been previously shown that twin gestations are at an increased risk for chromosomal abnormalities. Further, the rate of multiple births is increasing. Between 1980 and 1999, the overall multiple birth ratio increased 59% and by 1999 multiples accounted for 3% of all live births[14]. Women of advanced age have experienced the greatest increase in rates of multiples[14]. As these women who are already at an increased risk for chromosomal abnormalities at baseline become increasingly pregnant with multiples, it is imperative that we have an accurate estimate of the risks of amniocentesis in this setting. As data is limited on cell free fetal DNA screening (see later) in the setting of multiple gestations, amniocentesis remains important for genetic diagnosis in these patients[15].

In addition to its utility in genetic testing, amniocentesis has been used in the third trimester to test the amniotic fluid for biochemical markers suggestive of fetal lung maturity. This indication for amniocentesis has recently come under closer scrutiny. The issue of timing when delivery is indicated in the late-preterm or early-term time period is based mostly on expert opinion. To analyze the obstetric, fetal and maternal conditions that often lead to late-preterm or early-term birth, the National Institute of Child Health and Human Development (NICHD) and the Society for Maternal-Fetal Medicine held a workshop in February 2011. In this meeting, the issue of using amniocentesis for fetal lung maturity to guide decision-making was directly addressed. The consensus was that if there is an indication for delivery, amniocentesis to assess fetal lung maturity should not be used to assist in delivery timing[16]. There are several rationales for this recommendation. The first is that if significant fetal or maternal risk exists, delivery should occur regardless of fetal lung maturity. The second issue is that confirmation of fetal lung maturity with amniocentesis does not translate into maturity of organ systems other than the lungs[17]. A committee opinion from the American Congress of Obstetricians and Gynecologists (ACOG) supports this recommendation, based on the same two issues[18]. In an editorial statement arguing the dissenting opinion, Towers *et al*[19] asserts that fetal lung maturity testing provides more information than just lung maturity. He argues that many of the serious morbidities for preterm neonates born after 34 wk (intraventricular hemorrhage, necrotizing enterocolitis) are highest in those infants who are intubated, and that the risk of intubation in the neonatal period after an amniocentesis showing fetal lung maturity is < 1%[20-22]. Further, he argues that there is a role for amniocentesis when the clinical scenario is not clear. For example, in the setting of uncertain dates and a quasi-urgent fetal indication for delivery, such as suspected fetal growth restriction with less-than-optimal interval growth at < 34 wk. We believe that it is reasonable to use amniocentesis to assess fetal lung maturity in this setting, when there is an indication for an early delivery but no imminent danger to mother or fetus would likely occur while awaiting results.

Another use for amniocentesis is in the diagnosis of intraamniotic infection. This diagnosis can usually be made clinically, based on maternal fever often with associated maternal or fetal tachycardia, uterine tenderness, or foul smelling amniotic fluid[23]. There are situations, however, where infection is suspected or likely, but the clinical picture is not this clear. It is extremely important in these situations to clarify the diagnosis and obtain further information to guide management, as undiagnosed infection would put the patient at risk. One example of this is in the case of a candidate for a physical exam indicated cerclage, as subclinical intraamniotic infection is seen in 13%-28% of these women[24]. Amniocentesis can be utilized in these cases, as an amniotic fluid culture remains the most specific test for documentation of intraamniotic infection. There are several other tests that have been used to aid in the diagnosis, as it is not always practical to wait several days for final culture results in the setting of a possible infection. Romero et al. studied the diagnostic value of each of these tests and found that a high interleukin-6 (IL-6) level was 82% sensitive and a negative gram stain was 99% specific for the detection of amniotic fluid containing bacteria[25]. The correlation between high amniotic fluid IL-6 levels and chorioamniotic infection has been supported by other authors as well[26]. Analysis of amniotic fluid for these parameters remains an invaluable tool in detecting infection when the clinical picture is not straightforward.

Amniocentesis should also be used to diagnose intrauterine viral infections, such as Cytomegalovirus or Parvovirus. Whether there are ultrasonographic signs that a fetus has been affected by one of these viruses or maternal serum indicates infection, amniocentesis can be performed. Polymerase chain reaction studies for these viruses should then be performed on the amniotic fluid obtained[27]. This information is essential to guide further fetal assessment and possible intrauterine treatment, depending on the clinical scenario.

An exciting new application for amniotic fluid and,

thus, amniocentesis is its potential use for the ascertainment of stem cells. There has been much attention and research aimed at the potential clinical uses of stem cells from bone marrow, blood, embryonic tissue and umbilical cord blood. Their widespread use has been limited by small cell number, potential tumorigenesis, and some ethical concerns with the use of embryonic tissue. The use of amniotic fluid cells obtained from discarded fluid after second trimester amniocentesis has shown promise as a way to circumvent some of these limitations. The ability to expand these multipotent cells in culture and to cryopreserve them for delayed differentiation and use has already been documented[28]. They have been shown to differentiate along adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic pathways without giving rise to tumors[29]. These initial studies make these cells available to be used and studied for potential clinically significant therapeutic purposes.

There is one additional use for amniocentesis that is no longer widely used, though is worthy of discussion: spectral analysis of amniotic fluid (ΔOD450) to quantify the severity of fetal anemia. *In utero*, bilirubin from the fetal pulmonary and tracheal effluents is found in the amniotic fluid. The level of bilirubin in the fluid can be obtained *via* amniocentesis and then be used to estimate fetal hemolysis[30,31]. This technique was compared to middle cerebral artery (MCA) Doppler assessment in a prospective study by Oepkes *et al*[32]. MCA Doppler assessment was found to be 85% accurate, whereas ΔOD450 measurements were 76% accurate using the Liley curve and 81% accurate using the Queenan curve. Based on the findings of this study, MCA Doppler assessment has been widely accepted as the primary screening tool in the detection of fetal anemia[5]. We agree that amniocentesis should no longer be the first line surveillance tool in this situation, given that the noninvasive option has been shown to be superior.

COMPLICATIONS

Amniocentesis is a relatively safe procedure with minimal risk to the patient. With sterile technique, chorioamnionitis is seen in less than 0.1% of cases[11]. Other infrequent complications include transient vaginal spotting or leakage of amniotic fluid. Patients should be counseled that if leakage occurs, it usually occurs within 48 h and that fetal survival is greater than 90% in these cases[11].

Pregnancy loss is the most serious and feared risk to an amniocentesis. Generally quoted loss rates are primarily based on 3 main studies in the 1970s that were not randomized[33-35]. Based on these studies, the Centers for Disease Control and Prevention (CDC) promulgated a loss rate of 0.5% following amniocentesis. Despite the fact that these studies were not randomized and amniocenteses were not performed with the use of concurrent ultrasound guidance, this CDC estimation of loss after amniocentesis is still often quoted. There is only one randomized trial evaluating loss after amniocentesis published by Tabor *et al*[36] in 1986. This trial reports a 1% increased risk in the amniocentesis group when compared to the control group who did not undergo amniocentesis[36]. This study has been criticized for being carried out on young, low-risk women, which is generally not the group of women most commonly undergoing this procedure. The results from this trial, which was carried out 30 years ago, may not be applicable to our current practice, as their equipment was far inferior to what we have today. Nonetheless, this is the only randomized trial comparing amniocentesis to no amniocentesis, and it is likely to remain so due to societal pressures and ethical concerns.

Absent randomized controlled trials (RCT), researchers have sought to refine the reported risk of amniocentesis by utilizing non-randomized studies that, although not randomized, mitigate some of the criticisms of older studies. For example, Eddleman *et al*[37] used the large database of approximately 35000 patients who were enrolled in the FASTER trial. In this multi-center, prospective clinical trial, there was a 1% loss rate in the amniocentesis group and a 0.94% loss rate in the no amniocentesis group. This difference of 0.06% (1/1600) is the loss rate attributable to the amniocentesis. Another very large, contemporary study that included 51557 patients was done by Odibo *et al*[38], with loss rate of 0.13% (1/769) attributable to amniocentesis. A meta-analysis that included 21 studies performed after 2000 showed a 0.11% procedure-related loss rate[39]. This study only analyzed studies which included greater than 1000 procedures and only those who examined loss rates < 24 wk gestation in order to determine loss rates attributable to amniocentesis. Other recent trials have continued to demonstrate this trend in loss rates lower than previously seen by Tabor *et al*[38,40-43]: Typically in the range of 0%-0.5% loss rate attributable to amniocentesis. This more contemporary analysis of loss rates is reassuring and should be included in current patient counseling[38-42].

Overall, reported loss rates for amniocentesis in recent years are consistently low, but have been criticized for various reasons. Due to their nonrandomized nature, many of these studies do not have a control group that would provide a background loss rate. Even in the studies that do have a control group, they are often not appropriately matched in terms of baseline risk factors for the women in each group. Another issue with current literature is that there has not been a standard manner by which to report procedure-related loss rates. Studies to date have used varying definitions of pregnancy loss in terms of cutoffs for gestational age and length of time from procedure to loss. As mentioned earlier, there will likely not be any future RCT to assess contemporary loss rates. An RCT would require > 400000 patients in each arm to have adequate power to detect a difference

of 0.05% in loss rates between those who do and do not undergo amniocentesis[37]. Thus, using large scale, multicenter, prospective trials, such as the FASTER Trial, as a surrogate appears to be the best option. Given it was carried out in multiple centers and that there were no specifications as to the technique, the results are generalizable to the larger "national" community. Given that more recent literature suggests loss rates lower than seen in the 1970s, amniocentesis remains a safe option for genetic testing. We believe it is reasonable to counsel patients of an approximate 1/500-1/1000 risk of loss attributable to amniocentesis. All women should be offered genetic testing and we recommend a customized risk assessment for each individual patient, rather than using an arbitrary age cut-off to guide recommendation for amniocentesis. We also believe that patients today, with appropriate counseling, are able to understand the reasons that we cannot give them an exact number for the risk of loss and accept a "range of risk" as our best estimate.

The literature regarding loss rates after amniocentesis in twin gestation is even more limited[11]. There have been several published studies addressing this; however they are limited by small sample size[44-48]. Cahill published a retrospective review of a 16 year time period comparing women who underwent amniocentesis (n = 311) to a control group (n = 1623) who did not[49]. In this study, the attributable risk of pregnancy loss prior to 24 wk after an amniocentesis was 1.8%. The women who elected to have an amniocentesis were older, more likely to be ⩾ 35 years old and more likely to report alcohol exposure. It should be noted, however, that this increased risk of loss after amniocentesis remained after adjustment for maternal age, chorionicity, presence of anomaly on ultrasound, alcohol exposure, or race. Further, amniocentesis remained significantly associated with loss in patients who were younger than 35 years old and had normal ultrasound findings. In agreement with this finding, Yukobowich *et al*[47] found a statistically significant increase in fetal loss rate after amniocentesis (2.7% *vs* 0.6%). Conversely, other authors have found no difference in loss rates[45,46,50]. It is clear that additional studies are needed to further elucidate the true impact amniocentesis has on the loss rate in a twin gestation. The literature is extremely scant in regards to loss rates in the setting of higher order multiple gestations and further research is needed to guide counseling.

OTHER CONSIDERATIONS

The safety and accuracy of amniocentesis performed prior to 15 wk has been assessed in several randomized clinical trials. One study by the NICHD compared first trimester amniocentesis to first trimester transabdominal CVS[51]. They found an increase in spontaneous loss rate (RR-1.74) and a 4-fold increase in the rate of talipes equinovarus in the amniocentesis group. Those pregnancies which underwent CVS, however, are not an appropriate control group for those undergoing early amniocentesis. CVS is a different procedure that can be done at an earlier gestational age, which involves aspiration of the placental tissue and generally requires a larger gauge needle than used for amniocentesis. Nicolaides *et al*[52], found that the spontaneous loss rate after early amniocentesis (5.8%) was significantly higher than after CVS (1.8%). Other trials have been focused on comparing early amniocentesis to mid-trimester amniocentesis for a more direct comparison. Similarly, in a large study by the Canadian Early and Mid-Trimester Amniocentesis Trial Group[53] that included over 4000 amniocenteses, procedures done at 11-12 wk were compared to those done between 15-20 wk. The only difference between these two groups was the gestational age at the time of amniocentesis. There was a statistically significant increase in post-procedure spontaneous loss rate (2.6% *vs* 0.8%) and talipes equinovarus (1.3% *vs* 0.1%) in the early amniocentesis group. Given the established relative safety of amniocentesis in the mid-trimester, we recommend that these procedures be carried out after 15 wk gestation.

The safety of invasive procedures in the setting of maternal transmittable blood-borne illnesses is an additional concern, due to potential fetal contamination with maternal blood cells. Studies on amniocentesis in the setting of maternal human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) are limited. There have been several small studies evaluating the risk of transmission in mothers with HBV. In the limited studies analyzing transmission rates after amniocentesis in women who were HBsAg-positive, there does not appear to be an increased risk of vertical transmission after amniocentesis[54,55]. In these studies, all infants did receive HBV vaccine and immunoprophylaxis at birth. The data on women with HCV is extremely limited, as there has been one prospective trial including 22 women. The amniotic fluid was positive for hepatitis C RNA in one woman at the time of amniocentesis. None of the infants born to these 22 mothers were positive for hepatitis C on postnatal testing[56]. Due to lack of evidence, there is insufficient data to estimate the risk of transmission in women with high hepatitis B or C viral loads. This preliminary data seems to suggest low risk of vertical transmission, however further studies with larger numbers are needed to adequately assess safety.

There have been more reports on amniocentesis in the setting of maternal HIV infection. There are studies that have shown 2-4 fold increased risk of vertical transmission of HIV after amniocentesis in the second or third trimester[57-59], though these studies were performed prior to the widespread use of combination antiretroviral therapy (ART) and many women had received no treatment at all prior to the procedure. There have been subsequent, promising small series reported after the use of combination ART has been shown to be effective. The previously shown increased risk of vertical transmission was not seen in these

women who were effectively treated[11]; in fact, the risk of transmission was not increased when compared to women who did not undergo amniocentesis[60-62]. The United States Department of Health and Human Services guidelines were updated to account for these new studies, stating that the risk of transmission does not appear to be increased in women treated with effective combination ART[63]. They do caution that although there have been no cases of vertical transmission in the 159 reported cases of amniocentesis in women who are on effective combination ART, a small increased risk of transmission may still exist. Due to this potential risk of vertical transmission, it is recommended that women in whom amniocentesis is indicated should be started on combined ART and ideally should have an undetectable viral load at the time of the procedure. Women should be counseled on the potential risk for transmission as well as the risks and benefits of noninvasive testing alternatives[11].

Recently, some obstetrical care providers have opined that with cell free fetal DNA technology, there will no longer be a need for amniocentesis. This technology in the maternal circulation has received much attention and press recently for screening, however it is limited in the information it can provide. Comprehensive pretest counseling is prudent, as this remains a screening modality. Patients must understand that this is not a diagnostic test, a negative test does not ensure a normal pregnancy, and that all positive results should be confirmed by invasive testing[64]. It most commonly only tests for trisomies 21, 18, and 13 and sex chromosome abnormalities, with varying degrees of accuracy[15]. In a study by Bianchi *et al*[65] of patients with abnormal cell free fetal DNA screening, aneuploidy was confirmed by invasive testing in only 93% with trisomy 21, 64% with trisomy 18, 44% with trisomy 13, and 38% with sex chromosomal abnormalities. Similarly, patients must be counseled on the genetic disorders that are not tested for by these blood tests. Women must be counseled that major trisomies screened for with cell free fetal DNA make up only about 50% of the cytogenetic abnormalities that would be found by karyotype following CVS or amniocentesis[5]. Further, there is a chance of test failure using this technique, due to a low fetal fraction of cell free fetal DNA recovered from maternal blood. In this circumstance, we believe that diagnostic testing should be offered due to an increased risk of aneuploidy in this setting (OR = 9.2)[66]. Testing with amniocentesis or CVS remain the only ways to definitively obtain genetic information from karyotype and microarray. Thus, ACOG still recommends offering invasive testing to all women[15].

CONCLUSION

Amniocentesis is an essential tool in obstetrics. Invasive testing remains the only modality for diagnostic genetic testing and the only way to provide comprehensive testing for aneuploidy and microdeletions. Despite increasing use of cell free fetal DNA testing, this test should still be offered to all women who desire more complete and accurate genetic testing. Counseling patients on the limitations of screening tests is of the upmost importance and amniocentesis should continue to be recommended to confirm positive results from cell free fetal DNA testing, in the case of failed cell free fetal DNA test, or with a positive first or second trimester screen. As cell free fetal DNA screening has not adequately been studied in multiple gestations, its use is not recommended in this population and invasive testing should be offered.

Amniocentesis is also very useful in providing additional information in settings other than genetic testing the second and third trimester. If intraamniotic infection is suspected, but the clinical findings are not enough to guide management, amniocentesis can provide testing that can both immediately clarify the picture (IL-6, gram stain, glucose levels) and confirm or exclude the presence of infection (culture). Additionally, in cases where late preterm delivery is indicated, but exact timing is unclear, amniocentesis to test for fetal lung maturity provides useful information to guide decision-making.

Regardless of the indication, amniocentesis appears to be a relatively low risk procedure with minimal risk to the patient. Though additional RCTs are not likely, based on current literature, the risk of pregnancy loss from second trimester amniocentesis is low in both singleton and twin gestations. We counsel patients that technology has changed since the original studies in the 1970s and feel comfortable quoting a loss rate of 1/500-1/1000 based on contemporary data.

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MINIREVIEWS

Thyroid disease in pregnancy: A review of diagnosis, complications and management

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Abstract

Malfunction of the thyroid gland is the second most common endocrine disorder encountered during pregnancy. It is well known that overt disease of the thyroid gland, either hyper or hypo can adversely affect pregnancy outcome. There is also an ongoing debate surrounding the issue of subclinical hypothyroidism and its effect on the cognitive development of the unborn child. The goal of this paper is to present a systematic review of the literature and the current recommendations for diagnosis and treatment of thyroid disease in pregnancy and postpartum.

Key words: Pregnancy; Hypothyroidism in pregnancy; Hyperthyroidism in pregnancy; Thyroid; Thyroid cancer in pregnancy; Subclinical hypothyroidism in pregnancy

Core tip: Uncontrolled thyroid disease in pregnancy is associated with significant morbidity and mortality for both mother and fetus. Timely diagnosis and adequate treatment ameliorates the risk of complications. Treatment of subclinical hypothyroidism in pregnancy with the goal of improving the cognitive outcome for the fetus has not been shown to be useful and is not currently recommended.

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INTRODUCTION

The thyroid gland produces three hormones: Triiodothyroxine (T3), tetraiodothyroxine (T4), also known as thyroxine, and calcitonin. The amount of hormone production is controlled by thyroid stimulating hormone (TSH) which is secreted by the pituitary gland. Production of TSH is in turn regulated by a negative feedback loop to the hypothalamus which produces thyroid releasing hormone. The thyroid gland uses iodine, a trace element which is not produced in the body and must be ingested, and tyrosine to manufacture

Table 1 Recommended values of thyroid stimulating hormone for each trimester

First trimester	0.1-2.5 mIU/L
Second trimester	0.2-3.0 mIU/L
Third trimester	0.3-3.0 mIU/L

T3 and T4. The majority of production is T4 which is converted in target tissues to the active form T3. The majority of circulating hormone is bound to thyroid binding globulin (TBG) proteins. Only the unbound free hormone is active.

During pregnancy the size of the thyroid gland increases by 10%-40%. The upper end of this range is seen in iodine deficient areas. Production of T3 and T4 increases by 50% with a concomitant increase in iodine requirement. The hypothalamic-pituitary-thyroid feedback systems function normally during pregnancy. However there is a significant change in protein binding of T3 and T4. Additionally the hormone of pregnancy, human chorionic gonadotropin (HCG), acts like TSH on thyroid receptors. Due to the increase in TBG's during pregnancy total and bound levels of T3 and T4 will be increased but free T3 (FT3) and free T4 (FT4) are unchanged. TSH is typically low when HCG is high during the first 10 wk and will increase after 10-12 wk when the level of HCG falls. Serum FT4 is highest when HCG is high and falls when HCG levels decrease[1].

Screening

There is controversy regarding universal screening for thyroid disease in pregnancy. In 2005 a joint statement from the American Thyroid Association, The Endocrine Society and the American Association of Clinical Endocrinologists supported universal screening in pregnancy to detect subclinical hypothyroidism (SCH) which at the time had been linked to poor neurocognitive outcomes in offspring[2]. In 2011, the American Thyroid Association issued a guideline which did not address screening but recommended that pregnant women with SCH and thyroid peroxidase antibodies should be treated with levothyroxine[3]. In 2015 the American College of Obstetricians and Gynecologists advised against universal screening because treatment of SCH has not been shown to improve neonatal outcomes[4].

Serum TSH is considered the most accurate method of evaluating thyroid function during pregnancy[3,5]. If no gestational age specific values are available for local laboratories, the American Thyroid Association makes the following recommendations for TSH: In the first trimester 0.1-2.5 mIU/L; in the second trimester 0.2-3.0 mIU/L; and in the third trimester 0.2-3.0 mIU/L[3] (Table 1).

Women with a history of hypothyroidism, women currently on medication for hypothyroidism, women with a history of Graves disease, and women currently on medication for Graves disease should be evaluated with a serum TSH at the first prenatal visit.

Anti-thyroid antibodies

There are three types of TSH receptor antibodies (TRAb); TSH stimulating antibodies (TSI), inhibitory antibodies known as TSH binding inhibitory immunoglobulins (TBII) and neutral antibodies.

TSH stimulating immunoglobulins are IgG antibodies that bind the TSH receptors in the thyroid causing increased production of T4 and T3. TSI antibodies cross the placenta and may cause neonatal thyrotoxicosis. TSI antibodies are not routinely used for the diagnosis of Graves disease but should be evaluated in pregnancy because of the risk to the fetus[6]. TSH binding inhibitory immunoglobulins competitively inhibit TSH receptors. TBII may cause hypothyroidism and paradoxically are often present in patients with Graves disease. The clinical relevance of neutral TSH receptor antibodies is unknown.

Thyroid peroxidase is an enzyme that oxidizes iodide to iodine which is then added to tyrosine for production of T3 and T4. Antibodies to thyroid peroxidase (TPO-Ab) indicate autoimmune mediated thyroid disease (*i.e.*, Hashimoto's). Greater than 80% of patients with overt hypothyroidism and approximately 50% of women with SCH have circulating TPO-Ab antibodies.

Hypothyroidism

Approximately 2%-3% of pregnancies are complicated by hypothyroidism[1]. Overt hypothyroidism is defined as an elevated serum TSH (> 2.5 mIU/L) with a low serum FT4, or TSH ⩾ 10 mIU/L regardless of the amount of FT4. Hypothyroidism is associated with two-fold increased risk of ovulatory dysfunction. In pregnancy there is an increased risk of fetal demise, miscarriage, abruption and decreased fetal growth[3-5]. Symptoms may be missed because they are often nonspecific and mimic common complaints of pregnancy such as fatigue, dry skin, constipation and hair loss.

All pregnant women with hypothyroidism should receive hormone replacement in the form of Levothyroxine with the goal of keeping the TSH in the normal to high normal range. No available data supports the addition of T3 or thyroid preparations other than levoT4 (levothyroxine). Treatment has been shown to decrease the risk of adverse pregnancy outcomes[1,3]. Women who enter pregnancy on levothyroxine will need to increase their dosage. The average increase in dose during pregnancy has been reported between 45%-50%. The increased requirement is mediated by an increase in Thyroxin-binding globulin, increased maternal circulating volume and placental destruction of T4.

A starting dose of levothyroxine can be calculated as 1-2 μg/kg per day or 100 μg/d. Serial determinations of TSH should be followed every 4-6 wk and the dose of levothyroxine should be adjusted in 25-50 mcg increments until TSH is within the desired range. After

Table 2 Studies of subclinical hypothyroidism in pregnancy

Ref.	Design	Method	Result	Conclusion
Pop *et al*[23], 1999	Cohort study	220 children were evaluated at 10 mo of age. Maternal TSH, FT4 and TPO antibodies were measured at 12 and 32 wk of pregnancy	Children of women with FT4 levels less than the 5th and 10th centiles at 12 wk had lower scores on the Bayley Psychomotor Development Index at 10 mo. No differences were found at 32 wk	FT4 < 10% ile at 12 wk is a risk factor for impaired psychomotor development in offspring
Haddow *et al*[7], 1999	Retrospective	62 women with high TSH	Children of these women did less well on 15 tests of intelligence. Average decrease in IQ was 4 points	Undiagnosed hypothyroidism adversely affects the fetal neurodevelopment
Henrichs *et al*[8], 2010	Population based cohort	Women with normal TSH and FT4 < 5th and 10th centile. Expressive vocabulary of children was evaluated by mother at 18 and 30 mo	Maternal TSH not related to outcome. Both mild and severe low FT4 associated with higher risk of expressive language delay at all ages. Severe had higher risk of nonverbal cognitive delay	Maternal low FT4 is a risk factor for early childhood cognitive delay
Lazarus *et al*[11], 2012	Randomized prospective	Women in screening group were tested and treated Women in the control group had stored samples which were tested after delivery and received no treatment during pregnancy	No difference in cognitive function between the two groups at 3 yr of age	Screening and treatment for hypothyroidism did not improve neurodevelopmental outcomes in the offspring
Ghassabian *et al*[24], 2014	Cohort	3727 mother-child pairs with prenatal thyroid fxn tests before 18 wk. FT4 < 5% of normal. MRI of childrens brains and IQ test at age 6 yr	Children of mothers with low FT4 scored 4.3 points lower on nonverbal IQ test. No morphologic difference by MRI	Maternal hypothyroxinemia has adverse effect on children's non-verbal IQ at school age
Chen *et al*[13], 2015	Prospective	106 babies born to mothers with SCH and 106 babies born to euthyroid mothers	Babies from both groups had similar scores on the Gesell development test	No neurodevelopmental deficit detected up to 24 mo in babies of mothers with SCH

TSH: Thyroid stimulating hormone; TPO: Thyroid peroxidase; FT4: Free T4; SCH: Subclinical hypothyroidism; MRI: Magnetic resonance imaging; IQ: Intelligence quotient.

delivery, the dosage can immediately be returned to the pre-pregnancy amount and TSH should be checked at the 6 wk postpartum visit.

SCH defined as a normal FT4 with an elevated TSH has been the subject of much debate. In 1999, the *New England Journal of Medicine* published a study that evaluated the children of 62 women with high thyrotropin (TSH) and low thyroxine (T4) levels compared to the children of 124 controls with normal values[7]. Children in the study group had lower IQ scores (average 4 points lower) and performed less well on fifteen standard tests of attention, language, visual-motor performance and reading ability. They concluded that SCH had an adverse effect on the neurologic well-being of the fetus and that routine screening for thyroid disorders should be performed during pregnancy. After that initial publication, multiple observational studies reported a possible association between SCH and decreased intelligence in offspring[8-10].

In 2012 the Controlled Antenatal thyroid screening study was published which randomized pregnant women with SCH to treatment *vs* no treatment[11]. The primary outcome of interest was offspring IQ at age 3. The study found no difference in IQ between the two groups. The protocol for the second wave of the Controlled Antenatal Thyroid screening (CATS Ⅱ) study was published in 2014 and will assess the cognitive function of the same group of children between ages 7 and 10 years[12].

Chen *et al*[13] reported on a prospective study of 106 infants of mothers with SCH compared to 106 infants of euthyroid mothers. They reported no differences in neurodevelopment up to 24 mo between the two groups.

In 2013 the European Thyroid Association published a guideline on the management of SCH. They defined two categories of SCH based on the level of elevation of serum TSH; mild (4.0-10.0 mU/L) and severe (> 10 mU/L)[14]. They did not address SCH in pregnancy but recommended treatment for patients < 65 years of age with TSH in the severe range. They suggested a trial of treatment in patients with symptoms and mild elevations of TSH. Table 2 displays relevant studies addressing SCH in pregnancy.

Neither Screening for SCH, nor treatment of SCH in pregnancy with the goal of improving neurocognitive outcomes in offspring is supported by current evidence.

Fetal surveillance

No studies have addressed the frequency of growth scans or the need for antenatal surveillance in patients with hypothyroidism. In cases of overt disease, particularly those patients on medication, monthly growth scans may be a reasonable consideration.

Table 3 Diagnosis and treatment of thyroid disease in pregnancy

	TSH	FT4	FT3	Rx	Goal of treatment
Hypothyroid	↑	↓	↓	Levothyroxine starting dose 1-2 mcg/kg daily	Keep TSH normal range
Hyperthyroid	↓	↑	↑	PTU 50-150 mg TID in first trimester methimazole 10-40 mg BID or TID after first trimester	Keep FT4 high normal "watch for agranulocytosis"

TSH: Thyroid stimulating hormone; FT3: Free T3; FT4: Free T3; BID: Twice daily; TID: Three times daily; PTU: Propylthiouracil.

Breastfeeding

Thyroxine is a normal component of breast milk. Levothyroxine is considered safe during breastfeeding. Thyroid hormones are necessary for lactation and overt hypothyroidism is associated with a low milk supply. Thyroid hormone replacement has been shown to improve the milk supply in hypothyroid patients[15].

HYPERTHYROIDISM

Hyperthyroidism complicates up to 0.4% of pregnancies. Hyperthyroidism is defined as a suppressed TSH with elevated FT4. Left untreated in pregnancy there is an increased risk of miscarriage, stillbirth, low birth weight, and preterm delivery. Maternal complications include thyroid storm and a 5-fold increase in the risk of preeclampsia and congestive heart failure.

During pregnancy, it is important to differentiate Graves disease from gestational hyperthyroidism due to the effect of HCG on the maternal thyroid. Signs of overt hyperthyroidism include tremor, nervousness, heat intolerance, irritability and weight loss, and typically will not occur in patients with gestational hyperthyroidism. Absence of a goiter or ophthalmopathy also favors gestational disease. If there is doubt, thyroid receptor antibodies and T3 can also be obtained. If antibodies are present the diagnosis is more likely Graves disease.

Anti-thyroid mediation should not be used in patients with gestational hyperthyroidism. The serum T4 will normalize between 14-18 wk.

The goal of treatment is to keep FT4 levels in the high normal range with the minimum required dose of anti-thyroid medication. Two medications are commonly used in pregnancy: Methimazole and propylthiouracil (PTU). A third medication carbimazole is metabolized to methimazole. Both drugs cross the placenta and may suppress the fetal thyroid. Methimazole embryopathy has been identified in patients who took the drug during the first trimester and consists of choanal or esophageal atresia, cutis aplasia, minor dysmorphic features and developmental delay. Propylthiouracil has been associated with liver damage. The Food and Drug administration of the United States and the American Thyroid Association recommend PTU in the first trimester with a switch to methimazole in the second trimester. This avoids the teratogenic effect of methimazole in the first trimester and decreases the risk of hepatotoxicity from long term use of PTU[4]. The dose of PTU is 50-150 mg TID. The dose of methimazole is 10-40 mg 2 to 3 times a day.

Approximately 10% of women on antithyroid medication develop a transient leukopenia which does not require medication cessation. Agranulocytosis is a decrease in granulocytes (neutrophils, eosinophils and basophils) which can result in life-threatening infections. Agranulocytosis occurs in 1% of patients on medication; may develop suddenly and is an indication for medication discontinuation. Patients should be warned of this complication and instructed to discontinue medication if a fever or sore throat develops and to have a complete blood count for evaluation.

FT4 should be checked every four weeks during pregnancy. This is to ensure that target values are achieved and maintained. This information is summarized in Table 3.

Thyroid storm

Thyroid storm or crisis is a rare life threatening complication of uncontrolled hyperthyroidism. Thyroid storm is characterized by fever (> 103 F), tachycardia, hypertension and impaired thinking. Evaluation of TSH and FT4 will show suppressed TSH with elevated FT4 though the amount of FT4 does not correlate with symptom severity. Due to the life threatening nature of thyroid storm for both the mother and fetus, aggressive management is indicated. Admit to an intensive care unit with Ⅳ fluids and maintenance of electrolytes. Give Tylenol for hyperpyrexia. Antithyroid medication should be given immediately either 30 mg of methimazole or 300 mg of PTU every 6 h. An alternate dosing schedule recommended by the American Congress of Obstetricians and Gynecologists is a loading dose of 1000 mg of oral PTU followed by 200 mg orally every 6 h. The thioamides will block the synthesis of T3 and T4. One hour after starting medication give iodine either orally as 10 drops of Lugol's solution every 8 h or 1 g of sodium iodide Ⅳ every 8-12 h. Hydrocortisone 50-80 mg every 8 h for 3 doses or Dexamethasone 2 mg Ⅳ every 6 h for four doses should be given to block the peripheral conversion of T4 to T3. To control the tachycardia consider a beta blocker; esmolol, propranolol and labetolol are all equally efficacious (Table 4).

Radioactive iodine is commonly used to ablate the thyroid in cases of Graves thyrotoxicosis. This technique should not be used during pregnancy. The fetal thyroid begins to concentrate iodine at approximately 14 wk and is dependent on maternal sources. Radioactive

Table 4 Six steps for treatment of thyroid storm

1 Admit to intensive care unit	IV fluids and watch electrolytes
2 Tylenol 650 mg q6 h	For hyperpyrexia
3 Loading dose of 1000 mg PTU orally then 200 mg orally q6 h; alternate dosing 300 mg PTU q6 h	Will block synthesis of T3 and T4
4 Iodine supplementation 10 drops of Lugol's solution q8 h OR 1 g sodium Iodide IV q8-12 h Iodine allergy use lithium carbonate 300 mg PO q6 h	Blocks release of hormone from the thyroid gland
5 Hydrocortisone 50-80 mg q8 h for 3 doses OR Dexamethasone 2 mg IV q6 h for 4 doses	To block peripheral conversion of T4 to T3
6 Beta blocker Labetolol 300 mg TID may increase to a max dose of 800 mg TID but watch blood pressure	To control the tachycardia – use cautiously in heart failure

PTU: Propylthiouracil; TID: Three times a day.

iodine will destroy the fetal thyroid resulting in congenital hypothyroidism. However treatment prior to 12 wk is not associated with damage to the fetus.

If thyroidectomy is required during pregnancy, it is ideally performed in the second trimester due to a perhaps unjustified concern for teratogenesis of anesthetics in the first trimester, and possible onset of labor resulting in extreme prematurity during the third trimester. There is also less risk of supine hypotension from an enlarged uterus in mid trimester.

During an acute thyroid crisis, the fetal status will not be reassuring and fetal death is a significant risk. Nonetheless, delivery should not be undertaken until the maternal status is stable.

Thyrotoxic heart

Thyrotoxic heart failure is a direct result of the action of thyroid hormones on the heart. Common manifestations are left ventricular hypertrophy, abnormal rhythm usually sinus tachycardia or atrial fibrillation, pulmonary hypertension and diastolic dysfunction. Thyrotoxic heart is treated as thyroid storm with admission to the intensive care unit with cardiac monitoring, PTU to inhibit synthesis, and iodide to block release of hormone from the gland. Beta blockers should be used with caution with heart failure. Volume overload can be reversed with diuretics. The patient should be euthyroid before attempting cardioversion for atrial fibrillation because spontaneous resolution to sinus rhythm may occur and even if successfully cardioverted, atrial fibrillation is likely to recur if hyperthyroidism is the cause and has not been corrected[16].

Fetal surveillance

Titers of TSI antibodies should be measured by 24-28 wk in all women with a present or past history of Graves disease. Titers more than three times the upper normal limit are a significant risk to the fetus and warrant close follow-up. In the setting of high TSI titers the fetus should be followed with monthly growth scans for signs of hyperthyroidism which may include tachycardia, goiter, growth restriction and congestive heart failure. Weekly or twice weekly non-stress testing in the interval between growth scans can be considered.

In our practice we obtain monthly growth scans on all patients on anti-thyroid medication between 24-36 wk (Table 5).

Table 5 Frequency of antenatal surveillance

	Ultrasound	Antenatal testing (Nonstress test or Biophysical Profile)
Hyperthyroid	Monthly	Twice weekly if poorly controlled
Hypothyroid	No recommendation Consider monthly	No recommendation

Breastfeeding

Approximately 0.025% of the dose of PTU is excreted in breast milk. In contrast, the amount of methimazole excreted in breast milk is equal to maternal serum levels of the drug[17].

In a study of 42 breast fed infants of mother's on 30 mg of methimazole daily, all infants had normal thyroid function[18]. In a follow up study the authors looked at the same 42 children in comparison to breastfed infants of euthyroid mothers. They were followed up at 18 mo and at 86 mo of age. There was no difference in thyroid function or development between the two groups[19].

Breastfeeding is considered safe in women on doses of PTU less than 300 mg per day or methimazole 20-30 mg per day. It is recommended to take the medicine in divided doses immediately after feeding. Babies of mothers on these medications should be followed with thyroid function tests[3].

POSTPARTUM THYROIDITIS

Postpartum thyroiditis is an autoimmune mediated destructive thyroiditis occurring in the first year postpartum. It affects up to 21% of postpartum patients[20]. It is associated with the presence of anti-thyroid antibodies. The clinical course may vary but classically postpartum thyroiditis occurs in two phases. The first phase is a transient thyrotoxicosis occurring 2 to 6 mo postpartum, followed by a hypothyroid phase which may present between 3 mo up to one year postpartum. During the thyrotoxic phase actual symptoms are usually mild. The thioamides are not effective in treatment because the symptoms are due to autoimmune destruction of the gland. The goal of treatment is symptomatic relief usually with Beta Blockers. The thyrotoxic phase always resolves spontaneously. Patients may present with isolated hyperthyroidism (32%) or hypothyroidism (43%). It is estimated that 10% to 50% of women with postpartum thyroiditis remain hypothyroid at the end of the first postpartum year. Women who recover should

be screened annually for hypothyroidism[3].

THYROID CANCER IN PREGNANCY

The prevalence of thyroid cancer in pregnancy is estimated at 14.4/100000[3]. Thyroid nodules may be more common during pregnancy and the prevalence increases with parity. In the presence of a nodule, a serum TSH and FT4 should be drawn and an ultrasound of the thyroid and neck performed. If the ultrasound is suggestive of malignancy, fine needle aspiration should be performed. Thyroid function tests are usually normal in patients with thyroid cancer.

If the cytology is benign surgery is not indicated unless there is rapid growth that interferes with breathing or swallowing.

If cytology is suggestive of medullary, papillary, follicular or anaplastic carcinoma surgery should be offered. Women with well-differentiated thyroid carcinoma can be offered deferral of surgery until the postpartum period without concern that the delay will worsen the prognosis. If surgery is deferred ultrasound of the neck should be performed at least each trimester. Rapid tumor growth is a contraindication for surgery deferral[21].

Post-surgery, some patients require remnant ablation with radioactive iodine. As discussed previously, this should not be done during pregnancy. Women should not breastfeed while undergoing radioactive iodine treatment.

Thyroid hormone replacement should be initiated as soon as possible after surgery to maintain the TSH in normal range. Future pregnancies should be delayed 6 mo to one year to confirm remission of cancer and to achieve a stable dose of levothyroxine[21,22].

CONCLUSION

Diseases of the thyroid are common in pregnancy and knowledge of management is indispensable to anyone providing care to pregnant women. In this paper I have provided a brief review of diagnosis and management of thyroid disease during pregnancy and in the puerperium.

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MINIREVIEWS

Role of ultrasound imaging in advancing treatment of female patients with pelvic floor mesh complications

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Abstract

Application of vaginal mesh for stress urinary incontinence has seen widespread use due to its relatively short operative time in combination with its efficacy in treatment. However, vaginal mesh is not without its drawbacks and can lead to mesh erosion or extrusion, infection, dyspareunia, and recurrence of incontinence. Vaginal mesh complications can lead to feelings of hopelessness, isolation, shame, and emotional distress. Furthermore, failure to identify and address these complications in a timely manner can be permanently damaging to patient health. It is vital to be able to identify mesh complications early. Various imaging methodologies exist to visualize vaginal mesh placement and complications, including ultrasound, magnetic resonance imaging (MRI), and computed tomography (CT). This invited review paper focuses on the role of ultrasound in mesh visualization, mesh complication identification, and operative planning in the event of subsequent surgical mesh revision. Polypropylene mesh is echogenic on ultrasound, making it a useful tool for visualizing post-operative mesh placement. Transperineal, translabial and endovaginal ultrasound technique use has been described in the pre- and peri-operative setting to identify mesh in complex cases. Efficacy and practicality of CT and MRI use in identifying mesh in these cases is limited.

Key words: Mesh complications; Imaging; Ultrasound; Computed tomography; Magnetic resonance imaging; Mesh revision; Stress urinary incontinence; Erosion; Extrusion; Dyspareunia

Core tip: Pelvic ultrasound is a valuable and inexpensive technique that can be used both for localization, diagnosis, preoperative planning, and intraoperative guidance when dealing with mesh complications.

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INTRODUCTION

Urinary incontinence can affect up to 50% of women during their lifetime[1]. In the United States alone, the direct costs of urinary incontinence is upwards of $10 billion dollars per year with over $200 million directed towards surgical intervention[2,3]. Given the prevalence and cost of SUI, safe, effective and efficient treatment is imperative. Initial measures are generally conservative and involve some type of pelvic floor muscle training; other nonsurgical options include medical therapy, estrogen therapy, and injectable urethral bulking agents[4,5]. Begun in 1995, application of vaginal mesh for stress urinary incontinence has seen widespread use in recent years given its relatively short operative time in combination with its proven efficacy[6].

Vaginal mesh, although the preferred method of surgical intervention for stress urinary incontinence, is not without its drawbacks. In 2011, the Food and Drug Administration (FDA) issued an updated statement warning of potential side effects of vaginal mesh including mesh erosion or extrusion, infection, dyspareunia, and recurrence of incontinence[7]. Since the FDA warning, several studies have focused on mesh side effects and their etiologies.

Pain lasting longer than 6 wk after the initial operation is seen in about 2% of patients[8]. A retrospective study of 127 patients in 2009 showed transvaginal taping to have anywhere from 4%-10% incidence of mesh erosion (most commonly with anterior compartment repair)[9,10]. Mesh contraction (decreased mesh surface area) is another postoperative complication that occurs in roughly 5% of patients[11], though there is some evidence to suggest that this is actually due to mesh folding during surgical placement[12,13]. Commonly posited is the idea that surgeon skill is the most important factor in limiting mesh complications[13-15]. Regardless, reoperation for postoperative complications secondary to mesh insertion is not uncommon and has been estimated to occur at a rate of about 10% with some studies having reoperative rates as high as 29%[9,16,17]. Repeat operations can be very involved, ranging from mesh removal to abdominal cystorraphy or partial cystectomy depending on the exact mesh complications[18].

While initial physical symptoms such as irritation, vaginal or pelvic pain, and dyspareunia may be the first manifestations of complications, vaginal mesh complications in particular can lead to feelings of hopelessness, isolation, shame, and emotional distress. Failure to identify and address these complications in a timely manner can be permanently damaging to patient health[19]. Given the common nature of mesh side effects and high rate of surgical intervention for them, it is increasingly important to be able to identify mesh complications early. Various imaging methodologies exist to visualize vaginal mesh placement and complications, including ultrasound, magnetic resonance imaging (MRI), and computed tomography (CT). This paper focuses on the role of ultrasound in mesh visualization, mesh complication identification, and operative planning in the event of subsequent surgical mesh revision.

DIAGNOSTIC ULTRASOUND

Common postoperative complaints such as dysuria, pelvic pain, and dyspareunia are generally elicited through patient history and physical examination. Clinical examination alone, however, can be insufficient in determining mesh-related complications[20,21]. Polypropylene mesh is echogenic on ultrasound, making it a useful tool in visualizing post-operative mesh placement[22,23]. Ultrasound can aid in the visualization of the pelvic floor, sling positioning, and urethral length as well as mesh length and thickening[13,24-28]. Various different methods of sonography have been used clinically with some success including transperineal, translabial, and endovaginal ultrasound. It should be noted that there is very little data comparing these different sonographical approaches; as such, they will be described independently here.

Fleischer *et al*[29] describe in detail a combined 2D and 3D transperineal ultrasound technique that is effective in visualizing mesh location and length in uncomplicated transvaginal tape (TVT) as well as urethral angulation or stenosis in patients with postoperative lower urinary tract symptoms. Eisenberg *et al*[12] described similar success with transperineal ultrasound using a combined 3D/4D technique in visualizing mesh location, anterior and posterior mesh arms, and mesh folding after abdominal sacrocolpopexy. Both transperineal techinques involve dynamic ultrasound, observing patients at rest and maximal Valsalva.

Denson *et al*[28] describe a 3D, endovaginal technique that allows for adequate visualization of mesh location. Their method is non-dynamic and has resulted in recognizable sonographical patterns associated with mesh contraction and extrusion allowing for easier identification of postoperative complications. Velemir *et al*[27] utilized a 2D endovaginal technique with dynamic imaging that also allowed for localization of mesh, measurement of mesh thickness, and identification of mesh contraction. Other endovaginal techniques exist including the use of 4D proprietary software to aid in imaging with similar reliability in observing mesh dimensions[13]. It should be noted, however, that endovaginal ultrasound can be insufficient when attempting to identify mesh arm dimensions depending on sling location[20].

Other sonographical techniques include introital and translabial ultrasound. Introital ultrasound - placing

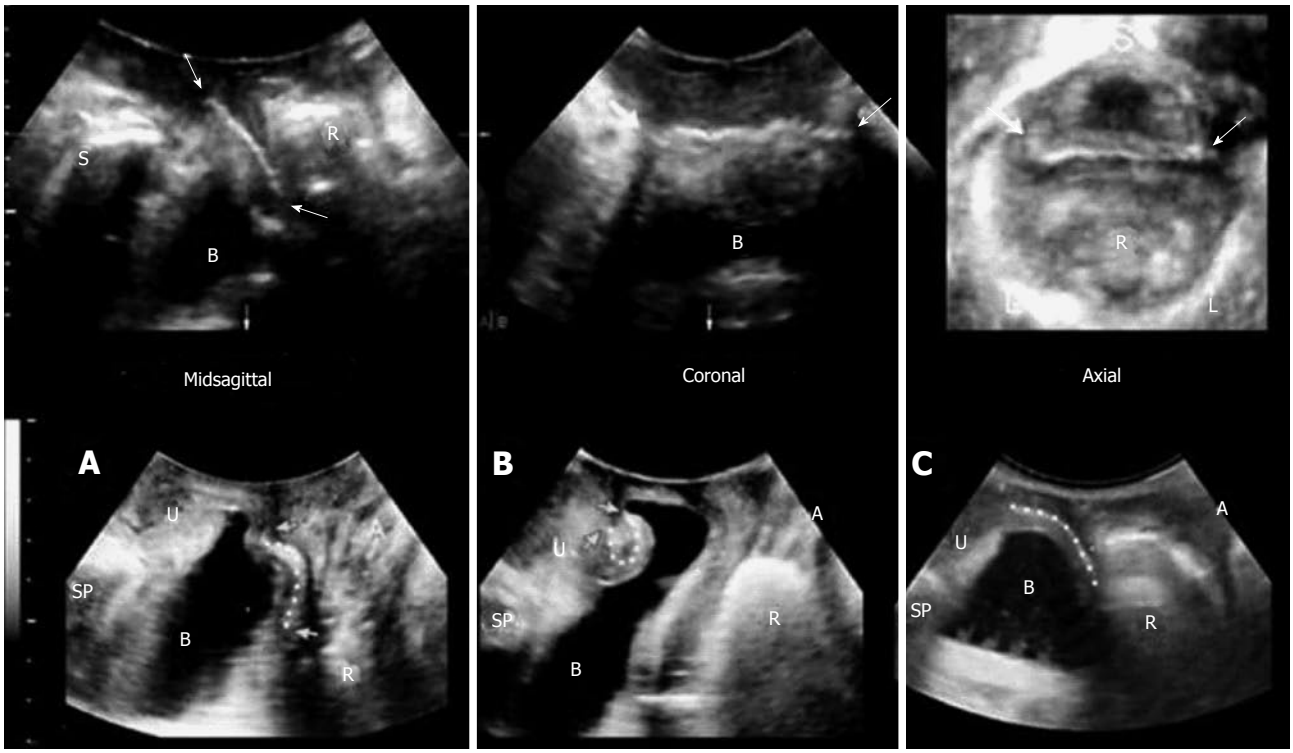

Figure 1 Pre-operative and intraoperative images of patients at University of Illinois-Chicagp Hospital with mesh complications. A: Anterior; B: Apical; C: Global recurrence after mesh repair.

the transducer over the external urethral orifice - can be used to evaluate for urethral pathology as well as to image retropubic slings with or in place of endovaginal techniques[26,30]. A combined 3D/4D translabial ultrasound technique has also been shown to reliably assess mesh location, length, and erosion in transobturator slings. This technique may also have benefit in its ability to detail mesh arm location indirectly *via* measuring mesh axis rotation[31].

DIAGNOSTIC ALTERNATIVES TO ULTRASOUND

While clinical examination and ultrasound are the most commonly reported methods of postoperative assessment in TVT and transobturator tape (TOT), an emerging modality involves the use of dynamic MRI (dMRI) in pelvic floor disorders. dMRI can be used diagnostically to evaluate preoperative pelvic floor dysfunction and pelvic organ prolapse[32]. A 2006 study of 20 women also showed MRI to be useful postoperatively in evaluating the retropubic portion of vaginal tape[23]. Most types of mesh used for TVT are not routinely visible on MRI, however, and require novel techniques such as MRI-visible mesh implants for visualization[33,34]. Thus, the role of MRI in postoperative evaluation is to provide information on the pelvic floor itself. dMRI can be used to locate the postoperative position of the Pouch of Douglas as well as any changes in vaginal axis[35]. dMRI also has utility in evaluating for prolapse recurrence and has been shown to be more sensitive than quality of life questionnaires in at least one study[36]. And while MRI is incapable of providing adequate mesh visualization, it has been shown to detect postoperative mesh inflammation and fibrosis due to increased intensity transmitted by these processes[37,38].

Routine evaluation of postoperative complications has not been recommended, however, and there is little data to suggest its use for this purpose[23,38]. Given the cost, time commitment, and potential for patient anxiety with dMRI when compared to sonography, it is a second line imaging modality. Presently, dMRI is useful only in the immediate post-operative setting to visualize the pelvic floor itself and is not used routinely in management of mesh complications.

Presently there is no role for CT imaging in postoperative evaluation of TVT or TOT and there is little to no literature discussing it (Figure 1).

ROLE OF ULTRASOUND IN RE-OPERATIVE PLANNING AND INTRAOPERATIVE GUIDANCE

In addition to its diagnostic value, ultrasound can be used for operative planning in cases where mesh revision is necessary. For postoperative pain, conservative therapy with short term rest and pain management is a reasonable first step in treatment. Intractable pain, however, may require more invasive measures. Mesh incision, mesh excision, or obturator/pudendal neurolysis are all options depending on the etiology of

the pain[15]. The ability to determine mesh dimensions with ultrasound can be used to assist in surgical decision making preoperatively[20,27].

Staack *et al*[21] first used preoperative translabial ultrasonography to determine sling type, location, and erosion into the urethra or bladder in 51 patients with previously placed suburethral slings and post-procedural lower urinary tract symptoms. Static, dynamic, and 3D techniques in conjunction allowed for visualization of the mesh in relation to the bladder neck and urethra, location of the mesh arms, and identification of urethral hypermobility and kinking or potential mesh folding. Sling location and type were then all confirmed intraoperatively. Thus, ultrasound had a 100% sensitivity in identifying sling location regardless of sling type even in patients without previous operative reports.

Intraoperative ultrasound is the next step in improving outcomes for patients with mesh complications. Though the literature is sparse, two case reports indicate that sonography could prove invaluable in difficult cases. Rostaminia *et al*[39] describes a case report of levator ani repair for a 33-year-old woman with bilateral levator ani separation after childbirth. With the aid of intraoperative 3D endovaginal ultrasound, the torn ends of the levator ani muscles were tagged with hooks to allow for identification and manipulation. Similarly, Mukati *et al*[40] reports the case of a 71-year-old woman with previous TVT surgery presenting 3 years later with incomplete bladder emptying requiring self-catheterization. Given the severity of voiding dysfunction, the patient underwent sling revision. As the previous sling could not be identified intraoperatively, a combined 3D-2D endovaginal ultrasound technique was used to localize and resect the sling. The patient's symptoms resolved after mesh resection.

At our institution, we routinely use pelvic ultrasonography for preoperative diagnosis and operative planning as well as intraoperative guidance. We have found that pre- and intraoperative ultrasound use can be used in complex revision cases to better delineate mesh position and thus reducing the extent of resection required to correct mesh-related complications. Given the high success rate of ultrasonographic visualization of mesh location as well as the comprehensive picture provided by this technique, translabial ultrasonography can be very valuable in preoperative planning and intraoperative guidance for surgical correction of suburethral sling.

CONCLUSION

Meshes have a vital role in the treatment of female pelvic organ prolapse as well as urinary incontinence. Despite improvements in surgical techniques and available mesh products, there is still a significant morbidity associated with complications of mesh surgery; serious complications are not rare. These complications and their clinical manifestations such as pain, urinary tract dysfunction, or sexual dysfunction can be difficult to manage. Two major challenges are early recognition of complications and their subsequent surgical management. Delayed recognition leads to patient dissatisfaction; delayed surgical management may make cases much more difficult. As such, innovative techniques are desired in the armamentarium for surgeons treating these complications. Pelvic ultrasound is a valuable and inexpensive technique that can be used both for localization, diagnosis, preoperative planning, and intraoperative guidance when dealing with mesh complications.

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MINIREVIEWS

Evaluation of sentinel lymph nodes in vulvar, endometrial and cervical cancers

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Abstract

Sentinel lymph node (SLN) biopsies are a sensitive tool in evaluating lymph nodes for multiple cancers, and in some diseases they decrease morbidity in both the short- and long-term. SLN detection in gynecologic malignancies has been studied extensively over the past decade. We review the current literature on SLN dissection in vulvar, endometrial and cervical cancers. Large, well-designed trials in each of the three types of cancer have demonstrated high sensitivity and low false-negative rates when SLN biopsy is performed in the correct patients and with an appropriate technical approach. In all of these cases the addition of ultra-staging to conventional pathology yields increased detection of micrometastatic disease. Biopsy of the sentinel nodes is feasible and safe in early vulvar malignancies, with multiple studies describing low recurrence rates in those women who have with negative SLNs. There does not appear to be a survival benefit to lymphadenectomy over SLN biopsy and quality of life is improved in women undergoing SLN biopsy. Optimal treatment strategies for women with positive nodal biopsies, particularly in cases with micrometastatic disease, remain unclear. Multiple large studies investigating the utility of SLN biopsy in endometrial malignancy have found that sentinel nodal status is a reliable predictor of metastases in women with low-risk disease. Prospective studies are ongoing and suggest sentinel nodal detection may soon become widely accepted as an alternative standard of care for select cases of endometrial cancer. In cervical cancer, SLN biopsy is accurate for diagnosing metastatic disease in early stage tumors ($\leqslant$ 2 cm diameter or stage $\leqslant$ IB2) where the risk of metastasis is low. It is unknown if women who undergo SLN biopsy alone will have different survival outcomes than women who undergo complete lymphadenectomy in these cases. In a specific population of women with vulvar cancer, SLN dissection is an effective and safe alternative to complete dissection. It can be offered as an alternative management strategy in these women. In women who do undergo SLN biopsy, it is associated with improved quality of life. Promising evidence supporting the utility of SLN dissection in endometrial and cervical cancer continues to emerge, and it may soon become a reasonable option for select patients. However, continued research and refinement of appropriate patient selection and long-term follow-up are necessary.

Key words: Gynecologic malignancies; Sentinel lymph node; Endometrial cancer; Cervical cancer; Vulvar cancer

Core tip: In a specific population of women with vulvar cancer, sentinel lymph node (SLN) dissection is an effective and safe alternative to complete dissection. It can be offered as an alternative management strategy in these women. Sentinel node biopsy is also associated with an improved quality of life. Promising evidence supporting the utility of SLN dissection in endometrial and cervical cancer continues to emerge, and it may soon become a reasonable option for select patients. However, continued research and refinement of appropriate patient selection and long-term follow-up are necessary.

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INTRODUCTION

Sentinel lymph node (SLN) biopsy has become the standard of care in multiple non-gynecologic malignancies. As a surgical technique, SLN biopsy was initial developed for the treatment of penile cancer in 1977, and was adopted into treatment strategies for breast cancer and melanoma shortly thereafter[1]. The SLN is the first node within a lymphatic chain which drains the primary tumor. As the first in a chain of lymph nodes, theoretically the sentinel node will be the first to receive metastatic disease. If the sentinel node is negative then, it is assumed that the remainder of the lymphatic basin is also without metastatic disease. One of the distinctive benefits of SLN biopsies is the opportunity to avoid "over-staging", the removal and dissection of non-diseased tissues in an effort to identify the extent of a patient's cancer. Furthermore, with fewer lymph nodes to examine, pathologists can performed more in-depth analysis on the relatively smaller volume of available tissues.

Application of SLN biopsy techniques in gynecologic malignancies has been studied extensively over the past decade as nodal dissections in these cancers can lead to long-term morbidities. In breast and vulvar cancers SLN biopsy is predictive of the disease status of the lymphatics and has demonstrated decreases in the significant short and long-term morbidities which are seen in complete lymphadenectomy. However, sampling of the SLN in other gynecologic malignancies is still investigational. We will review the continually growing body of literature supporting SLN biopsy in the treatment of vulvar cancer, as well as reviewing the current evidence behind the use of SLN biopsy in endometrial and cervical cancers.

VULVAR CANCER

Vulvar cancer is relatively uncommon, accounting for 5% of gynecologic malignancies[2]. Five thousand one hundred and fifty new cases of vulvar cancer and 1080 deaths attributable to the same are anticipated in the United States in 2015[2]. Traditional radical vulvectomy with en bloc inguinofemoral lymphadenectomy was associated with high morbidities including 70% of women suffering from chronic lymphedema[3]. Over time, in order to minimize surgical morbidity, the standard of care has shifted. It is now standard practice to perform vulvectomy or radical wide local excision, with deep or superficial inguinal femoral node dissection, instead of radical vulvectomy. Unfortunately, even with this less radical surgery complication rates remain significant. A 2013 review of complications in inguinal femoral nodal dissection reported lymphedema in 14%-48% of patients and formation of lymphocele in 7%-40% of patients. Furthermore they report wound infections in 21%-39% of patients, cellulitis in 21%-57% and wound breakdown in 17%-39% of cases[4]. This significant morbidity has lead to the development of less invasive and less morbid methodologies, particularly the use of SLN biopsy. Indeed, at experienced centers and with select patients SLN sampling is the new standard of care[5].

Survival rates in vulvar cancer are highly dependent upon inguinal femoral lymph node status; thus their evaluation is critical[6]. Five-year survival rates are 96%, 80% and 12% in women with negative inguinal femoral lymph nodes, two or less positive lymph nodes and more than two positive lymph nodes, respectively. Survival continues to falls significantly with increasing numbers of positive nodes beyond 2[7]. However, the majority of women that undergo lymphadenectomy will not have nodal disease. It has been shown that tumor size is prognostic of the risk of lymph node metastases with only 10%-15% of patients with vulvar tumors less than 20 mm having inguinal femoral metastases[6,8]. This suggests that up to 90% of patients could be spared the morbidity of complete lymphadenectomy if appropriately identified.

Levenback *et al*[9] first described the application of SLN biopsy in vulvar malignancy, adopting technical features from the melanoma literature[9,10]. Multiple subsequent studies have demonstrated the safety, feasibility and low false negative rates of SLN biopsy in these patients[10-15]. The majority of studies use a dual-injection approach with pre-operative radioactive tracer injection of technetium-99 sulfur colloid (^{99m}Tc) and isosulfan or methylene blue injection in the operating room[16].

A systematic review published in 2015 by Covens *et al*[17] reported high rates of sentinel node detection and low false-negative rates. Although they report significant variability among studies, the overall detection rate was 86.9%. Twenty five studies analyzed in this review reported SLN biopsy followed by inguinal femoral nodal dissection; the false negative rate for sentinel node biopsy among these studies was 6.6%[17].

GOG 173 was a large prospective multicenter trial comparing SLN biopsy to inguinal femoral lymphadenectomy. Four hundred and fifty-nine patients with tumors between 2 and 6 cm and without signs of affected lymph nodes on clinical exam were included in the trial. All women underwent lymphatic mapping using radioactive tracer and blue dye. SLN biopsy was performed when possible, followed by complete lymphadenectomy. One or more SLN was found in 412 women, and 132 (31.6%) had lymph node metastases. Sensitivity and false-negative predictive value (FNPV) were 91.7% and 3.7%, respectively. Both were impacted by the size of the tumor; in those lesions less than 4 cm in diameter the FNPV was 2%, while it rose to 7.4% when size ranged from 4-6 cm[18]. Another large multicenter study, conducted by Hampl *et al*[19], evaluated accuracy and feasibility of SLN biopsy in women with T1-T3 vulvar cancer. They reported a 98% detection rate, 92.3% sensitivity and 7.7% false negative rate[19].

This study included patients with large lesions (> 4 cm), and a wide range of experience in SLN biopsy among participating surgeons, likely contribute to the higher false negative rate.

One of the distinct advantages of SLN biopsies is the opportunity for ultra-staging. Levenback *et al*[18] found that the mean total of lymph nodes resected with complete inguinal femoral dissection was 8.94, as compared to a mean of 1.54 lymph nodes with sentinel biopsy. With fewer nodes, the pathologist can focus efforts on examining smaller, serial sections, a technique known as ultra-staging. Pathologic examination of a SLN is likely to identify smaller metastases to these nodes. Those metastases measuring 0.2-2 mm in size are referred to as micrometastases, and while their clinical significance is not entirely understood in all gynecologic cancers they have been identified as predictors of relapse in melanoma and breast cancer. In addition, techniques such as immunohistochemical staining and reverse-transcriptase polymerase chain reaction analysis for cytokeratin expression can be added to hematoxylin and eosin staining to potential increase tumor cell detection rates[20]. Studies evaluating the impact of these methods on detection of metastatic tumor cells are varied and report a range of results. However, this is at least partially due to a lack of uniform techniques used across institutions[21]. Current expert opinion argues that the potential benefit provided by ultra-staging and immunohistochemical staining of sentinel nodes outweighs the risks of increased time, cost, and identification and treatment of metastases of uncertain clinical significance[17].

Initial studies evaluated SLN biopsy followed by complete nodal dissection. However, the large multicenter GROningen International Study on Sentinel nodes in Vulvar cancer (GROINSS-V-I) was the first to evaluate the safety of SLN biopsy alone.

Inclusion in this multicenter observational study required that patients have unilateral and unifocal tumors of the vulva smaller than 4 cm in diameter; only squamous cell cancers were included. Women with negative SLN evaluation following completion of ultra-staging underwent serial surveillance, while those with positive SLN underwent inguinal femoral lymphadenectomy. Of the 403 patients enrolled, 276 had negative SLNs. During a median follow-up period of 35 mo there were 8 episodes (2.9%) of groin recurrence. Furthermore, the investigators found a decrease in morbidity for patients who had only SLN biopsy when compared to women who underwent complete nodal dissection. Perhaps the best illustration of this is in the incidence of postoperative lymphedema. Less than 2% of women who had only SLN biopsy experienced lymphedema, compared to 25.2% of women who underwent complete lymphadenectomy[22].

Unfortunately, groin nodal recurrence of vulvar cancer carries a dire prognosis, with 5 year survival rates ranging from 0%-17%[23,24]. In their meta-analysis, Covens *et al*[17] included an analysis of recurrence rates when women were followed after SLN biopsy, superficial inguinal nodal dissection, or complete nodal dissection (involving dissection of the deep femoral lymph nodes). Twenty-three studies were included, with a broad range of follow-up durations. They reported a 6.6% (4.4-9.0) recurrence rate in women undergoing superficial nodal dissection and a 1.4% (0.4-2.9) recurrence rate with complete inguinal femoral dissection. Comparatively, the recurrence rate with sentinel node biopsy was between these two values, at 3.4% (1.8-5.4)[17].

Identification of appropriate patients for sentinel node biopsy instead of complete inguinal femoral lymphadenectomy is another important factor. GROINSS-V-I reported an increased risk of recurrence in women with multifocal disease (11.8% *vs* 2.3%), suggesting that sentinel nodal biopsy is likely inadequate in this subset of patients[22]. Tumor size is another important predictor of nodal metastases. The largest studies evaluating SLN biopsy excluded patients with clinically suspicious nodes, and most would recommend complete groin lymphadenectomy in this group of patients[22]. GOG 173 demonstrated differences in both the rate of nodal metastasis and the false-negative SLN biopsy rate when comparing tumors of different sizes. In women with tumors measuring 2.0-3.9 cm the rates of nodal metastasis and false-negative SLN biopsy were 26.4% and 2%, respectively. Comparatively, women with tumors measuring 4-6 cm had nodal metastasis in 40.9% of cases and the false-negative rate was 7.4%[25]. Furthermore, tumors near the midline have increasing odds of bilateral lymphatic drainage, with

tumors located < 2 cm from the midline accounting for the majority of recurrences after SLN biopsy[26].

Much of the research on SLN biopsy began as an effort to decrease morbidity from the surgical management of vulvar malignancy, which raises the question "Is quality of life (QoL) better for women that undergo SLN biopsy alone?". While all studies have shown decreased treatment related morbidity with SLN biopsy, a few studies have also shown that SLN biopsy improves overall QoL for women who undergo SLN biopsy compared to women who undergo complete groin lymphadenectomy[27-29].

Questions of the cost-effectiveness of SLN biopsy have also been raised; the short-term increased costs associated with an additional surgical technique and possible increased risk of recurrence must be weighed against the longer term impacts that complete inguinal femoral lymphadenectomy have on both healthcare expenditures and quality of life. A cost-effectiveness model evaluating SLN biopsy in vulvar cancer found that SLN biopsy was both less costly and more effective than complete lymph node dissection. Only when the model was altered in such a way that lymphedema did not negatively impact quality of life, did complete inguinal lymphadenectomy become a cost effective option[30].

Although there is a significant body of literature to verify the safety and feasibility of SLN biopsy in vulvar squamous cell cancer, the appropriate treatment in women with a positive sentinel node remains uncertain. The currently recruiting GROINSS-V-Ⅱ/GOG 270 study aims to answer this question, treating women with positive sentinel nodes with radiation plus or minus chemotherapy, eliminating the complete inguinal femoral dissection. However, until those results become available, the standard of care remains complete dissection in the setting of nodal metastases. This is based upon GROINSS-V-Ⅰ data demonstrating that when there is sentinel node metastasis present there is an unacceptably high risk of additional metastasis beyond that node, regardless of metastasis size[31].

A 2008 statement issued by the International Sentinel Node Society states that SLN biopsy should be offered to patients with clinical stage Ⅰ-Ⅱ vulvar cancer when "the SLN biopsy is performed by a skilled multidisciplinary team in well-selected patients." We feel that SLN biopsy is appropriate when the tumor is ⩽ 4 cm in diameter**,** there is no clinical evidence of groin involvement, and invasion is > 1 mm[16,32]. Additionally, midline lesions necessitate bilateral SLN biopsy, and patients with multifocal tumors should undergo complete inguinal femoral dissection[22]. Furthermore, surgeons should demonstrate their ability to identify sentinel nodes before offering this technique to patients. This can best be accomplished by performing SLN biopsy with a standard technique followed by concurrent total lymphadenectomy[22,32]. The panel recommended that before utilizing SLN biopsy alone surgeons should successfully identify a SLN in ten successive cases, without any false-negatives[32]. Unfortunately, the infrequent occurrence of vulvar cancer may make the necessary volume difficult to achieve for many gynecologic oncologists. Due to this low volume, some suggest that vulvar cancer is best treated in a limited number of specialized referral centers where patients can best benefit from maximally trained and experienced surgeons[17].

ENDOMETRIAL CANCER

An estimated 54870 new cases of endometrial cancer will be diagnosed in the United States in 2015, making it the most common of the gynecologic malignancies[2]. For the majority of newly diagnosed patients management includes complete surgical staging, which includes pelvic and para-aortic lymphadenectomy. Lymph node status is an important prognostic element in endometrial cancer, making lymphadenectomy a central factor in the initial treatment[33]. However, lymphadenectomy is not without risk. Low-risk patients undergoing lymphadenectomy experience increased morbidity, cost and operating room time without associated survival benefit[34]. Only about 10% of women with clinical stage Ⅰ cancer will have disease-positive lymph nodes, and in women with superficial invasion and well differentiated tumors the rate of lymph node involvement falls to 3%-5%[35,36]. This indicates that 95%-97% of women with early stage cancer will have negative lymph nodes. However, inadequate staging often leads to increased postoperative therapy, particularly external beam radiation in "under-staged" individuals[37]. Given this clinical conundrum a less invasive approach for the evaluation of nodal basins may offer significant benefit.

A number of contemporary studies have now outlined the validity of lymphatic mapping in endometrial cancer. The SENTI-ENDO trial published in 2011 was a prospective multicenter cohort study assessing detection and accuracy of sentinel node biopsy in early endometrial malignancy. One hundred and thirty-three women underwent lymphatic mapping *via* intracervical injection of both ^{99m}Tc and blue dye, followed by complete pelvic nodal dissection. Sentinel nodes underwent more rigorous pathologic evaluation than non-sentinel nodes, with immunohistochemistry and ultra-staging. The negative predictive value and sensitivity for detection of metastatic disease in the lymph nodes were 97% and 84%, respectively. Of the three false-negative results, two were located in the contralateral pelvis and one was in the para-aortic nodes. There were no major adverse outcomes associated with the SLN biopsy approach[35]. This study successfully demonstrated that sentinel nodal status in endometrial cancer accurately predicts nodal metastatic disease.

More recently, Barlin *et al*[38] published the outcomes of their systematic and stepwise approach to lymphatic mapping and SLN biopsy. The algorithm involves universal evaluation of the serosa and peritoneum, excision of mapped SLNs, excision of any non-sentinel clinically suspicious nodes, and a hemi-pelvic complete

node dissection on each side where no SLN is identified. In their study of 498 patients, 81% had at least one sentinel node. Thus, 19% required bilateral pelvic nodal dissection and 30% required unilateral nodal dissection. Using this approach they reported a false-negative rate of 2%, sensitivity of 98.1% and negative predictive value of 99.8%[38]. This process for evaluating pelvic nodes provides a notable improvement in the yield of SLN biopsy while still leading to adequate lymph node evaluation in patients without successful bilateral lymphatic mapping.

In endometrial cancer, the SLN technique varies between institutions. There are three primary SLN injection protocols used. It is important to note that the lymphatic drainage of the uterus is bilateral, and as such identification of lymph nodes on both sides of the pelvis is a key factor in the potential success or failure of any SLN detection approach. Injection modalities include intracervical injection, injection into the uterine serosa, and injection into the endometrium *via* hysteroscopy. A 2011 meta-analysis found the greatest detection rates with intracervical injection, although this result was not statistically significant[39]. The majority of large studies published since employed intracervical injection. Ease of access, ease of injection, anatomic plausibility for accurate mapping, and low frequency of distorting factors such as myomas or scar tissue from cervical procedures in patients with endometrial cancer have all been cited as reasons to favor cervical injection[40].

Furthermore, three types of injected tracers, used alone of in combination with one another, provide variation between the published protocols and their results. Blue dye, ^{99m}Tc and indocyanine green (ICG) have all been shown to be efficacious in lymphatic mapping. The most prevalent strategies currently appear to be blue dye in combination with ^{99m}Tc, or ICG alone. Bilateral detection rates with the dye and ^{99m}Tc combination have ranged from 66%-69%[41-43], while ICG ranges from 60%-79%[44-46]. A recent cohort study comparing successful mapping with either blue dye or ICG found a significant improvement in bilateral SLN detection with ICG. Additionally, patient BMI was a predictor of failed mapping with blue dye, while BMI did not impact mapping with ICG[45]. This is an important difference given the prevalence of obesity in women with endometrial cancer. How *et al*[43] published on the approach of combining all three agents into one injection, and in a cohort of 100 patients reported a bilateral detection rate of 76%.

Importantly, lymphatic mapping can identify sentinel nodes in areas which would not have been sampled with conventional lymphadenectomy and are three times more likely to contain metastases than non-sentinel nodes[40]. Jewell *et al*[46] reported reported slightly more than 10% of patients in their study had sentinel nodes located outside of the pelvic basin, primarily in the para-aortic region. Others report identifying significant numbers of nodes in the pre-sacral region, parametria and the hypogastric vein with lymphatic mapping[43]. Expanding the field of dissection in the presence of variant drainage channels is generally done only when variance is identified, as is the case with lymphatic mapping.

The previously described studies pertain to patients with early stage disease. The Survival Effect of Para-aortic Lymphadenectomy in Endometrial Cancer study retrospectively evaluated overall survival in patients with endometrial malignancy who underwent either pelvic only or combined pelvic and para-aortic lymphadenectomy. Their results, published in 2010, found that more extensive lymphadenectomy improved survival in women with intermediate and high risk cancers, but not in women with low risk cancers[47]. Thus, SLN biopsy is likely only appropriate for women with early stage disease.

As previously described in the context of vulvar cancer, ultra-staging can provide added benefit when a select few nodes have been removed. Kim *et al*[48] report on 425 women who underwent SLN biopsy at the time of staging for low grade endometrial cancer. Ultra-staging was used when standard hematoxylin and eosin (H and E) staining did not identify metastatic disease, and the number of metastatic cancers diagnosed doubled with ultra-staging[48]. Others have also documented increased detection rates with ultra-staging[38,49]. The importance of these low-volume metastases should be underscored, as they are associated with worse outcomes and increased risk of recurrence[49,50]. However, the most appropriate management of these micrometastases remains unknown.

The low false negative rates and high sensitivity of sentinel nodal biopsy when done as part of a comprehensive algorithm make it a practical and appealing solution to the problem of staging women with early stage endometrial cancer. However, lymph node involvement is low for many women with early stage endometrial cancer and the survival benefit of adding SLN biopsy is unknown. At this time we feel SLN biopsy is investigational in women with endometrial cancer and should be done only on protocol. Routine pelvic and para-aortic lymphadenectomy should be performed on women at risk for lymph node metastasis. Additional information regarding long-term outcomes including overall survival among women undergoing SLN biopsy alone is still needed before we can determine which women will most benefit from the addition of a SLN biopsy and when we can omit pelvic and para-aortic lymphadenectomy.

CERVICAL CANCER

Despite not being an element of the FIGO clinical staging for cervical cancer, lymph node status is one of the most influential factors in disease free and overall survival for women with early stage disease[51]. The current standard of care for women with cervical cancer treated surgically includes bilateral pelvic lymphadenectomy plus or minus para-aortic lymphadenectomy. This

procedure however comes with significant morbidity, such as prolonged operative times, nerve and vascular injury, lymphocysts, and lymphedema[52]. However, only about 15% of women with early stage disease have lymph node metastases, meaning that the majority of the women are exposed to the increased morbidity of lymphadenectomy without an associated survival benefit[53]. This creates a prime opportunity for sentinel node biopsy.

There are a multitude of studies investigating the accuracy of sentinel node biopsy in cervical malignancy, and a recent systematic reviewed by Kadkhodayan and others analyzed the results of 67 such articles. They determined that the pooled detection rate of sentinel nodal mapping was 89.2% (95%CI: 86.3%-91.6%), with an overall sensitivity of 90% (95%CI: 88%-92%). When comparing the results of all included studies, they found that SLN detection rates were lower when using blue dye alone, and highest when the combination of blue dye and radiotracer. They determined that dilution of blue dye, superficial injections, and cervical injections were all associated with higher detection rates[54]. It has also been reported that SLN detection is best when done within 30 min of blue dye injection; when searching 50 min or greater after injection nodes are not able to be identified[55]. Others have found slightly improved detection rates when radiotracer is injected the day prior to nodal biopsy as compared to 2 d prior, although the difference is small and not statistically significant[56]. Furthermore, minimally invasive techniques (*via* either conventional or robotic laparoscopy) yield improved detection as compared to an open approach[54].

One important consideration in cervical cancer is the impact that positive lymph nodes have on indicated treatment. When lymph node metastases are detected during radical hysterectomy, para-aortic lymph node dissection is often performed and the remainder of the procedure is typically aborted, as the patient will subsequently require chemoradiation with the uterus and cervix *in situ*. Because of this, intraoperative detection of lymph node metastases is a very useful tool. Unfortunately the Kadkhodayan *et al*[54] review found that the pooled detection rate of lymph node disease with frozen section is low at 60%, a value which was influenced largely by the fact that frozen section analysis was not able to detect small macrometastatic and micrometastatic disease[54].

Cibula *et al*[53] examined the prognostic significance of low volume SLN disease in 645 women with early-stage cervical cancer who underwent SLN biopsy followed by complete pelvic lymphadenectomy. They found that isolated tumor cells were not independently associated with a decrease in overall or recurrence free survival. However, the presence of micrometastases was an independent prognostic factor for overall survival, and was equivalent to the survival effect of macrometastases. This serves to highlight the important role of ultra-staging in the management of these patients. Unfortunately, the most appropriate management for these isolated tumor cells remains unclear.

As cancer of the cervix is a midline disease, it must be assumed that tumors will drain to bilateral lymphatic basins. Failure of mapping on one side can be due to extensive tumor involvement on the un-mapped side, which in turn leads to significant false-negative rates with SLN detection. Cormier and others published an algorithm wherein all SLNs are removed, any suspicious nodes are removed whether they have mapped or not, and in the instance of only unilateral mapping contralateral pelvic nodal dissection and parametrectomy is done. They applied this method to a prospectively collected database of 122 patients who underwent SLN mapping followed by complete bilateral nodal dissection, and found that use of the algorithm would lead to detection of all cases of lymph node metastases, and avoid bilateral nodal dissection in 75% of cases[57]. Such an approach is likely the best way to optimize detection of metastatic disease while minimizing unnecessary complete nodal dissections.

Tumor size is also an important factor in the use of SLN biopsy in cervical cancer. When larger tumors are present there is a higher risk of replacement of lymph nodes with tumor, leading to decreased uptake of tracers. This can lead to either no identification of a sentinel node, or dye uptake by a non-sentinel node because of alterations in lymphatic drainage cause by tumor spread. Because of this a cutoff of SNL mapping only in tumors $\leqslant$ 2 cm or $\leqslant$ IB2 has been suggested[54].

To date, prospective studies on the survival outcomes of women who undergo SLN biopsy alone without concurrent pelvic lymphadenectomy are lacking. However, it is known that in a population of women with early stage disease those with positive lymph nodes do not see a survival advantage with more extensive lymphadenectomy. Conversely, women with negative lymph nodes do experience improved survival when a greater number of nodes are removed[58]. It is important to note that the study which reported those findings did not employ ultra-staging and it is possible that a portion of the "node negative" women who benefited from greater dissection would have in fact had micrometastatic disease detected with more advanced pathologic evaluation.

Based upon the above findings, SLN mapping in early stage cervical malignancy is a feasible and reliable approach for detecting metastatic disease. Given the morbidity of total pelvic lymph node dissection, and the relative infrequency with which metastatic disease is present in early cervical disease, SLN mapping has encouraging possibilities in select patients. However, larger prospective studies evaluating the long-term outcomes in patients who undergo SLN biopsy without subsequent complete lymphadenectomy are needed before clinical recommendations can be made.

CONCLUSION

SLN biopsy is a well-developed technique that is now

the standard of care in melanoma, breast cancer and penile cancer. In women with early vulvar cancer sentinel node biopsy should be considered a feasible alternative to total inguinal femoral lymphadenectomy. When undertaken by a qualified multidisciplinary team SLN biopsy is a safe approach that improves a woman's quality of life. In fact, SLN biopsy is the standard of care at some institutions for vulvar cancer patients.

In endometrial cancer, when using the appropriate technique lymphatic mapping demonstrates high sensitivity for detecting metastatic disease. While prospective studies applying these findings are ongoing, currently available data are promising that sentinel nodal detection may soon become widely accepted as an alternative standard of care for select cases of endometrial cancer.

In cervical cancer, sentinel node biopsy is practical for women with small lesions ($\leqslant$ 2 cm) and has the potential to spare a substantial proportion of women the morbidity of extensive nodal dissection. When used in conjunction with an algorithm which accounts for incomplete bilateral mapping the diagnostic yield is quiet high, however prospective data on survival and outcomes of women who undergo SLN biopsy are needed before it can be considered a viable alternative to complete lymphadenectomy.

While the process of ultra-staging lends additional information about the spread of disease, large-scale prospective data are needed in all three of these cancers to better understand the significance and proper treatment of micrometastatic malignancy.

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MINIREVIEWS

Apoptosis and endometrial receptivity: Relationship with *in vitro* fertilization treatment outcome

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Abstract

Apoptosis is an important process in the reconstruction of endometrium within the menstrual cycle. The balance between cell proliferation and apoptosis regulates the periodic repair and shedding of endometrial cells and leads to the menstruation or prepare the mucosal layer of endometrium for the implantation of the embryo. Many factors with pro- and antiapoptotic action, such as B cell lymphoma/leukemia-2 and inhibitors apoptosis proteins families, caspases, tumor necrosis factor receptors, phosphatase and tensin homolog, proliferator-activated receptor gamma, microRNAs and others are differently expressed in the endometrial tissue at phases of menstrual cycle. Receptivity of the endometrium at the period of "window of implantation" is associated with the significant increase of apoptosis in endometrium to allow the embryo to be successfully implanted. The impairment of apoptosis regulation in the endometrium at this period often is observed in infertile women with endometriosis, tubal factor, polycystic ovary syndrome, *etc*.. In many cases the impairment of apoptosis regulation in the endometrium is the main cause of *in vitro* fertilization (IVF) treatment failure in these patients. As of today, the exact mechanisms and factors mediating the apoptotic process in normal endometrium and in infertile women are not fully understood. Herein, the literature data concerning the endometrial apoptosis regulation in general, and in light of the influence of apoptosis upon IVF treatment outcome are reviewed. The possibility to use some parameters of endometrial apoptosis for prediction of the successful pregnancy achievement in women participating in IVF protocols also is discussed.

Key words: Apoptosis; Endometrium; Receptivity; *In vitro* fertilization

Core tip: Endometrial receptivity depends on many factors and apoptosis regulation as well. Compromised fertility and *in vitro* fertilization (IVF) failure is often associated with the impairment of endometrial apoptosis during "window of implantation". Understanding of the molecule mechanisms involved in apoptosis regulation in the infertile women might have a great value and let us to use them as predictors of endometrial dysfunctions

to improve implantation rate in IVF program.

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INTRODUCTION

In the last decades *in vitro* fertilization (IVF) is widely used for the treatment of infertility in couples with unexplained subfertility, male subfertility, endometriosis or tubal pathology[1]. The impact of different clinical parameters (infertility etiology, female age, time of infertility, thickness of the endometrial tissue, the quantity of follicles and amount of the progesterone on the day of hCG injection, *etc*.) on the outcome of the IVF treatment was proved[1-4]. Analysis of the data received from different clinical centers shows that maximum achievable pregnancy rate in IVF doesn't exceed 50% per embryo transfer cycle and the average clinical pregnancy rate is between 27% and 40%[5-8]. Despite some evident technical improvement of IVF protocol, it is clear now that the percentage of the pregnancy onset cannot be made higher due to the manipulation with the embryo transfer procedure and conditions of embryo cultivation or by optimal choice of blastocysts[9]. Many studies have demonstrated that the function and receptivity endometrium must be considered as the main limiting factors in the pregnancy onset after IVF, because for accomplishing an implantation, pregnancy and subsequent live child birth endometrium should be ready to accept the embryo[9-11]. One can see now that overcoming the difficulties in improvements to ART will require a deeper insight in the contact between the endometrium and embryo[12].

It is well known that the success of the embryo implantation depends on some morphologic and biochemical modifications of the human endometrium during menstrual cycle[9]. These processes are controlled by a plethora of different factors, including ovarian steroids and its receptors, cytokines, growth factors, adhesion molecules, transcriptional factors and many others[13]. Recent investigations have demonstrated an importance of apoptosis in the processes of endometrial tissue reconstruction during menstrual cycle[10]. The balance between cell proliferation and apoptosis regulates the periodic repair and shedding of endometrial cells and leads to the menstruation or prepare the mucosal layer of endometrium for the implantation of the embryo[14].

According to classical definition, given by Kerr *et al*[15] in 1972, "apoptosis, or programmed cell death, is characterized by the fragmentation and enfolding of cell compartments into membrane-covered apoptotic bodies that are removed without any immune response or damage of the surrounding cells". Caspases, or intracellular cysteine proteases, are the main enzymes in the process of apoptosis cascades execution[16]. At the beginning of the apoptotic pathway the initiator caspases, like capsase-8 and -9 are activated, and the later morphological changes of the apoptotic cell are mediated by the action of effector caspases, like caspase-3[16]. Now two apoptotic signaling pathways, the extrinsic and intrinsic, have been established. The extrinsic apoptotic cascade is activated by ligation of specific death receptors, expressed on the cell-surface, *i.e.*, Fas, tumor necrosis factor (TNF) receptor or TNF-related apoptosis-inducing ligand (TRAIL) receptor[17]. After binding with specific ligands these death receptors are oligomerized, involving Fas-associated death domain (FADD) and procaspase-8 with subsequent appearance of the death-inducing signaling complex, activation of caspase-8 and development of intracellular apoptotic signaling cascades. This results in morphological and biochemical changes in the cell, characteristic for apoptosis[18]. The intrinsic mechanism of apoptosis is activated by a large spectrum of intracellular death signals, such as damage of DNA, stress, and diminishing of the growth factors value[19]. In the result of these apoptotic signals, the mitochondrial membrane became permeabilized and a lot of pro-apoptotic proteins that are typically located in the intermembrane space, *i.e.*, cytochrome c, release into the cytoplasm to trigger the caspase-9 activation[20]. Subsequently both extrinsic and intrinsic apoptosis pathways result in the activation of the caspase-3. They are regulated by numerous molecules with pro- and anti-apoptotic action, among them the best known factors which are belonged to the B cell lymphoma/leukemia-2 (Bcl-2) and inhibitors apoptosis proteins (IAP) families.

The Bcl-2 family comprises 25 members with pro- and anti-apoptotic effects. Bcl-2-family members that have four Bcl-2 homology (BH) domains (Bcl-2, MCL-1, A1/Bfl-1, Bcl-B/Bcl2L10, and BCL-xL) possess the anti-apoptotic action due to inhibition of their pro-apoptotic counterparts *via* protein-protein interactions[21,22]. The pro-apoptotic family members are divided into two subgroups: those having multiple BH domains (effector proteins), such as Bcl-2-associated X protein (BAX), BAK, and BOK (Bcl-2 related ovarian killer), and containing only the BH3 domain (BID, BIM, PUMA, NOXA, BIK, BAD, HRK, and BMF)[21,22]. The members of IAPs family prevent the activation of caspases-3, -7 and -9 and thereby inhibit apoptosis[23]. Today 8 human IAP family members are identified and divided into 3 classes (1, 2 and 3) according to the presence or absence of a RING or caspase activation recruitment domain and the homology of their baculovirus IAP repeat domains[23]. The best known member of class 1 IAP family is X-linked IAP (XIAP), which is also known as hILP, MIHA and BIC4. It inhibits caspases-3, -7 and -9, but it does not influence on caspase-8[23]. The class 2 IAP family member NAIP is expressed in adult liver, placenta and central nervous system and inhibits caspases-3 and -7,

but not caspases-1, -4, -5, or -8. Class 3 IAP members such as survivin is mostly expressed in the fetal tissues, but not in most normal adult tissues[23]. Recently it was demonstrated that survivin is also expressed in the endometrium of healthy women[24]. In addition to these factors there are many others proteins and enzymes which are directly or indirectly involved in the apoptosis of different types of endometrial cells during menstrual cycle. Study of the distribution and expression of these apoptosis regulators can give us new data concerning endometrium functioning in normal and pathological conditions. And understanding of the molecule mechanisms involved in apoptosis regulation in the infertile women might have a great value and let us to use them as predictors of endometrial dysfunctions to improve implantation rate in IVF program.

LITERATURE RESEARCH

In this study we review the data of the most important factors regulating apoptosis in normal endometrium at proliferative and secretory phase of cycle and in endometrium of women with compromised infertility, who participate in IVF protocols. The literature data concerning the role of apoptosis in the establishment of endometrial receptivity during the "window of implantation" and association of the parameters of endometrial apoptosis with the IVF treatment outcome also has been described. To this purpose we conducted a search at http://www.ncbi.nlm.nih.gov/pubmed/electronic database using the following keywords: (1) "endometrium and apoptosis"; (2) "endometrial receptivity"; (3) "endometrium and IVF"; (4) "implantation window"; (5) "endometrium and IVF success"; and (6) "endometrium, infertility, apoptosis". A literature review of English-language papers published by May 2015 has been performed. The selection of articles was based on their titles and abstracts with subsequent analysis the texts of the related articles.

APOPTOSIS IN THE NORMAL ENDOMETRIUM

Endometrium is a mucosal inner layer of the myometrium[25]. Steroid hormones (estrogens, progesterone, androgens and glucocorticoids) tightly regulate endometrium function and morphological changes during menstrual cycle[25,26]. Noyes, Hertig and Rock had described these morphological changes in 1950[26]. These changes are characterized by the alteration of the cellular morphology and expression of some molecules on the cell membrane as well as a synthesis and production of different biologically active factors[26]. The dominant hormone within proliferative phase of the menstrual cycle is estrogen, the secretory phase is determined by high production of the progesterone[9]. There are several types of cells in the endometrial tissue, including luminal and glandular epithelial cells, stromal fibroblastic cells, immune cells and blood vessels[25]. The number, activity, structure and function of the endometrial tissue cells are significantly changed during the proliferative and secretory phases of menstrual cycle. It was established that endometrial cell proliferation and cell death are directly regulated by numerous factors, including cytokines, growth factors, proteolytic enzymes, transcriptional factors and different apoptosis-related factors as well[9,27,28].

The proliferative phase is associated with follicular growth and increased estrogen secretion, leading to endometrial reconstruction[29]. This phase of menstrual cycle is accompanied by the active cells proliferation and angiogenesis to provide the nutrition of the developing new endometrium[9]. All tissue components (glands, stromal and endothelial cells) show proliferation, with DNA nuclear and RNA cytoplasmic syntheses, which peak on days 8-10 of the cycle, reflecting maximum concentration of estrogen receptors in the endometrium[29]. It was demonstrated that insulin-like growth factors (IGF), vascular endothelial growth factor, epidermal growth factor (EGF) system, transforming growth factor (TGF)-α and amphiregulin are highly expressed in endometrium during proliferative phase[9,30,31]. The action of all these factors is directed towards the tissue proliferation, and very little apoptosis was detected in the endometrium during this period[32]. It was supposed that the high level of Bcl-2 expression by the glandular epithelial cells leads to the inhibition of apoptosis in the endometrial tissue during proliferative phase of cycle[32-34]. Bcl-2 as apoptosis inhibitor reduces oxygen free radicals, blocks the intracellular Ca^{2+} influx into cell organelles, decreases p53-dependent apoptosis, and antagonizes c-Myc[35-37]. High level of Bcl-2 expression likely mediates the reduction of mitochondrial-induced apoptosis of the endometrial epithelial cells and leads to proliferation of endometrial cells, thereby making the endometrial tissue thicker[33,34]. Estrogen increases the cellular proliferation during the proliferative phase of menstrual cycle due to inhibition of the tumor suppressor gene phosphatase and tensin homolog (*PTEN*)[9]. This gene increases the apoptosis of endometrial cells by down-regulating the Akt-depending signal pathway[38].

Progesterone plays the main role in the secretory endometrial transformation during the second half of menstrual cycle, suppressing proliferation and inducing cell differentiation[39]. After ovulation, the granulosa cells undergo luteinization and form part of the corpus luteum, which secretes progesterone[40]. The influence of the progesterone on epithelial cell proliferation is proved by the observation that progesterone completely inhibits estrogen-induced DNA synthesis and suppresses cell proliferation[41]. Progesterone also antagonizes the stimulatory action of many oncogenes that are likely to mediate estrogen-induced growth[29]. Progesterone inhibits the estrogen regulation of cyclin D1 nuclear translocation resulting in the hypophosphorylation of Rb and p107 proteins and a block in G1-S phase development[42]. The inhibitory action

of progesterone on Akt-depended signal pathway with subsequent suppression of cells proliferation was also demonstrated[43].

The apoptosis in the normal secretory endometrium is proposed to promote elimination of the senescent or dysfunctional cells and to provide the tissue repair at each menstrual cycle[44]. The abundance of the apoptotic cells is maximal in the glandular epithelium and much less in the stroma[44]. It was demonstrated that different apoptosis-related factors are involved in the regulation of secretory endometrial cell transformation. It was found that after entering the secretory phase, Bcl-2 expression in endometrium is declined[32,33]. At the same time the increase of the proapoptotic factor BAX expression was noted in the endometrium at this phase of cycle[33]. The function of BAX is opposite to that of Bcl-2. It can accelerate the apoptosis and the ratio of Bcl-2: BAX defines if a cell lives or dies[33]. The BAX proteins form BAX/BAX homodimers, which lead to cytochrome C release and caspases activation with subsequent induction of the endometrial epithelial cells apoptosis[45,46]. It was also shown that endometrial stromal cells apoptosis in the secretory phase of cycle was increased due to caspase activity, based on the up-regulation of specific receptors Fas and TRAIL-R2[47]. Different expression of DNA fragmentation factor of 45 kDa (DFF45) was found in endometrium during the menstrual cycle. In normal endometrium, a lowest DFF45 expression was detected in the late proliferative phase secretory endometrium and maximal endometrial tissue staining for DFF45 was observed in an early secretory phase of the menstrual cycle[48-50]. Much more DFF45-positive cells were found in the endometrial glands in comparison to stroma, irrespective of menstrual cycle phase[48,49]. It is known that the DNA damage during apoptosis is a consequence of activation of the DFF40/DFF45 complex. DFF40 (DNA fragmentation factor of 40 kDa) causes the direct DNA fragmentation while DFF45 acts as a DFF40 inhibitor and as its chaperone[49]. Therefore, the DFF45 is required for adequate DFF40 synthesis and the high level of DFF45 expression in the secretory endometrium can reflect the high intensity of endometrial cells apoptosis. Recently it was shown that the immunoreactivity of the peroxisome proliferator-activated receptor gamma (PPARγ) protein was more pronounced in secretory-phase endometrium than in that the proliferative endometrium[51]. PPARγ agonists can trigger terminal differentiation, suppress cell proliferation, increase apoptosis, and inhibit inflammation in many cancer models, so the high level of PPARγ expression can mediate the induction of apoptosis in the secretory endometrial tissue. Immunoelectron microscopic study of Fas and FasL molecules expression demonstrated their different expression in human endometrial glandular cells during the menstrual cycle[52]. Fas and FasL were found mostly on the Golgi apparatus during the late proliferative phase and on apical membranes and Golgi-transporting vesicles during the late secretory phase. Thus, it was shown that apoptosis of human endometrial glandular cells is suppressed by Bcl-2 expression on the proliferative phase and induced by the Fas/FasL system in an autocrine or paracrine way on the secretory phase[34].

Table 1 Regulation of apoptosis in normal endometrium during menstrual cycle

Proliferative phase		Secretory phase	
Increased expression	Inhibited expression	Increased expression	Inhibited expression
Bcl-2	p53	BAX	Bcl-2
Fas, FasL	PTEN	Fas, FasL	
	PPARγ	TRAIL-R	
	DFF45	PTEN	
		Caspase-3	
		PPARγ	
		DFF 45	

Bcl-2: B cell lymphoma/leukemia-2; PTEN: Phosphatase and tensin homolog; PPARγ: Peroxisome proliferator-activated receptor gamma; TRAIL: TNF-related apoptosis-inducing ligand; DFF45: DNA fragmentation factor of 45 kDa.

Thus, literature data clearly demonstrate the tight association of endometrial apoptosis intensity with the hormonally-dependent changes of endometrial tissue morphology. The summarized data, concerning the expression of pro- and anti-apoptotic factors during different phases of the menstrual cycle, is presented in the Table 1.

APOPTOSIS AND ENDOMETRIAL RECEPTIVITY

It is well known, that a short period of time during the mid- to late-secretory phase exhibits a highest readiness of endometrium for embryo implantation and this period is called as the "window of implantation"[9]. This period is extremely important for the preparation of endometrium to the acceptance of the implanted embryo. In humans the endometrium becomes susceptible to blastocyst implantation at 6-8 d after ovulation and remains susceptible for approximately 4 d (cycle days 20-24)[25]. Last years there have appeared a number of studies aimed at the identification of potential markers of receptivity[53]. These studied were devoted to the investigation of the molecular changes and apoptosis regulation that takes place in the endometrium during the "window of implantation". It was shown that apoptosis in the human endometrium plays an essential role for endometrial receptivity and early implantation. An imbalance of pro- and anti-apoptotic events in the secretory endometrium seems to be involved in implantation disorders and consecutive pregnancy complications[47].

It was established that at the beginning of the implantation window the changes of apoptosis in endometrium become most intensive. Increase of apoptosis activity may have significance for the decidualization processes establishment in endometrium

during the late secretory phase. It was shown that decidualization includes differentiation and apoptosis of epithelial as well as stromal cell compartments[53]. The morphological changes that characterize decidualization take place in the endometrium without respect to conception[9]. All the cells in the endometrium are influenced by these changes. Epithelial cells are undergoing glandular secretory transformation. Stromal cells decidualization is associated with production and secretion of numerous decidual proteins such as prolactin, IGF binding protein-1 (IGBP-1) and tissue factor[54,55]. Endometrium is infiltrated by a large amount of different types of immune cells, including NK, T-lymphocytes, and macrophages[9]. On the secretory phase, vascular remodeling takes place, with the main angiogenic mechanisms leading to coiling and intussusceptions of the spiral arteries[56]. All these changes prepare endometrium for the successful implantation of the blastocyst.

Successful implantation supposedly is associated with the apposition and attachment of the embryo to the endometrial epithelial cells and adequate invasion in the endometrial stroma[9]. It was established that several mechanisms facilitate the embryo adhesion. Pinopodes change the concentration of endometrial fluids at the implantation site[9]. Mucins, in particular MUC1, take part in the selection of implantation site, because MUC1 barrier on the luminal epithelium prevents the interactions between the embryo and the endometrium, but the blastocyst itself cleaves MUC1 and defines the most appropriate site of implantation[57]. Integrins, especially $\alpha_v\beta_3$, and their receptor osteopontinare are highly expressed in the endometrium at the period of the window of implantation[56]. The important role of leukaemia-inhibitory factor (LIF), matrix metalloproteinase's family, TGF-β, colony stimulating factor-1, transcription factor home box gene *HOXA10*, cytokines IL-1, IL-11, IL-15 in implantation also has been clearly demonstrated[9,58,59]. But during this step of embryo attachment endometrial stroma is not susceptible to the apoptotic events[9].

Apoptosis evidently takes part in the regulation of the process of blastocyst invasion. It was shown that on the initial steps of implantation the uterine epithelium of the implantation chamber undergoes apoptosis by the influence of the interacting blastocyst in mouse model[60]. With progressing of implantation, the regression of decidual cells results in a restricted and coordinated invasion of trophoblast cells into the maternal compartment due to the balanced expression of BAX, Bcl-2 and caspase-9 proteins in the decidual compartment and the high level of caspase-3 synthesis in the apoptotic uterine epithelium[60]. It was also shown that apoptosis-inducing factor might play an important role during mouse embryo implantation[61]. Tumor suppressor p53 is important for embryonic implantation because of transcriptional up-regulation of uterine LIF. It was reported, that simultaneous activation of p53 and estrogen receptor α took place in the endometrial tissues during implantation to coordinately regulate LIF production[62].

So, apoptosis is crucial for embryo implantation, rescuing the endometrium from apoptosis in the apposition phase and then providing the successful invasion of blastocyst by locally induced cells death in the site of the embryo and endometrial surface contact. But it is obvious that many apoptosis-regulating factors directly involved in the interaction between the embryo and endometrium have not yet been studied both in healthy women and in the infertile patients.

APOPTOSIS IN THE ENDOMETRIUM OF INFERTILE WOMEN

It is well established that in women with certain gynecologic diseases, including endometriosis, tubal disease, and polycystic ovary syndrome (PCOS), endometrial receptivity is compromised, leading to infertility and pregnancy loss[58]. Supposedly, regulation of apoptosis in the endometrium of infertile women also is impaired. It was reported that the endometrial Wilms tumor suppressor gene (*WT1*) in fertile women is highly expressed during the period of the window of implantation, but the endometrial WT1 expression was decreased in patients with PCOS during the secretory phase of the cycle[63]. The group of women with PCOS also was characterized by the higher stromal expression of Bcl-2 and p27, lower expression of EGF receptor in comparison with the fertile women. It was found, that apoptosis level was significantly reduced in the endometrial samples of women with PCOS[63]. These changes can hinder the success of decidualization and endometrial receptivity in infertile patients with PCOS[63]. In another work the changes in the apoptosis-related genes expression in the endometrium during the window of implantation were demonstrated in PCOS patients[64]. Five apoptosis-associated genes (including *Bcl-2*) were up regulated and four (including *FADD*) were downregulated. Authors concluded that the diminishing of the cell apoptosis at the period of the window of implantation in PCOS patients can result in the reduced endometrial receptivity[64].

Another gynecological pathology - chronic endometritis also can compromise human fertility and lead to abnormal uterine bleeding, pain, and reproductive failures. In women with chronic endometritis the expressions of Bcl-2 and BAX in endometrium were up-regulated, while caspase-8 was down-regulated[65]. Possibly the altered expression of these genes in the endometrium can be responsible for the impaired endometrial receptivity and the presence of endometrial hyperplastic lesions in women affected by chronic endometritis[65].

The spontaneous apoptosis, which is regulated by Bcl-2, BAX, p53, during the window of implantation in women with unexplained infertility was studied In another work[66]. It was found, that reduced amount

of apoptotic cells, weak immunoreactivity of p53 and strong immunoreactivity of Bcl-2 took place in the endometrium of infertile women compared with the fertile women. Authors suggest that this finding might be an important factor of defective implantation compromising fertility in this group of patients[66].

The changed endometrial microRNAs (miRNAs) expression profile was shown during the window of implantation for women with repeated implantation failure (RIF)[67]. In recent years miRNAs are intensively studied. Now miRNAs are considered as key posttranscriptional regulators. As members of small non-coding RNA family, miRNAs are transcribed from specific genes spread over multiple locations in all human chromosomes except the Y chromosome. The mature miRNAs incorporate into the RNA induced silencing complex and, through complementary binding to the 3′ UTR of specific target genes, post-transcriptionally regulate their expression[68]. It was identified 13 miRNAs, differentially expressed in endometrial samples of patients with RIF. Among these genes 10 miRNAs were more expressed (including miR 145, 23b and 99a) and 3 were less expressed[12]. These miRNAs participate in the regulation of p53 signaling and cell cycle pathways. It was also shown that during window of implantation in the natural menstrual cycle, endometrial miRNA 22 was considerably higher in patients with RIF in comparison with control group[12]. Simultaneously, experiments using animal models demonstrated that miRNA 22 up-regulation contributed to the inhibition of the embryo implantation in mice[69]. Thus, these promising results allow to conclude, that the RIF-associated miRNAs could be used as new candidates for diagnosis and treatment of embryo implantation failures[12].

Significant changes of endometrial apoptosis were noted in women with endometriosis. Now it is widely accepted that pathogenesis of endometriosis is directly connected with the resistance of eutopic endometrial cells to apoptosis-induced signals which enable endometrial cells to escape immunosurveillance in the peritoneal cavity and to be implanted and growing in ectopic location[28,32,70]. Numerous literature data support this hypothesis. About 20 years ago the high level of Bcl-2 expression was for the first time demonstrated in the endometrial stroma of women with endometriosis and later these data was confirmed by different groups[28,32,70,71]. It was also shown that increased expression of anti-apoptotic factors and decreased expression of pro-apoptotic factors took place in the eutopic endometrium from women with endometriosis compared with endometrium from healthy women[72]. Both mRNA and protein amounts of several members of IAP family with anti-apoptotic action (c-IAP1, c-IAP2, XIAP and Survivin) were more expressed in women with endometriosis then in healthy donors[73]. In the endometrium of women with ovarian endometriosis the lower level of DFF45 expression was observed then that in both normal eutopic proliferatory and secretory endometrium. These results also directly evidence in favor of reduced apoptosis in the endometrium of patients with endometriosis[48]. Our research group also studied the expression of some factors with pro- and anti-apoptotic action in the endometrium of infertile women with endometriosis. We have found that in the endometrium of women with endometriosis during "window of implantation" the level of XIAP mRNA expression was significantly higher than that in the endometrium of healthy women[74]. Evidently taking together all these data about the impairment of endometrial apoptosis can explain the severe deficiency of endometrial receptivity which was noted for patients with endometriosis[28,32].

We have studied the character of apoptosis in the endometrium of women with tubal factor infertility, because these patients represent the most numerous clinical group of women participating in IVF protocols, so the estimation of their endometrial receptivity is of grate clinical value for improvement of IVF success. We have studied 73 women with tubal factor infertility, which planned to participate in IVF program. In women with tubal factor of infertility the increased level of the expression of pro-apoptotic factor PTEN mRNA with simultaneously elevated expression of mRNAs of anti-apoptotic factors XIAP and HSP27 were noted comparing to that in healthy women[74]. It is well known that XIAP is one of the important inhibitors of apoptosis, which is able to bind with caspase-9, -2 and -7 and inactivate their activity[75]. The XIAP expression is strongly elevated in patients with different types of tumors and the high level of XIAP production is associated with the poor prognosis[75]. The elevated expression of HSP27 also effectively protects cells from apoptosis[76]. It was found that HSP27 interacts and inhibits components of both stress- and receptor-induced apoptotic pathways[76]. It was shown that HSP27 could prevent activation of caspases by direct sequestering cytochrome c released from mitochondria into cytosol[76]. As we have mentioned above, normally during the window of implantation the increase of apoptosis in endometrial tissue is noted, so the high level of the production of anti-apoptotic factors might be estimated as negative factor which can reduce the receptivity of endometrium of women with tubal factor infertility. But it must be noted that the synthesis of PTEN was considerably increased in endometrium of women with tubal factor infertility. The tumor suppressor PTEN is also known as mutated gene in multiple advanced cancer 1, was discovered independently by two groups in 1997[38]. Somatic mutations of PTEN were identified as a prevalent event in different type of tumors, including tumors of the endometrium, brain, skin and prostate[38]. PTEN is a non-redundant, evolutionarily conserved dual-specific phosphatase. PTEN is capable to remove phosphates from protein and lipid substrates[77]. The primary target

Table 2 Expression of apoptosis-related genes in the endometrium of women with fertility problems during window of implantation

Clinical group	Studied gene	Character of expression	Ref.
Chronic endometritis	Bcl-2	Up-regulated	Yan *et al*[64]
	BAX	Up-regulated	
	Caspase-8	Down-regulated	
Unexplained infertility	Bcl-2	Strong immunoreactivity	Vatansever *et al*[66]
	p53	Weak immunoreactivity	
RIF	miR 145, 23b,99a (involved in p53 signaling)	Over expressed	Revel[12]
PCOS	Bcl-2 and p27	Increased expression	Gonzalez *et al*[63]
	FADD	Down-regulated	Yan *et al*[64]
Tubal factor	PTEN, XIAP, HSP27	Increased expression	Antsiferova *et al*[74]
Endometriosis	Bcl-2	Up-regulated	Jones *et al*[71]; Harada[32]; Nasu *et al*[28]
	IAP-proteins (c-IAP1, c-IAP2, XIAP, Survivin)	Up-regulated	Antsiferova *et al*[74]; Uegaki *et al*[73]
	DFF45	Low level	Banas *et al*[48]

Bcl-2: B cell lymphoma/leukemia-2; RIF: Repeated implantation failure; PCOS: Polycystic ovary syndrome; FADD: Fas-associated death domain; PTEN: Phosphatase and tensin homolog; XIAP: X-linked inhibitors apoptosis proteins; DFF45: DNA fragmentation factor of 45 kDa.

of PTEN is the lipid second messenger intermediate PIP3 (phosphatidylinositol 3, 4, 5-trisphosphate). PTEN removes the phosphate from the tree-position of the inositol ring to generate PIP2 (phosphatidylinositol 4,5-bisphosphate) thereby preventing intracellular signaling through the PI3K/Akt pathway[77]. It is known that Akt is the main downstream effector of PI3K (phosphoinositide 3-kinase) signaling that can phosphorylate a wide range of substrates and, thus, activate cell growth, proliferation and survival[38,77]. Today it is well known that PTEN function affects different cellular processes such as cell-cycle progression, cell proliferation, apoptosis, aging, DNA damage response, angiogenesis, muscle contractility, and other[77]. Recently the participation of PTEN in trophoblast invasion and decidual regression during human pregnancy was demonstrated[78]. Taking into account these properties of PTEN we suggested that the high level of PTEN mRNA expression in the endometrium of patients with tubal factor infertility might be estimated as positive mechanism, which compensates the over expression of anti-apoptotic factors and facilitates the preparing of endometrium to the implantation[74].

Summarized data about the character of apoptosis regulation in the endometrium of women with infertility are present in the Table 2.

ENDOMETRIAL APOPTOSIS AND IVF TREATMENT OUTCOME

Now it is evident that endometrial gene expression during receptive phase is associated with the IVF treatment outcome in infertile women. Recently the comparison of apoptosis-related genes expression in the endometrium of patients who achieved a successful pregnancy and those of patients who was not successful after at least two failed ICSI cycles was performed. And this comparative analysis showed a significant different expression of genes, involving in apoptosis, such as caspase-8, -10, FADD, APAF1, and ANXA4 in the endometrium of women with different ICSI outcome[79].

We also studied the relationship between the character of apoptosis-related genes expression in endometrium during window of implantation and successes of IVF treatment in women with tubal factor of infertility and with endometriosis. We found that in women with tubal factor of infertility implantation failures were associated with the lowest amount of PTEN mRNA expression in the endometrium[74]. These results evidence that this factor is essential for implantation and estimation of PTEN synthesis in the endometrium and can be used as the predictor of endometrial receptivity at least in infertile women with tubal factor. In group of women with endometriosis the pregnancy ongoing was achieved in those patients who initially had the minimal level of anti-apoptotic factors XIAP and HSP27 expression in the endometrium. So, it can be concluded that the high level of the activity of anti-apoptotic factors negatively influence the endometrial receptivity in infertile women with endometriosis. Probably the future investigations will allow us to identify the best receptive endometrial gene expression profile which can be used as an effective prognostic tool for IVF patients[79].

CONCLUSION

Normally, in secretory phase of menstrual cycle, namely during the window of implantation, the increase of tissue apoptosis is noted. Probably, this phenomenon is important for the establishment of endometrial receptivity and provides the adequate invasion of the implanted blastocyst in the endometrial stroma. Regulation of apoptosis is impaired in infertile women. In most cases the impairment of endometrial receptivity is associated with the decrease of apoptosis in endometrium during period of "window implantation". The estimation of the activity of apoptosis in the endometrium during the window of implantation surely gives us the important information about the endometrial receptivity. The further investigations of this problem would likely let us to better understand the mechanisms of endometrial receptivity and to develop new predictors

of IVF outcome.

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Review of the current surgical management of vulval cancer

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Abstract

Currently in the United Kingdom, 1200 cases of vulval cancer are diagnosed per annum accounting for 6% of female genital cancers. Although classically a condition that affects older women and associated with lichen sclerosus, there has been a greater incidence of vulval squamous tumours in young women due to the increasing prevalence of promoting human papillomavirus (HPV). The advent of a vaccination programme against HPV 16 and 18, the main aetiological causes of vulval intraepithelial neoplasia and cervical intraepithelial neoplasia, may reduce the incidence in future generations. Primary surgery is the current gold standard treatment and although mortality rates have reduced by 40% since the 1970s, radical vulval resections are associated with significant morbidity such as wound breakdown, infection, lymphoedema and psychosexual consequences. Over the past decade there has been a move to less mutilating procedures in women diagnosed with early vulval cancer. This is in combination with the introduction of new surgical methods such as sentinel lymph node testing, more directed radiotherapy and chemotherapy options. These treatment methods are being assessed in clinical trials to determine their associated recurrence rates, survival rates and morbidity. Most vulval cancers are squamous cell in origin but, there are other histological subtypes including Paget's disease and vulval melanoma which can require different management approaches. The objective of this paper is to review the current literature on the management of vulval cancer, summarise the new treatments which are being developed and the associated evidence.

Key words: Vulval cancer; Paget's disease; Human papilloma virus; Sentinel lymph nodes; Inguinofemoral groin node dissection

Core tip: One thousand and two hundred cases of vulval cancer are diagnosed annually in the United Kingdom. Primary surgery is the current gold standard treatment but radical vulval resections are associated with significant morbidity so there has been a move to less mutilating surgical procedures. Sentinel lymph node testing, more directed radiotherapy and chemotherapy

are all currently being assessed in clinical trials and the advent of the HPV vaccination programme may reduce the incidence in future generations. The objective of this paper is to review the current literature on the management of vulval cancer and summarise the new treatments and the associated evidence.

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INTRODUCTION

Currently in the United Kingdom, 1200 cases of vulval cancer are diagnosed per annum accounting for 6% of female genital cancers[1]. Due to the increasing prevalence of promoting human papillomavirus (HPV), the incidence of vulval squamous tumours has been increasing in young women[2]. The advent of a vaccination programme against HPV 16 and 18, the main aetiological causes of vulval intraepithelial neoplasia (VIN) and cervical intraepithelial neoplasia (CIN), may reduce the incidence in future generations[3]. Primary surgery is the current gold standard treatment and although mortality rates have reduced by 40% since the 1970s, radical vulval resections are associated with significant morbidity such as wound breakdown, infection, lymphoedema and psychosexual consequences[1,4-6]. Over the past decade there has been a move to less mutilating procedures in women diagnosed with early vulval cancer and this review paper outlines the current trends in treatment of women with vulval cancer, both early and advanced.

RESEARCH

A systematic literature search was performed using PubMed, Cochrane and EMBASE, using search criteria of "vulval cancer - early and late", "Paget's disease of the vulva", "sentinel lymph node testing in vulval cancer", "HPV and vulval cance" and "surgery, radiotherapy and chemotherapy for vulval cancer". Fifty-four papers were identified and thirty were directly referenced in this review paper.

DISCUSSION

Treatment of early cancer

For the management of stage Ⅰ and Ⅱ (Table 1), current studies have advocated a policy of "less is more"; wide local excision (WLE) reduces the significant morbidity associated with radical vulvectomy and recurrence rates are low[7,8]. It is recommended that tumours should be excised, ideally with a 2-cm excision margin down to the inferior fascia of the urogenital diaphragm and the fascia over the symphysis pubis[9]. Studies suggest that microscopic excision margins beyond 8 mm are associated with low recurrence rates, whilst margins less than 8 mm carry a recurrence rate of around 50%[10]. Moreover, larger surgical excision margins allow for the reduction and contraction of tissues following pathological preparation techniques. The dissection of inguino-femoral lymph nodes is dependent upon the depth of invasion and the site of the tumour; lateral tumours have more than 1 cm of healthy tissue beyond the WLE which does not affect midline anatomy and drain to the ipsilateral groin node. Resection of these nodes alone is safe with similar recurrence rates to bilateral dissection if the contralateral vulva is disease free[7,11]. With midline lesions, which occur less frequently, bilateral groin node dissection is required but pelvic lymph node dissection is not necessary due to the recognised pattern of lymphatic drainage[11].

Table 1 Staging system for vulvar cancer of the international federation of gynaecology and obstetrics

IA	Tumour confined to the vulva or perineum, ≤ 2 cm in size with stromal invasion ≤ 1 mm, negative nodes
IB	Tumour confined to the vulva or perineum, > 2 cm in size or with stromal invasion > 1 mm, negative nodes
II	Tumour of any size with adjacent spread (1/3 lower urethra, 1/3 lower vagina, anus), negative nodes
IIIA	Tumour of any size with positive inguino-femoral lymph nodes (1) 1 lymph node metastasis greater than or equal to 5 mm (2) 1-2 lymph node metastasis(es) of less than 5 mm
IIIB	(1) 2 or more lymph nodes metastases greater than or equal to 5 mm (2) 3 or more lymph nodes metastases less than 5 mm
IIIC	Positive node(s) with extracapsular spread
IVA	(1) Tumour invades other regional structures (2/3 upper urethra, 2/3 upper vagina), bladder mucosa, rectal mucosa, or fixed to pelvic bone (2) Fixed or ulcerated inguino-femoral lymph nodes
IVB	Any distant metastasis including pelvic lymph nodes

Hacker *et al*[12] 1993 investigated the correlation between depth of invasion and nodal involvement (Table 2). The evidence suggests that women with early vulval cancer (FIGO Ia) are at very low risk of nodal involvement if the depth is less than 1 mm and WLE alone is appropriate in this cohort. Women with a FIGO stage IB and above have a significant risk of groin node metastasis and therefore bilateral resection is recommended.

A Cochrane Review has assessed randomised control trials which have compared primary radiotherapy of the inguinofemoral lymph nodes to inguinofemoral lymph node dissection in women with early stage vulval cancer. This review concluded that groin node recurrence rates were increased (RR = 10.21, 95%CI: 0.59-175) with primary radiotherapy but lymphoedema was reduced (RR = 0.06, 95%CI: 0.00-1.03) and so were life-threatening cardiovascular complications (RR = 0.08, 95%CI: 0.00-1.45). Even though the review only included one study, which had a small number of participants (n = 52), it appears safer to excise the lymph nodes at present until the outcome of larger

Table 2 Correlation between depth of invasion and nodal metastasis in vulval SCC

Depth of invasion	Percentage of positive nodes
< 1 mm	0%
1-2 mm	7.6%
2-3 mm	8.4%
3-5 mm	26.7%
> 5 mm	34.2%

randomised control trials are available[13].

Lymph nodes

The method of lymph node dissection has also moved towards more conservative approaches: *En bloc* "butterfly" dissection has been replaced by the triple incision technique[14]. Additionally, there is some evidence that preservation of the fascia lata and saphenous vein can reduce the post-operative morbidity of lymphoedema without jeopardising outcome[15].

Recent advances in the management of vulval cancer have focused on sentinel lymph node testing. As lymph node metastases are only present in 30% of women with early vulval cancer, a significant number of women will be at risk of substantial postoperative morbidity and have negative lymph nodes. Different techniques to identify sentinel nodes have been used in trials, based on experience with different cancer types such as breast cancer and cutaneous melanoma. The best detection rates are seen when a combination of blue dye and technetium-based tests are used[16]. The GROINSS-Ⅵ trial concluded that patients with early stage vulval cancer patients and a negative sentinel node have low rates of groin recurrence (2%-3%), excellent survival rates at 3 years (97%) and minimal morbidity[17]. Groin recurrence with positive nodes was reported as 9%. However, other studies have suggested that detection rates and recurrence rates can vary significantly and the trial concluded that an element of quality control is required to ensure that an adequate level of surgical expertise and experience is present within an institution. Sentinel node techniques are therefore best suited to larger centres with higher case numbers. Additionally, data on longer-term recurrence is limited[18]. The GROINSS-Ⅶ trial is ongoing and seeks to treat patients with metastatic sentinel lymph nodes directly with radiotherapy instead of inguinofemoral lymphadenectomy followed by radiotherapy, thus theoretically reducing the morbidity of dual modality treatment[17]. In this trial, radiotherapy alone is restricted to patients with sentinel node metastases measuring ≤ 2 mm due to the known poor prognosis associated with sentinel node metastases measuring over 2 mm[19].

Adjuvant therapies

Adjuvant radiotherapy is offered to all patients who are found to have positive lymph nodes. The recent AGO-CaRE-1 study indicated that prognosis is improved in node positive patients, although when compared to those patients who are node negative the overall prognosis is poorer. Progression-free survival at 3 years was 39.6% in lymph node positive patients receiving adjuvant radiotherapy, 25.9% in LN positive patients without adjuvant treatment and 75.2% in lymph node negative patients[20]. It has been suggested that prognosis may be improved with the use of chemoradiation rather than radiotherapy alone, as this has been successful in the management of other squamous cell cancers such as anal and cervical cancer. No randomised controlled trials have yet been performed, but initial studies have indicated good clinical responses with acceptable toxicity[2].

Treatment of advanced vulval cancer

FIGO stage 3 and 4 tumours are characterised by local extension, pain, discharge, bleeding and odour. Management can be very difficult, particularly as lymph node metastases are present in approximately 50% and can ulcerate into the groin or can be fixed to the femoral vessels[14]. These patients have the option of ultra-radical surgery involving partial or total pelvic exenteration with the burden of a urostomy or colostomy, or treatment with chemotherapy or radiotherapy[21,22]. Results and prognosis can be poor, with symptom control often being the preferred option.

Post-operative complications and psychosexual consequences

The drive for a move towards more conservative surgery is related to the significant morbidity associated with excision of vulval lesions and lymph node dissection. Patients often have post-operative wound infections or dehiscence, prolonging hospital stays and require ongoing outpatient care. Moreover, inguinofemoral lymph node removal is associated with wound breakdown, lymphocyst formation, infection, lower limb lymphoedema and chronic cellulitis[23].

Excision of the clitoris, distortion of the vulval anatomy and changes to bowel and bladder function can also have significant psychological effects[24]. Many patients will become sexually inactive, and as expected, this trend seems to increase with the extent of surgical excision[25].

Paget's disease of the vulva

Extramammary Paget's disease can rarely affect the vulva (1%-5% of vulval malignancies), and is defined as an intraepidermal adenocarcinoma. The management is primarily surgical, but can be particularly complex to manage due to a multicentric pattern and irregular margins, resulting in up to 44% local recurrence rates[26]. Paget's disease can be sub-classified into intraepithelial and invasive types, with a significantly greater recurrence rate in invasive lesions of 67% compared with 9% in intraepithelial lesions over a median follow-up period of 69 mo in a recent study by

Nomura *et al*[27]. Distant metastases tend to be rare, with management focusing on surveillance and repeat surgical excisions. Alternative treatments that have been used with varying effects are local and systemic chemotherapy, radiotherapy, photodynamic therapy, laser therapy and Moh's micrographic surgery.

Impact of a HPV vaccine

The development and implementation of a vaccine against the highly oncogenic HPV genotypes 16 and 18, which account for 70% of CIN and VIN, have been projected to decrease the incidence and morbidity of pre-cancerous and cancerous genital disease. Indeed, the Future II trial, a randomised control trial which compared women given a HPV vaccine to placebo, showed a vaccine efficacy of 75% for low grade warty or basaloid VIN[28].

Despite these promising results, HPV is the main aetiological cause in only 30%-40% of invasive vulval cancers[29]. Differentiated VIN is responsible for 65%-80% of vulval cancers and arises in older women (55-85 years old) with concomitant vulval dermatoses such as lichen sclerosis, lichen planus or lichen simplex. Other histological subtypes include melanomas (3%), Bartholin's gland tumours (5%), adenocarcinomas (< 1%) secondary to Paget's disease and sarcomas (1%-2%)[3]. It seems apparent that the predicted decrease in incidence of cervical cancer following the introduction of the vaccine may not have such significant effects on the prevalence of vulval cancer, particularly in the aging population. Management of this cohort needs to be individualised to their medical comorbidities and therefore topical treatments such as Imiquimod and Cidofovir are being investigated to determine their efficacy in treating VIN so the morbidity of surgery, as outlined in this review, can be circumvented. Studies have shown a reduction of 25% in lesion size over 20 wk of treatment but this has to weighed against the acute discomfort following application of the cream and the lack of long term data assessing recurrence rates[30].

CONCLUSION

This overview of vulval cancer management summarises the current surgical approach, with an emphasis on conservative surgery that minimises the risk of recurrence whilst maintaining body function. There are important developments being made in the introduction of new management methods, most notably sentinel node testing. Current practice is limited by the availability of evidence and therefore further trials investigating existing and novel surgical management of vulval cancer will be vital. The results of ongoing sentinel node trials will give more information on longer-term recurrence rates and streamlining treatment modalities. Furthermore, reviewing resection margins in a more formal trial setting would also be valuable, whilst other trials researching chemo-radiation and the role of HPV may further change the direction of vulval cancer management.

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MINIREVIEWS

Imperative for improvements and international convergence of intrapartum fetal monitoring: A bird's eye view

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Abstract

Intrapartum fetal monitoring has been criticized for the lack of evidence of improvement in fetal outcome despite causing increased operative intervention. Paradoxically, cardiotocography (CTG) has been a major driver for litigation for neonatal neurological injury. This analytical review tries to explore why extensive clinical studies and trials over 50 years have failed to demonstrate or bring about significant improvement in intrapartum fetal monitoring. There seems a need for significant reform. International congruence on most aspects of CTG interpretation [definitions of fetal heart rate (FHR) parameters, CTG recording speed, 3-tier systems, *etc*.] is highly desirable to facilitate future meaningful clinical studies, evaluation and progress in this field. The FHR changes are non-specific and poor surrogate for fetal well-being. As a compromise for maintaining low false-negative results for fetal acidemia, a high false-positive value may have to be accepted. The need for redefining the place of adjuvant tests of fetal well-being like fetal blood sampling or fetal electrocardiography (ECG) is discussed. The FHR decelerations are often deterministic (center-stage) in CTG interpretation and 3-tier categorization. It is discussed if their scientific and physiological classification (avoiding framing and confirmation biases) may be best based on time relationship to uterine contractions alone. This may provide a more sound foundation which could improve the reliability and further evolution of 3-tier systems. Results of several trials of fetal ECG (STAN) have been inconclusive and a need for a fresh approach or strategy is considered. It is hoped that the long anticipated Computer-aided analysis of CTG will be more objective and reliable (overcome human factors) and will offer valuable support or may eventually replace visual CTG interpretation. In any case, the recording and archiving all CTGs digitally and testing cord blood gases routinely in every delivery would be highly desirable for future research. This would facilitate well designed retrospective studies which can be very informative especially when prospective randomised controlled trials are often difficult and resource-intensive.

Key words: Cardiotocography; Electronic fetal moni-

toring; Fetal heart rate decelerations; Intrapartum fetal monitoring; Intrapartum fetal surveillance; Fetal electrocardiography; Computerised cardiotocography

Core tip: Intrapartum fetal monitoring remains unsatisfactory because of the variability and errors in the interpretation of cardiotocographs and lack of proven benefit. Extensive clinical studies over the last 50 years have given divergent results because of the heterogeneity. There is a need for a significant reform of electronic fetal monitoring (EFM) with every aspect critically examined and debated. This analytical essay presents an overview of the current and newer modalities of EFM. It examines controversies and opportunities for improvement in American and European practice. Avoidance of biases, international congruence in terminology/standardization and refinement of adjunctive tests of fetal well-being are explored.

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INTRODUCTION

Cardiotocography (CTG) or the interpretation of fetal heart rate (FHR) patterns has been the most widely accepted and practiced method of intrapartum fetal monitoring for over 50 years. However, it remains the most controversial and problematic issue in Obstetrics despite being the commonest medical procedure in the western world and also the most extensively studied[1,2]. Controlled prospective trials show that caesarean section and operative delivery rates are increased with continuous CTG without improvement in fetal/neonatal short or long term outcomes irrespective of low or high risk labours[3]. Nevertheless, the CTG has become a "standard of care" expected especially in the presence of common risk factors. Severe perinatal hypoxia remains rare but can lead to distressing catastrophic outcomes like perinatal death or permanent neurological damage. Most developed countries spend big sums of money on litigation for cerebral palsy. For example the National Health Service in England paid out £3.1 billion (49% value of all claims) for negligence linked to maternity care in the past decade, mainly for cerebral palsy and errors in the interpretation of CTGs[4]. The term electronic fetal monitoring (EFM) is generally used to encompass techniques other than simple auscultation of FHR with a stethoscope or Doppler device. Although perceived to be a "defensive" practice, it is the EFM that has been the main driver for the increasing litigation for neonatal neurological injury and cerebral palsy[5]. This financial imperative (rightly or wrongly) pushes the issue of "intrapartum fetal monitoring" to the top of the patient safety agenda. This of course highlights the need, opportunity and potential to improve intrapartum fetal monitoring and patient safety. There is a strong desire and wish for some other different modern technology to replace CTG. However, presently it is difficult to see which cutting edge technology would be user-friendly, relatively non-invasive, and at the same time cost-effective and suitable for mass-application to the physiological process of childbirth. National Professional bodies have devised 3-tier systems of CTG interpretation which utilise multiple FHR parameters in different combinations with increasing degrees of abnormalities with an aim to achieve acceptable positive and negative predictive values to detect fetal academia[1,6-11]. However, the North American 3-tier has been found wanting[2,12,13] with a major drawback that almost 80% of all FHR tracings fall in the Category Ⅱ of indeterminate significance[12]. "Early" decelerations are extremely rare and all other FHR decelerations (late and all variable decelerations) fall in the category Ⅱ[12]. The clinicians can "continue to observe", "evaluate further" or "deliver" on individualized basis as no management algorithm can be prescribed for category 2, which has been a major criticism[12,13]. In the United Kingdom, there have been no significant clinical trials of the 3-tier system but the concept of "atypical variable decelerations" (with major impact on classification of CTGs) has been found to be flawed[4]. The National Institute for Health and Clinical Excellence (NICE) has recently abandoned the sub-classification of variable decelerations into "typical" and "atypical"[6]. Moreover, the CTG has a 99.8% false positive rate in predicting CP (cord pH < 7.00)[3] and there is scant evidence if at all that EFM has improved neonatal well-being. The very high disappointment with EFM has led Sartwelle *et al*[5] to argue that EFM is a "junk science". They propose that during medico-legal proceedings, the evidence from EFM should be considered invalid and inadmissible, based on the "Daubert doctrine" which excludes "junk science" from the courtrooms. They make a strong reasoned argument for a "change in course or abandonment of the ship (*i.e.*, EFM)"; a wake-up call for Obstetricians[5].

This brief analytical review is mainly directed at Obstetricians and midwives and intended to encourage a wide debate on the current perspectives, possible deficiencies, remedies, and future developments. It is not a "systematic review" of EFM which has already been presented by NICE and other national professional bodies[1,6,8,10,14,15]. This limited review is not intended to be entirely comprehensive or all-inclusive. Indeed some facets of EFM, not essential to the thesis presented, would be outside the remit of this paper. The issues and opinions described remain controversial by very nature but need to be debated and some experts may hold different views. The main focus is "CTG and FHR patterns especially decelerations". Other techniques

like "intermittent auscultation (IA) of FHR", fetal electrocardiography (ECG) (STAN or ST analysis), fetal oximetry and computerised CTG interpretation will be discussed briefly and not in-depth. The 3-tier systems of CTG interpretation in specific or proposing an alternative "proven" system are not the subject or purpose of this review.

EFM AND EVIDENCE BASED MEDICINE

EFM (CTG) became a routine clinical practice following pioneering work of Hon and Caldeyro-Barcia in 1950s[16,17]. Following the revolution of "evidence based medicine" (EBM) since 1980s, EFM has been subjected numerous clinical studies and trials. The International Federation of Gynaecology and Obstetrics (FIGO)[11] first proposed a 3-tier system of graded FHR abnormalities, variations of which have been adopted by most national guidelines to standardise the terminology as well as clinical intervention. But none of the national 3-tier classifications were published with the estimate of their sensitivity or false positivity[18]. A recent high quality study found no correlation of American 3-tier system to neonatal acidemia[13]. Although there are studies showing good correlation between CTG and neonatal acidemia, the overall quality of available evidence of reliability of CTG can be summarized in the words of NICE as, "The evidence is of moderate and low quality that there is moderate to low degree of association between different FHR parameters and neonatal acidosis"[6]. The extensive experience, evaluation and application of EBM has done very little to lessen these controversies. The reasons seem to be as follows: (1) the significant variations in definitions and grading of FHR parameters in different studies over the years make it extremely difficult to draw valid conclusions. Moreover, different and variable benchmarks of fetal outcome like pH (7.05 to 7.20); base deficit (-8.0 to -12.0), lactate, Apgar scores, *etc*. further complicate comparisons; (2) outcomes of importance (*e.g*., hypoxemic ischemic encephalopathy) are very rare so that large numbers of cases would be needed to show a difference; (3) it is almost impossible to separate "treatment effect" because the intervention in presence of abnormal CTG modifies the neonatal outcome. It is unethical and impractical to conduct truly blinded randomised controlled trials (RCTs); (4) the fetal heart rate is only a surrogate for fetal hypoxia and not a very good one[6]; (5) complex tasks of "pattern recognition" together with clinical evaluation may not be captured in simple algorithms and not reflected in the research trials and reviews[6]. Previous studies have often produced contradictory results owing to methodological and logistical limitations. This also means that definitive evidence from clinical studies (hard to come) need not be a precondition for the reform of CTG interpretation if prompted and supported by careful systematic observation, deliberation and restoration of physiological principles. There is a place for Bayesian approach with variable emphasis on observational data[19]; and (6) framing and Confirmation biases: These poorly recognised biases seem important correctible factors. "Anchoring/framing bias" is the tendency to create coherent initial picture without examining all available information[20-22]. "Confirmation bias" follows when we selectively focus upon evidence that supports our beliefs, while ignoring more comprehensive evidence that disproves these idea[20-22]. These biases are said to be very common and may particularly apply to interpretation of FHR decelerations[22] which are centre-stage in interpretation of CTG[23].

Despite the lack of good quality evidence of improvement in neonatal outcome overall, many Obstetricians believe that failure to use EFM may lead to bad outcome. The CTG generates an explicit confirmation and documentation of FHR which can be reassuring to patients and health workers, but has a potential to provoke anxiety as well. Recently the author conducted a survey of personal preferences of clinicians in our Institute with existing liberal culture of "IA of FHR". They were reminded of the evidence that CTG is not reliable in preventing CP or intrapartum hypoxia even in the presence of risk factors but does increase the operative delivery rate. All 14 Obstetricians and 11 of 15 midwives still chose to have CTG for themselves or their partners in the presence of risk factors. Thus, there seems a disconnect between the "evidence" and the beliefs/experience of birth-attendants themselves. Secondly, they seem to judge any harm from EFM in a different context/balance.

POSSIBLE REMEDIES TO IMPROVE CTG INTERPRETATION

There is a scope to make significant improvements and international congruence in CTG interpretation although one could not expect it to become a perfect test. The remedial measures could start by addressing variations and flaws/biases in categorization of FHR decelerations, variations in 3-tier CTG interpretation systems, standardization of CTG recording speed and refining the place of confirmatory tests of fetal well-being like fetal scalp blood sampling (FSBS).

VARIATIONS IN CATEGORIZATION OF FHR PARAMETERS

There is relative consistency and uniformity in defining normal and abnormal baseline FHR, baseline variability and accelerations in different countries on both sides of the Atlantic over the last 50 years. However, the opposite is true when it comes to FHR decelerations which are more complex to interpret by very nature. Unfortunately, unambiguous and specific standardised definitions of FHR decelerations are missing in NICE guidelines[5,6] thus leaving several different sources to propose their own definitions often based on arbitrary and sometimes unscientific or implausible (fictional)

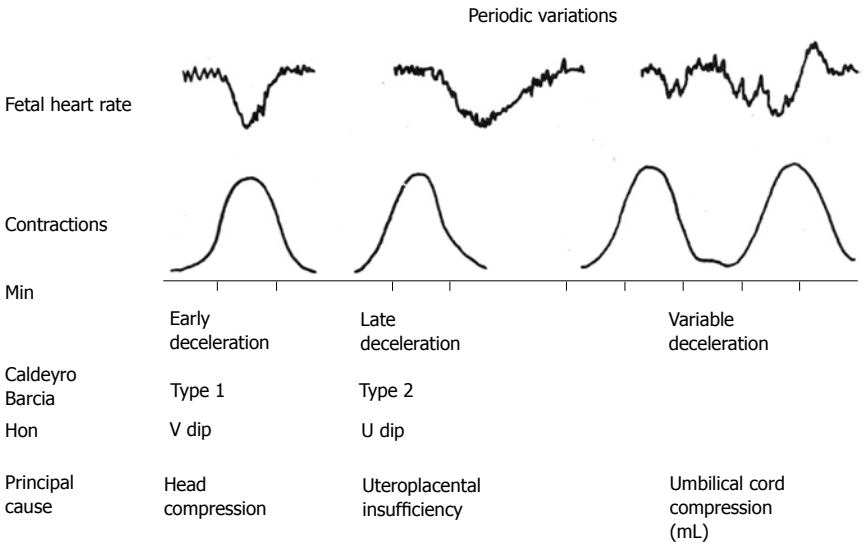

Figure 1 Diagrammatic representation of early, late and variable decelerations as practiced in British Obstetrics before 2007 (Reproduced with kind permission from "Principles of Obstetrics" by Bryan Hibbard, 1988)[26]. Note the apparent rapid descent of early decelerations. CTG paper speed 1 cm/min.

criteria[4]. This can constitute major framing biases which can go on to corrupt subsequent systems of interpretation incorporating them. FHR decelerations are the most common aberrant features and thus often deterministic in classifying CTGs into the 3-tier systems. Indeed the FHR decelerations were the "low hanging fruit" (generally with major correlation to outcomes) which were immediately picked up by the pioneers like Hon *et al*[16] and the group of Caldeyro-Bracia[17] based on clearly discernible observational evidence[22]. They categorised FHR decelerations based on time relationship to contractions only as indeed reflected in the terminology itself[16,17]. As a secondary hypothesis, they proposed that the early decelerations may be the result of head compression and those with variable time relationship to contractions may be due to cord compression although their classification was not primarily "etiological"[16,17,24]. Similar categorization was rooted and practiced in British Obstetrics until very recently which meant "early" decelerations were the most common variety (Figure 1)[25-28]. FHR decelerations can be said to be of two main types, one due to benign parasympathetic (vagal) reflex and other due to hypoxic (chemoreceptor) vagal reflex or direct suppression of myocardium in later stages[24]. The clue to differentiating this is in the "timing" rather than "shape" since hypoxia during contraction has a lag time to develop or worsen[24]. FHR decelerations which start recovering immediately after the peak of contraction (early timing) are not likely to have hypoxic component and hence it would be important to appropriately recognise them as benign ("early"). On the other hand the etiology of decelerations would always remain putative and possibly multifactorial with one of the causes predominant[24]. Although the current American and European categorizations of FHR decelerations seems to claim its foundation and legitimacy from the pioneering work and terminology of Edward Hon, they constitute a significant departure from Hon's original description[16]. Sometime in the late 20th century the American classification of decelerations seems to have become primarily "etiological" despite the many pitfalls. All decelerations with rapid descent were presumed to be due to cord compression even though head-compression could also cause rapid decelerations[23-25]. Thus all rapid decelerations were by definition called "variable" even though majority of them started early during contractions with nadir corresponding to the peak of contraction (early timing). This paradoxically made "early benign decelerations" extremely rare in the recent American practice. Does this represent a framing bias in need of correction? Terms that are specific, precise and truly descriptive (embody what it says on the tin) tend to be useful or convey meaningful information. Misleading (ambiguous) terminology can lead to loss of meaning. Moreover, the major focus on putative "repeated cord compression" as a main cause for development of fetal hypoxemia lacks clinical evidence and ignores the most likely cause of hypoxemia namely the repeated drop in maternal uteroplacental perfusion during contractions (especially on the background of reduced uteroplacental reserve or excessive uterine action). Etiological classification (misconstrued?) placing vast majority of FHR decelerations in the category of "variable decelerations" - based on unscientific hypotheses and misjudged application of animal experiments[22,24] - does not seem to have worked and indeed has been suggested to lead to loss of meaning. The pathophysiological hypotheses proposed for "cord compression decelerations" have several contradictions and "rapid" descent of decelerations does not discriminate between decelerations due to cord or head compression[22,24]. The heterogeneity in categorization of FHR decelerations and the interpretation of their significance (pathological nature) has been so great over the years and in different studies

in various countries that it has become impossible to draw any valid conclusions from the huge amount of clinical studies available in the literature. Although, more meaningful research is always welcome, it has been hard to come in this field, and should not be a precondition for examining validity of every aspect of CTG interpretation. Debating and arguing in a deliberative and interactive context can also help us to reach valid conclusions or closer to the scientific truth[22]. There is a substantial observational and experimental evidence that shape or rate of descent of FHR decelerations does not correlate to etiology of decelerations or fetal condition[17]. It would be greatly beneficial to reform the categorization of FHR decelerations in the USA and Europe correcting the framing/confirmations biases and flaws - the compatibility of which with scientific practice can be debated. Such a reform could go a long way in improving the reliability and further evolution of 3-tier systems. Simply standardization/uniform adoption and application of EBM principles on their own are unlikely to compensate for fundamental framing and confirmation biases.

CTG RECORDING SPEED

On the surface, it may seem unimportant that there is a difference in the CTG recording speed in different countries *viz* 3 cm/min in North America and 1 cm/min in United Kingdom and Australia-New Zealand. However, the CTG speed represents the horizontal scale and determines the "apparent" shape of the FHR waveforms especially decelerations. Thus gradual decelerations (U shaped) on an American CTG would appear rapid (V shaped) on British CTG. In fact the faster speed of CTG (3 cm/min) may have (erroneously?) drawn more attention to the shape of the deceleration waveform. Baseline variability, accelerations and decelerations can be judged quite well on CTGs with both speeds. With abandonment of reliance on so called atypical features of FHR decelerations, it is no longer necessary to look for FHR variability during a deceleration, which may have been possible with faster CTG speed of 3 cm/min. In any case this was never found practically useful. The slower CTG speed (1 cm/min) does seem to have one distinct advantage that FHR patterns over much longer time periods, *e.g*., 30-60 min can be visually examined and interpreted at a glance whether on a paper tracing or on a monitor screen. Since visual characterization and analysis of FHR waveforms has been of such critical importance, it would be highly desirable to adopt one uniform speed of CTG tracing across the globe to reduce heterogeneity in description and interpretation.

CONFIRMATORY/ADJUNCTIVE TESTS OF FETAL WELL-BEING

FHR is a relatively non-specific and poor surrogate for fetal condition[6]. Thus, with the current CTG interpretation, to achieve a very low false negative value for fetal acidemia, one has to settle for high false positive rate. In some clinical situations abnormal CTG may be enough to expedite delivery. But many times an adjunctive test may be necessary. In the United Kingdom, FSBS is a widely accepted and practiced test. Even FSBS is no stranger to controversy. There are reviews which propose that addition of FSBS to CTG interpretation does not improve outcomes or reduce operative intervention[29]. However, such meta-analyses are fraught with significant flaws and biases arising from dubious and variable quality of studies included. This seems another example as to how evidence from meta-analysis of several clinical studies runs counter to practically observed benefits of a long accepted practice. Vast majority of British hospitals use FSBS and find it practically useful in every day practice and hence it is unlikely that FSBS will be abandoned in British practice any time soon. FSBS following an abnormal CTG is quite often shows normal result thus allowing continuation of labor and often achieving vaginal delivery. It seems possible that FSBS result may be falsely positive (acidemic) because of stasis of blood flow in peripheral tissues especially in the presence of significant caput; but it is the extremely low "false negative" rate of FSBS that makes it very useful in day to day practice. FSBS is uncommon in American practice, but that probably leaves a gap to be filled. Fetal scalp stimulation test and vibroacoustic stimulation test seem promising[15] but they need to be more extensively and systematically studies. The role of fetal ECG (STAN) is discussed later.

IA

NICE suggests that about 45% of all labors are at low risk for fetal hypoxia and strongly recommends IA for these labors with fairly specific criteria to switch over to CTG[6]. The RCTs of IA *vs* CTG have shown equivalent perinatal outcome with reduced operative intervention in low risk labours[3]. However, even meta-analysis of these trials is underpowered to show possible differences because of rarity of serious adverse outcomes. Fortunately, the incidence of significant birth asphyxia in the absence of risk factors or an acute intrapartum adverse event is very low. Hence, a few regimes of IA can be quite loose or relaxed without frequent noticeable adverse events. For example, in Netherlands where all home births receive IA only, no structured guidelines are followed and as a convention FHR is auscultated every 2 h or so in the active first stage (personal correspondence with Jonge A de, 2015)[30]. NICE on the other hand recommends counting FHR with Doppler or Pinard stethoscope for 60 s after a contraction every 15 min in first stage and every 5 min in second stage of labor[6,7]. The intention is to detect/suspect late FHR decelerations[6]. In developing countries like China and India, the vast majority of labors (low and high risk) are monitored by IA and local protocols are often unstructured and variable; but are likely to be increasingly modelled on

NICE guidelines (personal correspondence). With the increasing use of IA, it is hoped that there may be future refinements[31,32]. However, it seems unlikely that more RCTs of IA *vs* CTG (requiring very large number of subjects) will be conducted in future.

FETAL ECG

Fetal ECG is recorded with a fetal scalp electrode. The ST segment analysis (STAN) has been practiced for over a decade mainly in Nordic countries but also in a few centres in United Kingdom and United States. Being a new arrival, STAN has undergone relatively ample evaluation in well-designed clinical trials. But, Five RCTs and five systematic reviews with meta-analyses have shown very divergent results[33]. Does this suggest that we may be looking for marginal gains here? Moreover, an absence of clear background, lack of transparency and a sense of Magic Black Box have been associated with STAN[33]. Most importantly and perplexingly, an ST event is supposed to lose its significance if the CTG is "normal". Hence, STAN results (unlike FSBS) seem dependent on the traditional CTG interpretation. Thus any major changes in the CTG interpretation[2,6] would further complicate the interpretation of the trials of CTG + STAN *vs* CTG alone by changing the "starting line" as well as the "finishing line". The largest RCT in United States on 11108 women did not show improved fetal outcomes or reduction in operative delivery unlike some of the trials in Europe[15,34]. The authors of this trial highlighted the need for caution when extrapolating results from studies outside the United States. This seems a major weakness in application of STAN, because the interpretation of "non-reassuring CTG" (*e.g.*, Category Ⅱ of American 3-tier system) varies markedly between United States, Europe, United Kingdom, Australia and New-Zealand. Moreover, the Category Ⅱ of American 3-tier system has been shown to be clinically unhelpful and already an additional algorithm[2] has been proposed to identify the "real" non-reassuring CTGs within the Category Ⅱ, which may become part of ACOG guidelines. At the same time there are some Obstetric units in Europe[35] and Australia (personal correspondence) which have abandoned the use of STAN because of serious adverse outcomes. All these seem major challenges to STAN and a different strategy which evaluates STAN results independent of CTG may need to be considered.

INTRAPARTUM FETAL PULSE OXIMETRY

This technology involves attachment of light emitting sensor to fetal scalp or temple to measure the proportion of haemoglobin that is carrying oxygen: Thus oxygenation. A recent Cochrane review found that intrapartum fetal pulse oximetry (IFPO) as adjunct to CTG (thus again dependent on correct interpretation of CTG) did not improve neonatal outcome or reduce the overall incidence of caesarean[36]. Again IFPO in parallel to CTG and FSBS may need to be subjected to more extensive clinical studies.

COMPUTERISED CTG INTERPRETATION

It is hoped that the long anticipated computer aided analysis of CTG will be more objective and reliable (overcome human factors) and may eventually replace visual CTG interpretation. However, despite the exponential increase in analytical and functional power of digital technology in the last decade, the development and adoption of "computerised CTG" has remarkably lagged behind. The difficulties are providing good quality evidence of its reliability or superiority over visual CTG interpretation, licensing and medicolegal considerations. Secondly, although the computerised CTG interpretation takes away inter-observer variation, there can be variations in FHR signal sampling and processing[15], and there may be a need for standardization probably supported by clinical trials, a challenging task in the field of EFM. Many singular instruments/parameters like "total deceleration area", "short-term variability", "approximate entropy" and "phase-rectified signal averaging" have been shown to correlate to fetal status to variable degrees. But a much "stronger" correlation with useful positive and negative predictive values would be required for clinical application. It is worth noting that the FHR patterns during the expulsive second stage are quite different from first stage (more frequent and deeper decelerations and higher variability). A mental adjustment is made for this difference during visual CTG interpretation which may not occur in computerised analysis. Hence, separate/different computerised analysis criteria (*e.g.*, deceleration area) for the first and second stage of labour would be highly desirable or indeed may improve correlation with fetal status[24]. One could argue that the computerised analysis should emulate the principles of visual CTG interpretation. The results of the "INFANT study"[38] evaluating a computer based "Intelligent Fetal Assessment" system (K2MS, Plymouth)[37] *vs* continuous CTG are eagerly awaited. The "Infant" (Intelligent Fetal Assessment) software emulates the principles of visual interpretation and provides 4 colour-coded categories[37]. Will such softwares need to be recalibrated and re-evaluated when visual CTG interpretation is changed significantly[2,6]? These are major challenges. Another software PeriCALM Patterns™ (PeriGen, Princton, NJ, United States) attempts to recognise EFM patterns based on baseline, baseline variability, FHR decelerations and contractions[18]. The "Infant" as well as PeriCALM™ do not claim to replace human visual CTG interpretation but propose to provide additional support at present. In any case, the recording and archiving all CTGs digitally and testing cord blood gases routinely in every delivery would be highly desirable. This would facilitate well designed retrospective studies which can be very informative especially when prospective RCTs are often impractical and resource-intensive.

COMMON PITFALLS IN INTRAPARTUM FETAL MONITORING

Errors in CTG interpretation are possible at any of the three stages involved namely signal acquisition, interpretation of signal (*e.g.*, FHR pattern) and clinical intervention[18].

Signal acquisition

The technology of obtaining FHR record with external Doppler and fetal scalp electrode has improved remarkably. Both maternal and fetal heart rates should be recorded and displayed which should eliminate mistaking maternal heart rate signal as FHR. A good record of timing and duration of contractions should be obtained in order to correlate them to any FHR decelerations.

Interpretation of FHR patterns

Variation in interpretation of CTG remains a problem although a lot of standardisation of terminology and analysis has been achieved over last 15 years by most national guidelines[1,6-11,14]. The current 3-tier systems are a graded classification of increasing abnormality of combinations of many FHR parameters like baseline, baseline variability and types of decelerations. Experts admit that CTG interpretation still has an element of "art" (expertise and intuition) in addition to "science based rules"[6]. Moreover, if standardisation is of the wrong sort then it is likely to be misleading and counterproductive. Framing/confirmation biases should not be dismissed as only of "academic interest"[39]. A major or critical framing bias would corrupt all consequent structures (*e.g.*, 3-tier systems) and developments. Thus the guidelines for CTG interpretation are still evolving and could undergo further material change. Secondly, similar CTG abnormalities have a more serious implication (higher positive predictive value) in the presence of high risk factors like growth retardation, thick meconium staining, infection, *etc*. Lastly, several human factors can affect interpretation like tiredness, tunnel vision, and failure of situational awareness *etc*[18]. These should be addressed by working hours regulations, appropriate staffing levels, fresh eyes approach and regular training updates/skills drills.

Clinical intervention

This final step is directly responsible for improving fetal outcome and safety[18]. The type and speed of clinical intervention has to be fine-tuned to the degree and evolution/progression of CTG abnormality in the given clinical scenario. It is a complex balance to undertake appropriate action without unduly increasing operative intervention. Automated warning systems when based on more reliable computerised CTG interpretation criteria would be very useful to improve patient safety.

CONCLUSION

Visual analysis of complex FHR patterns in response to uterine contractions (CTG) in the context of clinical setting remains the most widely practiced method of intrapartum fetal monitoring. National guidelines, (scientific) standardisation of terminology and structured systems of interpretation are important. However, there has been major criticism and disappointment associated with CTG. Hence, it seems urgent and important to have a fresh unbiased thorough assessment, reform CTG interpretation and eliminate any obvious framing/confirmation biases to restore scientific/physiological basis supplemented by clinical studies. International congruence on most aspects of CTG interpretation (definitions of FHR parameters, CTG recording speed, 3-tier systems, *etc*.) is highly desirable to facilitate future meaningful clinical studies, evaluation and progress. The birth attendants should apply critical thinking and reflection to all techniques of EFM and clinical cases/context, so that they can develop the ability (science and art) to make that final all-inclusive management decision. They should actively participate in the debate in this very practical and clinical subject thus contributing to the future knowledge and developments.

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MINIREVIEWS

Overview of embryonal rhabdomyosarcoma of cervix in women over 40-year-old

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Abstract

The literature on cervical embryonal rhabdomyosarcoma (RMS) is reviewed here to identify management guidelines for middle-aged women diagnosed with this rare type of gynecologic cancer. Specifically, the PubMed, Web of Science and Google Scholar databases, were searched to find published case series on cervical embryonal RMS reporting on four or more patients, of whom at least one was > 40-year-old. The χ^2 test was used to assess heterogeneity. Five articles published between 1986 and 2013 were identified, reporting on a total of 47 patients, of whom 22 (46.8%) were older and 25 (53.2%) younger than 40-year-old. Although the two age groups did not differ significantly by stage of disease or radiotherapy treatment, the older age group

received less chemotherapy (55% *vs* 90%, *P* = 0.008) and had more hysterectomy (86% *vs* 43%, *P* = 0.009). Follow-up data was missing for 18/47 (38.3%) patients. Among the 29 patients with follow-up data, survival was shorter in the older group, with 8/12 (67%) alive and 3 with disease at a median follow-up of 2.6 years, as compared with the younger group that had 15/17 (88%) alive and none with disease at a median follow-up of 3.5 years. The longest survivals among the older women were observed in those who received radiotherapy, including one case with a resected lung metastasis. A prospective multi-institutional collaboration and better follow-up are needed to determine the optimal management of cervical embryonal RMS. Long-term survival appears feasible if management is accompanied by chemotherapy and radiotherapy.

Key words: Embryonal rhabdomyosarcoma; Botryoid sarcoma; Cervix; Middle-aged adults; Chemotherapy; Radiotherapy; Review

Core tip: The present review of adult cervical embryonal rhabdomyosarcoma case series summarizes 5 studies reporting on 47 female patients, 22 of whom were > 40-year-old. Women over 40 had worse disease-free survival but also received less chemotherapy administration. Although radiotherapy was not often used, the longest survival in the > 40 age group was observed among those who received radiotherapy, including a case with resected lung metastasis. The review argues for multimodality management in this rare disease.

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INTRODUCTION

Rhabdomyosarcoma (RMS), a mesenchymal tumor with the potential to differentiate into skeletal muscle, is the most common soft tissue sarcoma in children[1,2]. It is a complex disease that can originate in any part of the body, from the head or neck to the limbs. The major histologic subtypes are the alveolar rhabdomyosarcoma (ARMS) and the embryonal rhabdomyosarcoma (ERMS). ARMS is frequently characterized by the translocation of t (2; 13) PAX3-FOXO1 and t (1; 13) PAX7-FOXO1, while ERMS typically has a loss of heterozygosity of the short arm of chromosome 11[1,2]. ERMS is further divided into two classifications for the sarcoma botryoid variant or spindle cell variant[2]. Considerable improvements in the survival of ERMS patients have been attributed to intensive chemotherapy[3]. While RMS is rare, cervical location is exceedingly rare in adults, but a well-known entity. Thus far, only 10 adult cases of women > 40-year-old have been reported in the literature[4]. The present review aims to define the presentation of cervical RMS in adult women. We examined how outcomes of women > 40-year-old compared with younger women diagnosed with ERMS.

Table 1 Intergroup Rhabdomyosarcoma Study Group pretreatment clinical staging system

Stage	Sites of primary tumor	Tumor size, cm	Regional lymph nodes	Distant metastases
1	Orbit, non-parameningeal head/neck; genito-urinary non-bladder/prostate; biliary tract	Any size	N0 or N1 or Nx	M0
2	All other sites	≤ 5 cm	N0 or Nx	M0
3	All other sites	≤ 5 cm	N1	M0
		> 5 cm	N0 or N1 or Nx	
4	Any site	Any size	N0 or N1 or Nx	M1

N0: Absence of nodal spread; N1: Regional nodes involved; Nx: Unknown nodal status; M0: No metastases; M1: Distant metastases at diagnosis.

LITERATURE ON ERMS

The PubMed and Web of Science databases were searched for publications on ERMS. The key word combinations used were "rhabdomyosarcoma and embryonal" or "sarcoma and botryoid*" and "cervical or cervix". The search was expanded manually using Google Scholar. Articles selected were case series reporting on four or more patients with cervical ERMS, among whom at least one was 40-year-old or older. The cutoff age of 40-year-old was chosen because fertility might be less of a concern than in younger women. We also took into consideration a bimodal age distribution around 40 years[5,6]. We focused on ERMS because of its preponderance among cervical sarcomas, representing 64.0% of all cervical sarcomas[7].

Two classification systems most commonly used were the pre-treatment TNM-based staging classification (Table 1), and the Intergroup Rhabdomyosarcoma Study Group (IRSG) clinical grouping based on operative pathology (Table 2). Tumor-node-metastasis classification has 4 stages (1-4) varying on the site of primary tumor, tumor size (T), regional lymph node involvement (N) and presence or absence of distant metastases (M). The IRSG classifies tumors in four groups (I-IV). Group I tumors are localized and completely resectable; however, based on whether they are confined to an organ/muscle or infiltrate outside is further classified as groups IA or IB, respectively. Tumors in group II are subdivided into three subgroups (A-C). In these tumors, the microscopic disease remains at margins and/or regional nodes. Group III (A-B) tumors

Table 2 Intergroup Rhabdomyosarcoma Study Group surgical grouping system

Group I	Localized disease, completely resected (clear margins, negative regional nodes)
IA	Confined to organ or muscle of origin
IB	Infiltration outside organ or muscle of origin
Group II	Microscopic disease remaining (at margins or in regional nodes)
IIA	Grossly resected tumors with microscopic residual tumor
IIB	Regional disease, completely resected, with nodes involved and/or tumor extension into an adjacent organ
IIC	Regional disease, with involved nodes, grossly resected, but with evidence of microscopic residual tumor
Group III	Incomplete resection or biopsy with gross residual disease remaining
IIIA	After biopsy
IIIB	After major surgical resection
Group IV	Distant metastases present at onset

are characterized by an incomplete resection or biopsy with gross residual disease remaining. Group Ⅳ tumors have distant metastases present at onset.

Data extracted from the publications were cross-tabulated by age group, the source of publication, and clinical grouping. For the analyses, we considered a pooled survival analysis using established methods[8]. However, upon browsing the publications, we realized that firstly there were no randomized trials, and secondly data were too scarce to allow advanced analyses[9]; providing inferential statistical outcomes would be misleading. We restricted the analyses to ad-hoc descriptive statistics, using the χ^2 test only to indicate heterogeneity of the data. If patients in a group received significantly more treatment than the other group, we would report the *P* value to indicate the heterogeneous treatment attribution. However, if survival outcome differed, we would not test the survival difference. We detailed each retrieved publication as a "vignette", so as to preserve a direct link with the source. We designed, after the fact, the cross tabulation to go one step beyond the narrative, without overextending to the pretense of a pooled review or a meta-analysis[10].

Review of ERMS case studies

A total of five eligible publications out of 6180 links from the three internet databases were identified (Figure 1, flowchart according to Moher *et al*[11]).

(1) Montag *et al*[5]: 4 cases of cervical ERMS; 18-42-year-old. One cervical ERMS patient was 42-year-old; (2) Brand *et al*[12]: 5 cases of cervical ERMS; 0-48-year-old. One cervical ERMS patient was 48-year-old; (3) Ferguson *et al*[13]: 15 cases of RMS embryonal and non-embryonal, of which 8 were cervical; 17-58-year-old. Five cervical ERMS patients were over 40-year-old; (4) Kriseman *et al*[14]: 11 cases; 3-52-year-old. ERMS in 10 cases, undifferentiated RMS in 1 case. Two cervical ERMS patients were > 40-year-old; and (5) Li *et al*[15]: 25 cases of ERMS, of which 20 were cervical; 20-89-year-old. Thirteen cervical ERMS patients were > 40-year-old.

Three additional publications also reported adult case series; however, there were no patients > 40-year-old[16-18]. Hence, these three publications were not selected for the review.

There were 47 patients with cervical ERMS from the five eligible case studies, with a mean age of 36-year-old at presentation (median, 33 years; range, 1-89 years). Table 3 summarizes patient characteristics according to age group. Table 4 provides the distribution by age and the IRSG grouping.

Montag *et al*[5] reported 6 cases of ERMS, diagnostic period unspecified, 4 of which involved the cervix or the cervix and uterus, and 2 of which had involvement of only uterus[5]. Five patients had a history of vaginal bleeding starting at 6 wk to 16 mo before the diagnosis. The ages of patients with a cervical origin were 18-, 21-, 26-, and 42-year-old. One patient had a vaginal protrusion of the tumor. The 42-year-old patient had a history of vaginal polyp excision, which had occurred 4 mo earlier. She was treated with a total abdominal hysterectomy (TAH), bilateral salpingo-oophorectomy (BSO), omental biopsy, and pelvic and paraaortic lymph node biopsies. There was no evidence of extra-uterine disease. The patient received external postoperative radiation therapy consisting of 4500 cGy to the whole pelvis and an additional 4000 cGy to the vaginal cuff with radium brachytherapy. Nine months later, the patient developed a metastasis in the right lung. No other site of recurrence could be documented. The patient underwent resection of the upper lobe of the right lung followed by intravenous chemotherapy with nine cycles of doxorubicin and dacarbazine, then nine other cycles combining vincristine, actinomycin-D and cyclophosphamide (VAC), duration unspecified. The patient was alive without evidence of disease 10 years after her initial surgery. The younger patients were treated with radical hysterectomy and bilateral pelvic lymph node dissection (PLND). The 3 younger cervical ERMS patients all received postoperative chemotherapy, and 2 of them also preoperative chemotherapy. They were alive without evidence of disease 17 mo, 7 years, and 8 years after their surgery, respectively. The authors insisted on the importance of initial staging, which included cystoscopy, radiological exams and scans, as the single most important factor affecting prognosis and survival. For non-metastatic locally advanced disease, chemotherapy and radiotherapy have been used to convert inoperable RMS into a resectable disease. The authors argued that surgery followed by adjuvant chemotherapy and, occasionally, radiotherapy for residual disease produced the highest survival rates. An interesting point was the aggressive management of oligometastases in the patient with the longest survival.

Brand *et al*[12] reported 4 cases of ERMS of the uterine cervix diagnosed between 1960 and 1986. Patients were 17-mo-old, 2 were 17-year-old, and 1 was 48-year-old. The patients had a history of vaginal bleeding for 1 to 8 mo prior to the diagnosis of ERMS. The patient over 40 was treated with TAH-BSO. She received eight

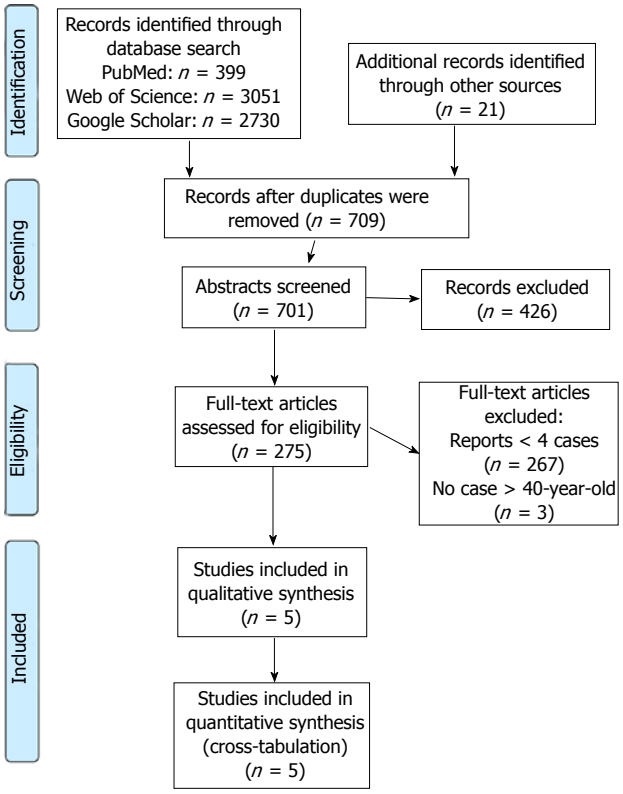

Figure 1 Flow diagram of studies selection.

cycles of cyclophosphamide over a 3-mo period. Vaginal ERMS recurrence was diagnosed 2 mo later, for which she received whole pelvis external radiation (5400 cGy) and intravaginal radium therapy (3500 cGy). She was alive without evidence of malignancy 22 years after the initial treatment. Among the ⩽ 40-year-old patients, the 17-mo-old infant presented with pulmonary, bone and brain metastases (Group IV, definition in Table 2). She received vincristine, dactinomycin and doxorubicin over 3 years and was reported to be alive and free of disease 8 years after the diagnosis of ERMS. The two other ⩽ 40-year-old patients, who were both 17-year-old, presented with Group IA disease (definition in Table 2, localized disease), and received both TAH-BSO. One received postoperative radiotherapy and was diagnosed with bone metastasis at 1 mo after the radiotherapy; she died with metastatic disease 1 year after the initial diagnosis of ERMS. The other 17-year-old received adjuvant chemotherapy with dactinomycin and vincristine; she was alive and free of disease at 1 year after the initial diagnosis of ERMS. In the series, the outcome of the 48-year-old patient was comparable to that of the younger patients. Brand and colleagues stressed the importance of neo-adjuvant chemotherapy to conserve the function of the bladder, rectum, vagina, and ovaries when feasible. The author considered the role of radiotherapy unclear and did not recommend it for the Group Ⅰ patients.

Ferguson *et al*[13] reported 8 cases of cervical ERMS treated between January 1963 and December 2003. Five patients were > 40-year-old at diagnosis (range, 46-58 years). All women older than 40 years presented with early-stage disease. The primary therapy for these women was surgery with TAH/BSO/PLND, except for 1 patient who had no BSO. Four of the women received adjuvant therapy, 2 with radiation alone and 2 in combination with chemotherapy. Three patients were alive without evidence of disease at 7, 27, and 37 mo after the diagnosis. Two patients who did not receive chemotherapy died of the disease. Among the 3 younger patients (age at diagnosis: 17-, 22-, and 32-year-old), 2 had a recurrence of the disease. Time to progression was 7 and 9 mo, and these 2 patients died of disease at 5 and 8 mo after recurrence, respectively. The authors stated that survival in women with gynecologic ERMS was not as favorable as compared to the pediatric population but emphasized that ERMS in adults is responsive to chemotherapy. They argued that adequate initial treatment should be multidisciplinary with surgery, radiation, and combination chemotherapy to offer the best chance of survival.

Kriseman *et al*[14] reported 11 cases of cervical ERMS between 1980 and 2010. Vaginal bleeding or discharge was present in all 11 patients, and 4 patients also had a protruding vaginal mass as a presenting symptom. Two patients had no staging information available. The other 9 had disease classified as IRSG group Ⅰ, for whom the authors also provided International Federation of Gynecology and Obstetrics (FIGO) staging information intended for carcinoma rather than sarcoma[19,20]. All patients received surgery at some point during the treatment. Two patients were > 40-year-old at diagnosis (49 and 52 years). The 49-year-old patient had FIGO stage IB1 disease (tumor ⩽ 4 cm). She was treated with TAH-BSO and received adjuvant chemotherapy (cyclophosphamide, doxorubicin, vincristine, and dacarbazine) and radiation beginning 6 mo after the diagnosis (4600 cGy delivered over 31 d). Six years later, this patient was diagnosed with an unresectable high-grade adenocarcinoma of the right parotid which metastasized to the lungs and caused her death. The 52-year-old patient had FIGO stage IB2 disease (tumor > 4 cm). She was treated by cone biopsy and did not receive adjuvant therapy. The patient was in complete remission 19 mo after the end of the treatment. The 9 younger patients presented with stage Ⅰ B1 disease (2 cases), stage IB2 disease (3 cases), stage ⅡA disease (tumor involving upper 2/3 of the vagina, without parametrial invasion; 1 case), and stage unknown (2 cases). Eight patients were treated by cone biopsy, and 1 had a TAH. Neo-adjuvant chemotherapy was given in 2/9 cases. Post-operative chemotherapy was given in 6/9 cases. Five patients were alive without evidence of disease at 4, 6, 23, 35, and 121 mo after completion of therapy. One patient was alive with the disease following a local recurrence, 1 died of the disease, 1 died of unknown cause, and 1 died of complications related to neutropenic fever. The authors stressed the importance of multimodal therapy, even at an early stage. They found no differences in

Table 3 Case-series of cervical embryonal rhabdomyosarcomas reporting on > 4 patients, of whom at least one was > 40-year-old

Author Year Cases	Women ≤ 40-year-old				Women > 40-year-old			
	n	Age, yr	1st treatment (*n*)	Follow-up (yr)	*n*	Age, yr	1st treatment (*n*)	Follow-up (yr)
Montag *et al*[5] 1986 *n* = 4	3	18, 21, 26	TAH (3), BSO (2), LND (2); CT (3)	Alive NED at 1.4 yr, 7 yr, and 8 yr	1	42	TAH-BSO-LND; RT + BT	No local recurrence, but lung metastasis at 9 mo, resection and CT; Alive NED after 10 yr
Brand *et al*[12] 1987 *n* = 4	3	1.4, 17, 17	TAH (2), BSO (1), LND (1), no surgery (1); CT (2), RT (1)	Died WD at 0.5 yr (case no RT); Alive NED at 1 yr and 8 yr	1	48	TAH-BSO; CT	Vaginal recurrence at 2 mo, pelvic RT + intravaginal brachytherapy; Alive NED after 22 yr
Ferguson *et al*[13] 2007 *n* = 8	3	17, 22, 32	Unknown (1), TAH (2), BSO (1), LND (2); CT (2), RT (2)	Died WD at 1.8 yr (case CT + RT); Alive NED at 0.2 yr and 10 yr (RT)	5	46, 51, 52, 56, 58	TAH-BSO-LND (4); TAH-LND (1); CT (2); RT (2); BT (2)	Dead WD (2) 1 yr and 1.4 yr after; Alive NED (3) at 0.6 yr, 2 yr, and 3 yr
Kriseman *et al*[14] 2012 *n* = 11	9	3, 12, 13, 17, 18, 18, 27, 33, 34	No surgery (2), cone biopsy (6), polypectomy (1), TAH-BSO (1), CT (8), RT (1)	No FU (4), Died at date not reported (1) Alive NED (5) at 0.3 yr, 0.5 yr, 2 yr, 3 yr, and 10 yr	2	49 52	TAH-BSO; CT; RT Cone biopsy; no adjuvant therapy	No recurrence, died after 6 yr from the second primary tumor, adenocarcinoma of parotid; Alive NED after 1.5 yr
Li *et al*[15] 2013 *n* = 20	7	20, 21, 23, 26, 29, 29, 31	Unknown (2), polypectomy (4), TAH (1) CT (3)	No FU (5); Alive NED (3) at 3.5 yr, 6 yr, and 8 yr	13	54 46 73 43, 44, 46, 47 48, 49, 51, 56, 56, 89	TAH TAH; CT Polypectomy Polypectomy (7); TAH (2); Unknown (1)	Alive WD (vaginal recurrence) at 0.4 yr Alive NED at 3 yr Died at 0.4 yr with lung metastasis and a second primary tumor, ductal breast carcinoma No FU data
Summary *n* = 47	25	Median age 21 yr (range 1-34 yr)	Surgery: TAH 9/21 (42.9%), CT: 18/20 (90.0%), RT: 4/19 (21.1%)	No FU: 8 (32.0%), Alive: 15/17(88.0%), 0 WD, Median FU, 3.5 yr Died: 3/17 (17.6%), at median 1.1 yr	22	Median age 50 yr (range 42-89 yr)	Surgery: TAH 12/14 (85.7%) CT: 6/11 (54.5%) RT: 4/11 (36.4%)	No FU: 10 (45.5%) Alive: 8/12 (66.7%), 3 WD, Median FU 2.6 yr Died: 4 (33.3%), at median 1.2 yr

TAH: Total abdominal hysterectomy; BSO: Bilateral salpingo-oophorectomy; LND: Lymph node dissection; NED: No evidence of disease; WD: With disease; CT: Chemotherapy; RT: External radiotherapy; BT: Brachytherapy; FU: Follow-up.

treatment or survival among women > 19-year-old and younger patients.

Li *et al*[15] reported 20 cases of cervical ERMS. Eleven patients were > 40-year-old. A cervical polyp was the most common clinical presentation. The diagnosis was made by biopsy (1 case), polypectomy (8 cases), and hysterectomy (2 cases). Therapeutic modalities were not available for all patients. Of the 3 patients with follow-up, 1 was alive without evidence of disease 3 years later; this case had received chemotherapy. One patient was alive with the disease at 5 mo after treatment. One patient died with pulmonary nodules at 5 mo after the treatment. Seven patients < 40-year-old were diagnosed by biopsy (2 cases) and polypectomy (5 cases). Follow-up data was available for 3 cases. These patients were alive without evidence of disease at 3.5, 6, and 8 years after chemotherapy. Li *et al*[15] compared the microscopic diagnosis of cervical ERMS in adults but reported no particular age-related morphological pattern. Their series' age distribution showed a bimodal pattern with a peak at 40-49 years, but the authors did not comment. The authors discussed the importance of considering ERMS in the differential diagnosis of uterine corpus or cervical spindle cell tumors, regardless of the patient's age.

Synthetic overview

Both adult and pediatric patients with cervical ERMS presented with vaginal bleeding and sometimes tumor protruding out of the vagina. However, in adults, symptoms can last up to 16 mo[5]; in contrast, in pediatric series, the vaginal bleeding and spotting lasted for 1 wk or less[18].

Histologically, ERMS tumors are characterized by myxoid stroma or edematous hypocellular spindle cell proliferation with cellular condensation beneath epithelial surfaces (cambium layer)[5,12,13,15]. Pathological diagnosis is based on the demonstration of skeletal muscle differentiation, morphologically, and with the use of immunohistochemistry for the coexpression of desmin and myogenin[5,13,15]. Ki-67 expression is usually elevated, and estrogen and progesterone receptors are

absent[15]. ERMS differs from ARMS in that the stroma in ERMS is variably loose and shows dense cellularity within a myxoid matrix, whereas ARMS cells tend to cluster into nests that may be separated by fibrous septa[2].

Genetic alterations have yet to be clinically explored in cervical ERMS. In pathology series, there are frequent allelic losses, most notably in the chromosomal region 11p15.5[2]. Loss of heterozygosity on the short arm of chromosome 11 suggests inactivation of tumor suppressor genes[21]. Diverse gene fusions have been reported in subsets of ERMS[22,23], fusion-positive PAX3/7-FOXO1 in 7 of 31 ERMS, and hyperploidy associated with gains of chromosomes 2, 8 and 12[24]. While a discussion of genetic lesions is beyond the purpose of the present review, the lesions may potentially explain heterogeneity of cases and the increased risk for multiple primary cancers that have been reported by several authors[14,16,18]. FOXO1 translocation status will be used as a risk stratification criterion in future pediatric RMS studies[3].

Staging did not seem to differ across different age groups; however, diagnostic work-up was missing in half of the cases reviewed (Table 4). The case series spanned across a long time period, from as early as 1960, whereas current imaging procedures differ considerably. Patients who had a negative positron emission tomography (PET) following induction chemotherapy and radiotherapy had a better local relapse-free survival than RMS patients who had a positive PET (94% *vs* 75%, $P = 0.02$)[25]. PET might replace conventional staging imaging studies[3,26,27]. None of the case series reported the use of PET.

There were substantial differences in the management between the two age groups in the pooled data, though this could not be ascertained within each paper (Table 3). Data on hysterectomy was more often missing in older women than in younger women [36% (8/22 patients) *vs* 16% (4/25 patients)]. Among documented cases, hysterectomy rates were higher in older women [86% (12/14 patients) *vs* 43% (9/21 patients)]. In older women, chemotherapy was used less often (55% *vs* 90%), but radiotherapy more often (36% *vs* 21%). These differences may be due to the desire to preserve fertility in younger patients.

Chemotherapy and radiotherapy data were missing more frequently for the older than the younger patients (50% *vs* 22%). Among those with available information, the most commonly used chemotherapy regimens in 20 patients > 40-year-old included VAC (13 patients, 65.0%), VA (2 patients, 10.0%), and vincristine, doxorubicin and cyclophosphamide (VDC) (2 patients, 10.0%). One patient received VDC plus dacarbazine and cyclophosphamide. Two patients received a non-VAC regimen (etoposide alone[13] or cisplatin and doxorubicin combination[14]). Overall, 85% of the chemotherapy regimens followed a pediatric protocol, regardless of age group[28-30].

The death rate was twice as high in patients > 40-year-old than in the younger age group. Older patients who survived had more residual disease than the younger patients. The poorer outcome might be explained by lower chemotherapy administration rate (Table 3). The follow-up data was missing across all age groups, but was especially high in the older group (45% *vs* 32%), precluding reliable comparison. In the most recent publications, the frequency of surgery appeared to decrease, and radiotherapy was infrequently given, even in women over 40 (Table 3). The median time of death or the last follow-up among younger patients who received radiotherapy was 1 year, which raises the question whether or not these patients had been selected because of more advanced disease. The longest survivals were observed among those who received external beam radiotherapy and brachytherapy. The reported survival times in that subset ranged between extremes, from 1.4 and 2 years[13] through 6[14], 10[5], to 22 years in a patient who received early salvage radiotherapy and brachytherapy[12].

Table 4 Intergroup Rhabdomyosarcoma Study Group clinical grouping of the 5 case-series of cervical embryonal rhabdomyosarcomas, overwhelming missing data

IRSG clinical stage	Women ≤ 40-year-old *n* = 25 *n* (%)	Women > 40-year-old *n* = 22 *n* (%)
NA	11 (44.0)	13 (59.1)
I	11 (44.0)	9 (40.9)
II	1 (4.0)	0 (0.0)
III	1 (4.0)	0 (0.0)
IV	1 (4.0)	0 (0.0)

IRSG: Intergroup Rhabdomyosarcoma Study Group; NA: Unknown, not available, or could not be inferred from the reports.

The limitations of our study have been a small sample size and a retrospective approach; nevertheless, EMRS is sensitive to chemotherapy and radiotherapy. Since ovarian preservation is less of a concern in women who are over 40 years old, radiotherapy could be applied in those who have metastatic pelvic and/or paraaortic lymph nodes or residual disease following surgery. In patients whose tumors are locally unresectable, chemoradiation may be given either preoperatively or to the patient who is not a surgical candidate because of associated co-morbidity. In patients who present with distant metastases, induction chemotherapy and reassessment for loco-regional treatment after chemotherapy may be an option, depending on the response to chemotherapy. In the era of modern radiotherapy techniques such as stereotactic body radiotherapy and image-guided radiotherapy, long-term remission may be feasible even when the patient has distant metastases[31]. Because of the rarity of the disease, the feasibility of prospective studies is questionable. An international registry for rare tumors could be used as a depository and a resource on treatment data and long-term follow-up.

CONCLUSION

ERMS is an exceedingly rare disease among middle-aged women. An international registry could help determine the optimal treatment of patients at various disease stages. Long-term survival is possible with multimodality treatments, as the tumor is chemo- and radio-sensitive. A multidisciplinary team approach is essential for optimal therapy. In the future, genomics might provide new therapeutic opportunities.

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ORIGINAL ARTICLE

Basic Study

Alpha1B adrenoceptor expression is a marker of reduced survival and increased tumor recurrence in patients with endometrioid ovarian cancer

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Abstract

AIM: To investigate the expression patterns of different adrenoceptor isoforms in ovarian cancer and their association with survival and tumor recurrence.

METHODS: The protein expression levels of α1B, α2C and β2 adrenoceptor were assessed in unselected ovarian cancer using immunohistochemistry on micro-arrayed archival tissue samples. A database containing clinical and pathology parameters and follow-up was used

to investigate the association between adrenoceptor isoform expression with ovarian specific survival and tumor recurrence, using univariate and multivariate statistical analysis.

RESULTS: Expression of α1B showed an association with reduced ovarian specific survival (P = 0.05; CI: 1.00-1.49) and increased tumor recurrence (P = 0.021, CI: 1.04-1.69) in the whole patient group. On sub-analysis the expression of α1B in endometrioid cancers (χ^2 = 5.867, P = 0.015) was found to predict reduced ovarian specific survival and increased tumor recurrence independently of tumor grade, clinical stage and chemotherapy. An association with clinical outcome was not seen for α2C or β2 AR.

CONCLUSION: Alpha1B adrenoceptor protein was found to predict increased risk of tumor recurrence and reduced mortality in patients with endometrioid type ovarian cancer and should be investigated as a biomarker for identifying patients at increased risk of disease progression. Furthermore, α adrenergic receptor antagonists with α1B selectivity should be investigated as a possible adjuvant therapy for treating patients with endometrioid cancer. Proof of principle could be tested in a retrospective population study.

Key words: Alpha adrenoceptor; Beta adrenoceptor; β-blockers; α-blockers; Ovarian cancer; Prognosis; Cancer therapy

Core tip: Epidemiological studies suggest that β-blockers might have a role in reducing metastatic spread and tumor recurrence and thereby prolong patient survival in some cancer types. In this novel study we found α1B adrenoceptor is a biomarker of tumor recurrence in endometrioid ovarian cancer. Further studies are needed to test if selective α1 adrenergic receptor antagonists inhibit tumor recurrence and prolong survival in patients with this type of cancer.

Deutsch D, Deen S, Entschladen F, Coveney C, Rees R, Zänker KS, Powe DG. Alpha1B adrenoceptor expression is a marker of reduced survival and increased tumor recurrence in patients with endometrioid ovarian cancer. *World J Obstet Gynecol* 2016; 5(1): 118-126 Available from: URL: http://www.wjgnet.com/2218-6220/full/v5/i1/118.htm DOI: http://dx.doi.org/10.5317/wjog.v5.i1.118

INTRODUCTION

Ovarian cancer is the fifth leading cause of cancer related death amongst women in the United Kingdom and the second most common gynaecological malignancy. The lifetime risk of developing ovarian cancer is estimated at 1 in 68 for women in the United States[1]. It arises with non-specific symptoms, thus about 70% of patients are diagnosed with late stage disease with a 5-year survival rate of less than 30%. In contrast, patients diagnosed with early-stage disease have a > 90% 5-year survival rate[2]. Endometrioid type tumors account for 10%-20% ovarian cancers and compared to other forms have a relatively better 5-year survival rate. Patients would benefit from earlier cancer detection improved therapies for preventing metastasis and disease recurrence.

Increased levels of catecholamine hormones are linked with poor prognosis in ovarian cancer[3-5] possibly explained by their ability at promoting cell invasion and proliferation *via* activation of adrenergic receptors (adrenoceptors, AR)[5,6]. Recent laboratory studies suggest that beta adrenergic receptor antagonists (β-blockers) abrogate cancer cell migration[7-9], an essential element of metastasis, and disrupt the stress/inflammatory/cancer pathway interactions[10]. Sloan *et al*[10] showed that epinephrine-induced beta2 (β2) adrenoceptor activated cancer cells produce mediators that recruit tumor associated macrophages, and the process is inhibited by the β-blocker propranolol[11].

There are 9 adrenoceptor subtypes classified in two major classes, α and β, belonging to the G-protein coupled receptor family[12]. Promiscuous AR coupling results in the activation of multiple cancer cell signalling pathways[13]. Included among these, β2 activation induces phosphokinase A (PKA) and ERK[14] cell signalling in response to upregulation of cyclic AMP (cAMP)[15], leading to migration in some cancer cell types. ERK1/2 phosphorylation may occur following α1B adrenoceptor activation in ovarian cancer cells[15-17] but opposing this, α2C adrenoceptor can preferentially inhibit *cAMP* and *PKA* gene transcription[17]. For this reason it can be hypothesised that activation of α2C adrenoceptors may have an anti-cancer regulatory role compared to α1B and β2 receptors.

A small number of conflicting population studies have investigated the impact of β-blocker usage on survival in ovarian cancer patients. These have either shown potential benefits[7,18] or no effectiveness[19,20]. Such differences could possibly be explained by AR expression heterogeneity and so the current study is designed to assess the distribution and pattern of α1B, α2C and β2 AR proteins expressed in ovarian tumors and association with clinical outcome. Knowledge of this information will improve the study design when assessing the feasibility of using adrenergic receptor antagonists for targeted adjuvant cancer therapeutics. It was found that α1B rather than β2 adrenoceptor expression correlated with poor survival and tumor recurrence in epithelioid tumors.

MATERIALS AND METHODS

The protein expression of α1B, α2C and β2 was chara-

cterised in formalin-fixed paraffin-embedded tissue microarrays of ovarian cancer from unselected patients attending Nottingham University Hospitals Trust. Damaged tissue cores and those that did not contain invasive carcinoma were censored. The study was approved by the Derbyshire ethics committee (07/H0401/156).

Patient selection

Following a diagnosis of ovarian cancer, patients were selected for chemotherapy treatment according to the East Midlands Cancer Network ovarian cancer treatment algorithm (http://www.eastmidlandscancernetwork.nhs.uk/Library/OvarianTreatmentAlgorithm.pdf). Tissue microarrays were produced by incorporating cores of archival formalin fixed ovarian tumor tissue from Nottingham patients presenting in 1991-2006. The presence of cancer was confirmed by a pathologist. Clinicopathological data was available for patients up to 240 mo post-diagnosis and was categorised as poor (< 60 mo) or better (> 60 mo) prognosis.

Immunohistochemistry

Four micron thick TMA sections had immunohistochemistry performed using a linked streptavidin peroxidase/biotinylated AB technique in accordance to the supplier's recommendations (DAKO, Cambs, United Kingdom). Microwave antigen retrieval was performed in 0.01 mol/mL citrate buffer (pH6). The primary antibodies were previously optimized in full face breast cancer tissues as previously described[21] and in ovarian TMA sections with negative controls. Primary rabbit polyclonal_antibodies against α1B (ab13297, Abcam, Cambs, United Kingdom), α2C (ab46536, Abcam), and β2 (ab13163, Abcam) AR were used diluted at 1:50, 1:750 and 1:450, respectively.

Statistical analysis

Statistical analysis was performed using statistical software, SPSS 20.0 (SPSS Inc., Chicago, IL, United States). Levels of adrenoceptor protein expression were microscopically assessed for staining intensity in malignant epithelium only and patients categorised into negative (negative or weak intensity) verse positive (moderate to strong intensity). Patients with missing clinical data were censored. Association between AR expression and different clinicopathology factors was evaluated using the non-parametric χ^2 test. Survival and tumor recurrence analysis was modelled using the Kaplan-Meier method with a univariate log rank test to assess significance. Patients that died due to causes other than ovarian cancer were censored during survival analysis. Multivariate Cox proportional hazard regression (95%CI) was used to evaluate the independence of adrenoceptors for predicting survival and tumor recurrence compared to other clinical variables. A *P* value of < 0.05 was considered to indicate statistical significance.

Table 1 Distribution of adrenoceptor protein expression according to age, tumor type and tumor grade in patients with ovarian cancer

		Adrenergic receptor	Absent	Present	χ^2	*P*
Age at diagnosis	< 49	α-1B	25 (66)	13 (34)	1.076	0.584
	50-69		55 (59)	38 (41)		
	> 70		24 (54)	20 (46)		
	< 49	α-2C	11 (29)	27 (71)	2.600	0.273
	50-69		38 (42)	52 (58)		
	> 70		19 (45)	23 (55)		
	< 49	β2	9 (31)	20 (69)	1.956	0.376
	50-69		36 (43)	47 (57)		
	> 70		13 (33)	26 (67)		
Tumour type	High grade serous	α-1B	49 (55)	40 (45)	14.648	0.005
	Low grade serous		4 (67)	2 (33)		
	Endometrioid		25 (60)	17 (40)		
	Clear cell		16 (94)	1 (6)		
	Mucinous		4 (29)	10 (71)		
	High grade serous	α-2C	44 (48)	47 (52)	11.038	0.026
	Low grade serous		4 (67)	2 (33)		
	Endometrioid		8 (21)	31 (79)		
	Clear cell		6 (35)	11 (65)		
	Mucinous		5 (33)	10 (67)		
	High grade serous	β2	25 (32)	54 (68)	13.559	0.009
	Low grade serous		1 (17)	5 (83)		
	Endometrioid		20 (54)	17 (46)		
	Clear cell		8 (61)	5 (39)		
	Mucinous		1 (9)	10 (91)		
Tumour grade	1	α-1B	11 (42)	15 (58)	3.472	0.176
	2		6 (55)	5 (45)		
	3		81 (62)	50 (38)		
	1	α-2C	9 (35)	17 (65)	0.456	0.796
	2		4 (36)	7 (64)		
	3		54 (41)	77 (59)		
	1	β2	4 (18)	18 (82)	4.236	0.12
	2		4 (44)	5 (56)		
	3		47 (41)	68 (59)		
Mortality (5 yr)	No	α-1B	55 (69)	25 (31)	4.821	0.028
	Yes		47 (52)	43 (48)		
	< 5 yr	α-2C	31 (39)	48 (61)	0.001	0.969
	> 5 yr		34 (39)	52 (61)		
	< 5 yr	β2	25 (36)	44 (64)	0.284	0.594
	> 5 yr		32 (41)	47 (59)		

Proportion of patients showing each adrenoceptor (percentage).

RESULTS

Patient characteristics and adrenoceptor expression

Tissue cores were available for assessment in 168 patients stained for α1B and α2C expression and 146 for β2 adrenoceptor protein. The reduced number of cores available for β2 assessment was due to detachment during immunohistochemistry processing. Adrenoceptor protein appeared localised in the cytoplasm of malignant ovarian tissue (Figure 1).

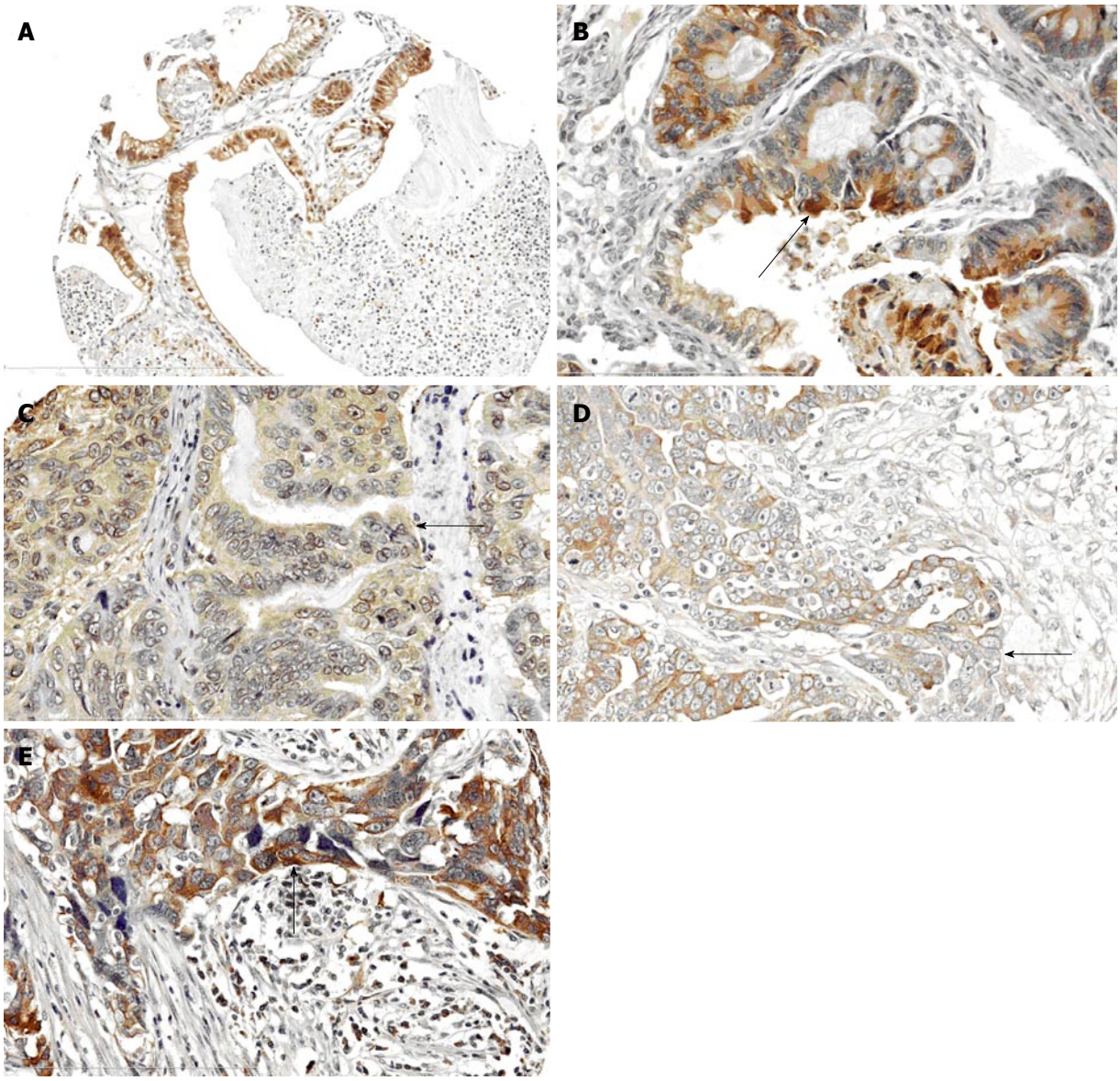

Figure 1 Following immunostaining, adrenergic receptor proteins were seen as brown staining in ovarian cancer tissue. A: Strong α1B staining in a mucinous tumor; B: Strong α1B - high grade serous tumor; C: Moderate α2C - endometrioid tumor; D: Weak β2-AR - high grade serous tumor; E: Strong β2-AR - high grade serous tumor. AR: Adrenergic receptor.

The median age at cancer diagnosis was 61 years (range 31-87) and the proportion of different cancer subtypes is shown in Table 1. Just over half (53%) of the patient cohort investigated in this study were categorised with poor survival (less than 5 years post-diagnosis) and the remainder survived 5-20 years.

The distribution of adrenoceptor expression differed by tumor type with 41%, 60%, 62% of the full cohort showing expression of α1B, α2C and β2 respectively (Table 1). Increased α1B expression was more frequently seen in mucinous cancers but in contrast was reduced in low grade serous and clear cell tumors. Levels of α2C were increased in endometrioid, clear cell and mucinous tumors. β2 expression was more frequently increased in high and low grade serous tumors and mucinous cancers (Table 1). No association was found between individual adrenoceptor types and tumor grade or clinical stage (Table 1). Patients with tumors expressing α2C adrenoceptor showed an association with low stage clinical disease (Table 2).

Clinical outcome

A Kaplan-Meier technique with a log rank test was used to model the independence of adrenoceptor protein expression in predicting ovarian cancer specific survival and tumor recurrence in the full patient cohort. High α1B protein expression was associated with reduced survival across the full patient cohort due to ovarian cancer specific mortality (P = 0.05; 95%CI: 1.00-1.49), resulting in a reduction in the median survival time from 63.5 to 44 mo (Figure 2 and Table 3). Similarly, α1B adrenoceptor protein expression was associated with increased tumor recurrence (P = 0.021, 95%CI: 1.04-1.69). A subanalysis of survival (χ^2 = 3.907, P =

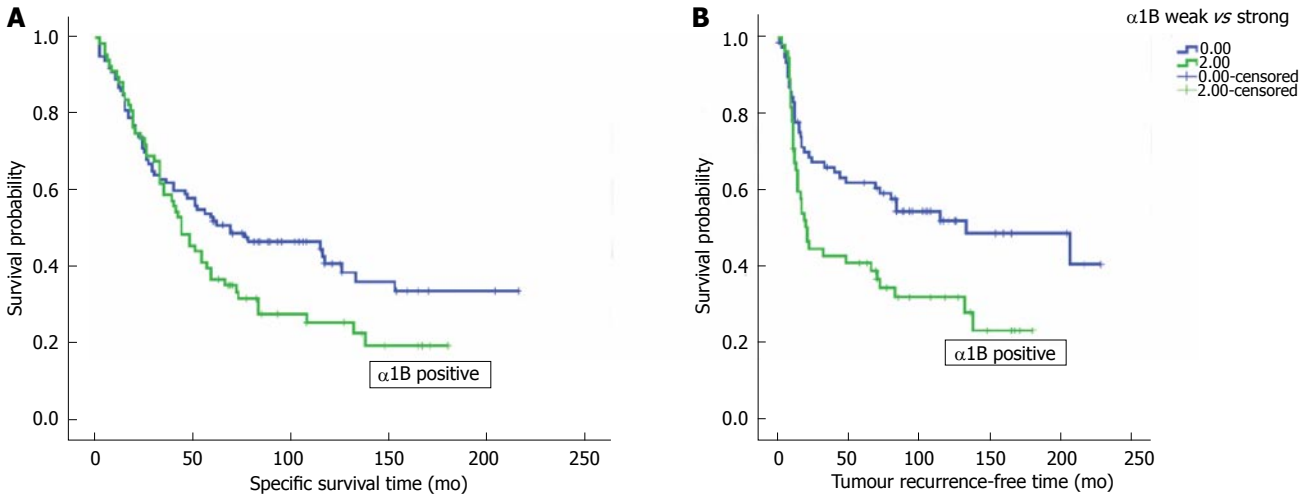

Figure 2 High alpha1B protein expression (green curve) showed an association with poor survival (*P* = 0.045) (A), and (B) tumor recurrence (*P* = 0.007) in the whole cohort of patients with ovarian cancer.

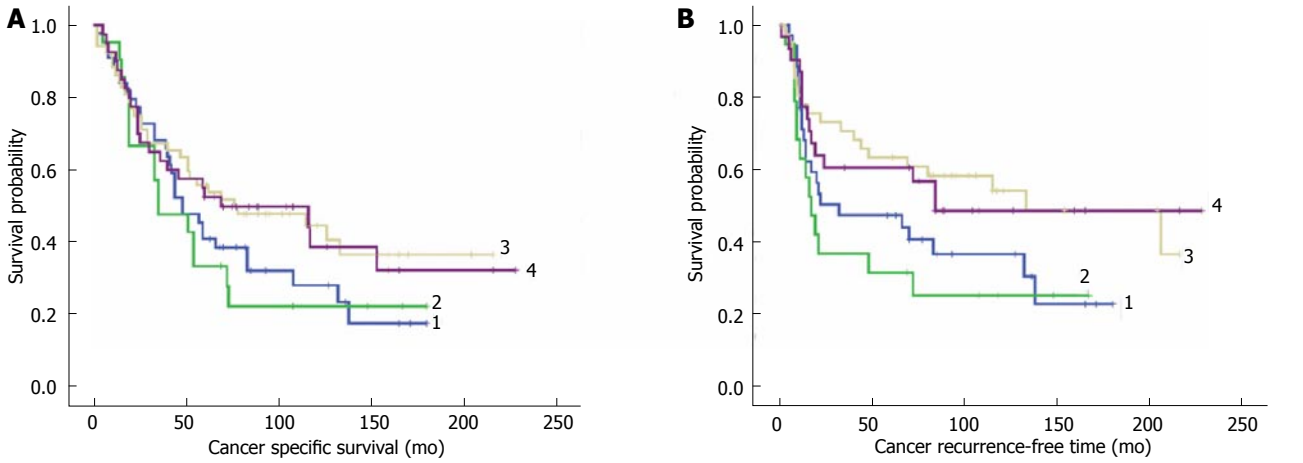

Figure 3 Four adrenoceptor groups were modelled for (A) ovarian cancer specific survival (*P* = 0.284) and (B) tumor recurrence (*P* = 0.061) according to the relative expression of α1B and α2C. Group 1: $\alpha1B^{positive}/\alpha\ \alpha2C^{positive}$; Group 2: $\alpha1B^{positive}/\alpha2C^{negative}$; Group 3: $\alpha1B^{negative}/\alpha2C^{positive}$; Group 4: $\alpha1B^{negative}/\alpha2C^{negative}$. Patients showing α1B expression (Groups 1 and 2) showed worse survival compared to patients that had α2C positive tumors without α1B expression.

0.048) and tumor recurrence (χ^2 = 5.867, *P* = 0.015) in the different cancer subclasses showed an association with endometrioid type cancer. No association was found for α2C or β2 AR with survival or tumor recurrence.

In the full patient cohort, tumor adrenoceptor protein expression showed no association with chemoresistance or responsiveness. A subanalysis of patients with endometrioid tumors showed similar results.

Relative expression of α1B and α2C adrenoceptor proteins: Association with survival

To test the hypothesis that patient clinical outcome might be influenced by the balance of G_s-adrenoceptor proteins (α1B and β2 are proposed biomarkers of disease progression) compared to those with G_i-protein affinity (α2C is proposed as a biomarker of good prognosis), patients were categorised into different groups according to their relative expression levels of tumor-stimulating α1B and tumor-inhibitory α2C. Four patient groups were defined comprising: Group 1: $\alpha1B^{positive}/\alpha\ \alpha2C^{positive}$; Group 2: $\alpha1B^{positive}/\alpha2C^{negative}$; Group 3: $\alpha1B^{negative}/\alpha2C^{positive}$; and Group 4: $\alpha1B^{negative}/\alpha2C^{negative}$.

No significant difference was identified between the four adrenoceptor groups with ovarian cancer specific survival (χ^2 = 4.211, *P* = 0.240) or tumor recurrence (χ^2 = 7.361, *P* = 0.061). For tumor recurrence, the best separation between plots was achieved between the singly positive Group 2 ($\alpha1B^{positive}/\alpha2C^{negative}$) and Group 3 ($\alpha1B^{negative}/\alpha2C^{positive}$) ($\chi^2$ = 5.136, *P* = 0.023). Groups showing co-expression of α1B/α2C showed intermediate risk of tumor recurrence suggesting the presence of α2C expression has a modifying effect on α_{1B} expression population studies (Figure 3).

Similarly, the relationship involving relative expression between β2 and α2C was tested by defining 4 patient subgroups: Group 1: $\beta2^{positive}/\alpha2C^{positive}$; Group 2: $\beta2^{positive}/\alpha2C^{negative}$; Group 3: $\beta2^{negative}/\alpha2C^{positive}$; and Group 4: $\beta2^{negative}/\alpha2C^{negative}$.

The effect of clinical outcome was assessed using

Table 2 Distribution of adrenoceptor protein expression in patients with ovarian cancer according to clinical stage, chemotherapy received and chemotherapy failure

		Adrenergic receptor	Absent	Present	χ^2	*P*
Clinical stage	1	α-1B	39 (65)	21 (35)	2.486	0.478
	2		12 (50)	12 (50)		
	3		47 (59)	33 (41)		
	4		4 (44)	5 (56)		
	1	α-2C	17 (29)	41 (71)	6.014	0.111
	2		10 (42)	14 (58)		
	3		38 (49)	40 (51)		
	4		2 (25)	6 (75)		
	1	β2	18 (37)	31 (63)	6.508	0.089
	2		4 (20)	16 (80)		
	3		31 (42)	43 (58)		
	4		5 (71)	2 (29)		
Stage 1 *vs* other stages	Stage 1	α-1B	37 (63)	22 (37)	0.894	0.344
	Other stages		59 (55)	48 (45)		
	Stage 1	α-2C	17 (29)	42 (71)	4.376	0.036
	Other stages		49 (45)	59 (55)		
	Stage 1	β2	18 (36)	32 (64)	0.090	0.764
	Other stages		37 (39)	59 (61)		
Chemotherapy regimen	Before surgery	α-1B	11 (58)	8 (42)	0.734	0.693
	After surgery		84 (57)	62 (43)		
	Before surgery	α-2C	8 (42)	11 (58)	0.702	0.704
	After surgery		58 (40)	87 (60)		
	Before surgery	β2	8 (47)	9 (53)	1.316	0.518
	After surgery		46 (36)	80 (64)		
Chemotherapy resistance	Refractory	α-1B	8 (80)	2 (20)	2.647	0.266
	Resistant within 6 mo		4 (44)	5 (56)		
	Responsive		72 (58)	52 (42)		
	Refractory	α-2C	2 (25)	6 (75)	2.342	0.310
	Resistant within 6 mo		6 (60)	4 (40)		
	Responsive		50 (41)	73 (59)		
	Refractory	β2	5 (56)	4 (44)	1.010	0.604
	Resistant within 6 mo		4 (40)	6 (60)		
	Responsive		40 (38)	64 (62)		

Proportion of patients showing each adrenoceptor (percentage).

Kaplan-Meier with a log rank test. No significant difference was seen between the patient subgroups when considering survival (χ^2 = 2.253, *P* = 0.689) or tumor recurrence-free time (χ^2 = 0.463, *P* = 0.927).

α1B is an independent prognostic biomarker

Multivariate Cox regression hazard analysis was used to test the independence of α1B as a prognostic biomarker for predicting ovarian cancer specific survival and tumor recurrence in patients with ovarian cancer. Tumor grade, clinical stage and systemic chemotherapy were included in the model. α1B adrenoceptor expression was found to contribute significant prediction ability concerning survival (HR = 1.221, *P* = 0.05, 95%CI: 1.000-1.493) and tumor recurrence (HR = 1.326, *P* = 0.021, 95%CI: 1.043-1.687) over and above the routinely used clinical parameters included in the model (Table 3).

Table 3 A Cox regression analysis was performed to test the independence of tumor alpha1B protein expression as a biomarker compared to tumor grade, clinical stage and chemotherapy treatment

	HR	95%CI		*P* value
Cancer specific survival				
Alpha1B expression	1.221	1.000	1.493	0.050
Tumor grade	1.353	0.992	1.844	0.056
Clinical stage	1.922	1.529	2.416	< 0.001
Chemotherapy	0.556	0.283	1.090	0.087
Tumor recurrence				
Alpha1B expression	1.326	1.043	1.687	0.021
Tumor grade	1.390	0.949	2.035	0.091
Clinical stage	2.469	1.825	3.340	< 0.001
Chemotherapy	0.614	0.257	1.467	0.272

Tumor α1B protein is an independent marker of reduced cancer-associated survival and increased tumor recurrence.

DISCUSSION

Ovarian cancer has a complex pathogenesis but recent studies have focused on the association between cancer progression and stress[4,22,23] involving the catecholamine hormones epinephrine and norepinephrine, and activation of β2 adrenoceptors. A recent paradigm proposed for a mouse model of breast cancer suggests cross-talk between cancer cells and macrophages triggering pro-metastasis cell signalling[10]. This paradigm might also extend to ovarian cancer because macrophages are implicated in ovarian metastasis, immunosuppression, angiogenesis and poor clinical outcome[24,25]. Consequently, it has been proposed that blockade of adrenoceptors using β-blockers could inhibit tumor progression in a number of cancer types including ovary[7], breast[26-28], prostate[29] and skin[30,31]. In addition, there is increasing evidence from patient[21] and *in vitro*[32] studies that alpha adrenoceptors are also implicated in breast cancer progression and for this reason we sought to identify the distribution and pattern of different adrenoceptor types in ovarian cancer patients.

The pattern of adrenoceptor expression was altered in different ovarian tumor types but overall, no association was found with tumor grade. Serous and endometrioid tumors generally differ in their prognostic outlook with the endometrioid type having better prognosis. A significant difference in pro-(β2) and anti-migratory (α2C) adrenoceptor expression patterns was identified. Compared to serous tumors, endometrioid cancers less frequently expressed β2 receptors (46% *vs* 68%) and were more likely to show α2 receptor

expression (79% *vs* 52%). But a subset of patients (40%) with endometrioid tumors expressed high levels of α1B protein and this correlated with poor prognosis due to a significantly shortened survival time and reduced tumor recurrence-free interval, independently of chemotherapy, tumor grade and clinical stage. The pathogenesis of endometrioid tumors is thought to be associated with endometriosis[33] and notable gene mutations in the phosphoinositide-3-kinase (PI3K) cell signalling pathway including PIK3CA and *PTEN* genes[34] in endometrial-derived cancer[35]. Activation of adrenoceptors provide a route to PI3K upregulation via the intermediary cAMP. Our findings suggest that α1B is a candidate biomarker and here it identified 17% of patients with endometrioid type cancer that require more intensive therapy and follow-up surveillance. Moreover, our findings suggest adrenoceptor antagonists and PI3K inhibitors provide potential for a targeted adjuvant therapy approach to complement existing therapies. In considering candidate anti-α adrenergic receptor drugs consideration has to be given to their selectivity and adverse effects. Alpha AR antagonists are used in the treatment of benign prostatic hyperplasia (*e.g.*, Prazosin, Doxazosin), urinary tract symptoms and hypertension. The non-selective drugs phenoxybenzamine and phentolamine would not be advocated, whereas some current tricyclic antidepressants could be considered but a recent study found amitriptyline, nortriptyline and imipramine are relatively weak α1B antagonists[36]. More promising is the recent development of a new family of 8-OMe benzodioxane analogues of the research drug WB4101 which has been shown to have high affinity for α1B AR[37]. The side effects of α1 antagonists including postural hypotension (Prazosin, Doxazosin), arrhythmia and CNS disturbances (tricyclic antidepressants) can be reduced by careful titration and active monitoring.

Cancer cell line studies have shown that norepinephrine activates α AR resulting in HIF1α dependent vascular endothelial growth factor transcription, required for angiogenesis[38]. Interestingly, Park *et al*[38] found that the α1 adrenoceptor inhibitor prazosin blocks the angiogenic pathway in the epithelial-to-mesenchymal type MDA-MB-231 breast cancer cell line, but not in liver (SK-Hep1) or prostate (PC3) cancer cells[38]. To translate this to ovarian cancer, we tested the proposal that α1B and 2C adrenoceptors might have an opposing promoting and inhibitory affect respectively on cancer progression and survival. To do this, patients were sub-classified according to α/β adrenoceptor phenotype, by comparing survival in patients with tumors expressing only one adrenoceptor (α1B, α2C or β2 positive) to those with co-expression of α2C. Although Kaplan-Meier models suggest α2C expression improves tumor recurrence-free times the finding was insignificant. Further studies are needed to better stratify patients for assessing possible therapeutic response to adrenoceptor antagonists.

No significant association between β2 protein expression levels and clinical outcome was found in this study. Recent laboratory studies show a significant pathologic role for neouroendocrine-induced progression in ovarian cancer (reviewed by Kang *et al*[39]), mediated by the β adrenoceptor activated cAMP - PKA cell signalling pathway. Increased cAMP activates Rap guanine-nucleotide-exchange-factor 3 (EPAC) leading to increased cell: Matrix adhesion needed for cancer cell implantation. Cancer growth is maintained due to enhanced cell survival resulting from γSrc-FAK signalling and STAT3 induced angiogenesis. These mechanisms explain the murine *in vivo* observation that the β2 antagonist propranolol inhibits ovarian cell growth[40]. However, other *in vitro* studies suggest that β-blockers would not be effective. In some instances, β2 agonists have been found to reduce cell proliferation[32,41,42] and migration[43]. In the latter case, it is proposed that β blockers could actually increase ovarian disease progression by promoting cell migration. Another explanation is that β blockers induce increased cell proliferation leaving unopposed α2C AR activity[42] in contrast to our findings suggesting that α1B is moderated by α2C in ovarian cancer. Recent proof-of-concept population studies of β-blocker users among ovarian cancer patients have produced conflicting findings. One study showed an association between increased progression-free survival (PFS) in users (n = 23) compared to non-users[7]. In contrast, a more recent study found no benefit in PFS or overall survival in platinum-sensitive patients prescribed β-blockers (n = 8)[5]. Clearly, larger studies are needed allowing for possible confounders and tumor receptor typing. Although previous studies have focused on the use of β-blockers to retard disease progression, the results presented here and in a recent breast cancer study[21] suggest that the possible therapeutic benefits of alpha adrenoceptor antagonists should be investigated.

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COMMENTS

Background

Many cancers are treatable by surgery, radio-/chemotherapy, or targeted drug treatments, or any combination of these. In some instances a cancer can spread (metastasise) to tissues distant from the original site. This process can place a patient at increased risk of disease progression and demise and in addition, it can present clinicians with more challenging medical management of a patient's disease. Knowledge about the biological process involved in cancer spread is increasing but there remains an unmet need to develop new treatment approaches to prevent it. Being able to identify patients that are at increased risk of metastasis can rationalise clinical management by focusing extensive treatment on those that will best benefit. Laboratory experiments have shown that some cancer cells are stimulated to migrate when adrenergic receptor proteins (stress receptors) are activated by stress hormones. Drugs are available that inhibit adrenergic receptor function and could be used to neutralise certain cancer cell functions.

Research frontiers

Identifying the expression pattern of adrenergic receptors (AR) in different cancer

types and their association with disease progression and survival could provide insight into using AR inhibitor drugs for targeted anti-cancer treatment.

Innovations and breakthroughs

The expression pattern of 3 AR proteins (αB1, α2C and β2) was investigated and its significance statistically related to survival and metastasis outcome in patients with different types of ovarian cancer. Only αB1 was found to predict shortened survival and increased risk of tumor recurrence, especially in patients with endometrioid type cancer, independently of tumour grade, clinical stage and chemotherapy treatment.

Applications

The results suggest that adrenergic receptor antagonists with anti-α1B selectivity could be used to limit disease progression in patients with endometrioid type tumors expressing α1B AR.

Terminology

Metastasis development is a major cause of mortality in patients with cancer and involves a multistep biological pathway resulting in tumor cells leaving the primary cancer and disseminating to distant body tissues. AR belong to a family of G protein-coupled receptors comprising 9 members.

Peer-review

This is a well written paper.

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ORIGINAL ARTICLE

Observational Study

Variation in use of menopausal hormone treatment on risk of health outcomes

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Author contributions: Khoo SK contributed to the concept and design of the study, assisted in patient supervision and wrote the manuscript; Tripcony L provided management of the data base and statistical analysis, and assisted in writing the manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of the Royal Women's Hospital and subsequently by the Royal Brisbane and Women's Hospital (ref RWH 99/17) (see attached).

Informed consent statement: All study participants were given information about the study and provided informed written consent before recruitment and enrolment. All data were de-identified.

Conflict-of-interest statement: Both authors declare no conflict of interest in the findings of the study.

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Abstract

AIM: To determine the relative risk of selected serious outcomes with variations in use of menopausal hormone treatment (MHT).

METHODS: A cohort of 489 women, randomly recruited at age 40-79 years, from a longitudinal study of urbanised population was a study group and was followed for 14 years. Four selected outcomes (coronary artery disease, stroke, peripheral artery disease, breast cancer) were tested. Each woman on entry to the study was interviewed by a dedicated medical practitioner, and data on menstrual and menopausal history and health status were obtained. Outcome information was ascertained by questionnaire and medical reports from attending medical practitioners. In case of death, cause of death was checked with the Registry of Births, Deaths, Marriages and Divorce. This information was available for all women. An ever-user of MHT was defined as use for 6 mo or more at any time during the study. A late start of MHT was defined as 3 years or more from onset of menopause. The generalised linear statistical package was used to examine the data; univariate logistic regression models were used to describe the relationship between patient characteristics and a disease outcome, followed by stepwise multi variate analysis, controlling for age, lifestyle factors and co-morbidities.

RESULTS: The risk of ever-use of MHT was significantly increased only for peripheral artery disease (RR = 2.16; 0.99, 4.71; P = 0.05), and not for coronary artery disease, stroke and breast cancer. A late start of MHT (three years or more from onset of menopause) was

associated with significantly increased risks for coronary artery disease (RR = 2.56; 1.15, 5.72; *P* = 0.02) and peripheral artery disease (RR = 4.42; 1.55, 12.64; *P* = 0.005), and use after age 60 years with significantly increased risks for coronary artery disease (RR = 4.98; 2.19, 11.55; *P* < 0.001), stroke (RR = 2.99; 1.11, 8.08; *P* = 0.03) and peripheral artery disease (RR = 4.18; 1.24, 14.14; *P* = 0.02). Use up to 10 years was not associated with significant risk for all outcomes. These risks were confirmed by stepwise multi variate analysis, adjusting for age at recruitment, body mass index, smoking, physical activity and alcohol use, and existing diabetes, mellitus, hypertension and hypercholesterolaemia. Regardless of variations in use, risk for breast cancer was not found.

CONCLUSION: The study confirms ever-use of MHT affected only risk of peripheral artery disease; but some use variations could have adverse effects.

Key words: Menopausal hormone treatment; Variation in use; Risk outcomes

Core tip: In contrast to larger studies, this small observational study examined effects of various ways of use of menopausal hormone treatment (MHT) when given for clinical indications. Of the four selected outcomes available at 14 years follow-up, overall risk was only increased for peripheral artery disease but not for coronary artery disease, stroke and breast cancer. However, risk was increased for coronary artery disease and peripheral disease when MHT was started more than three years after menopause in women over 60 years.

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INTRODUCTION

Since the findings of the Women's Health Initiative (WHI) were reported in 2002, there has been considerable research activity in analyses, re-analyses and meta-analyses of available and new data on the risks of use of hormone therapy at and after menopause. The reason for termination of the combined oestrogen plus progestogen arm of the WHI was a significant increase in risk of several health outcomes, namely breast cancer, coronary artery disease, stroke and deep vein thrombosis[1]. However, the reason for termination of the oestrogen-only arm of the same study, was different - the increase in risk was only significant for one outcome - stroke - and the risks of breast cancer and coronary artery disease were not increased[2]. Other studies such as the Million Women Study (MWS)[3] and the California Teachers Study[4], have added more information to an emerging complex plethora of data which gives rise to a variety of opinions and recommendations from international expert bodies. An excellent and comprehensive review of the risks and benefits of use is given by Davey[5].

Much attention has been given to obtain a better understanding of the vascular risks, especially in the heart and brain, and to reconcile the differences in outcomes of menopausal hormone treatment (MHT) between observational and randomised controlled studies. Lessons from monkey models suggest variable effects due to timing of treatment[6]. Whereas surgically-induced oestrogen deficiency results in premature coronary artery atherosclerosis, the process is prevented when replacement oestrogen is given early but not so when given late after the deficiency. This is the concept of a "window of opportunity" to explain the variable findings in women. It is proposed that oestrogen has a beneficial effect on arteries in younger women by preventing or delaying atherosclerosis; but when given to older women with advanced plaque formation, the effect may be absent or even, deleterious. However, the Kronos Study failed to show a good effect[7], whilst a Danish Study reported reduced risk of mortality and myocardial infarction[8].

In view of these variable findings, clinicians need to be aware that MHT may have different effects and risk outcomes when given in different ways and at different times of the woman's life. In this study, the effects of variations in use of MHT were tested in a cohort of women from a Longitudinal Study of Ageing in Women (LAW Study). Use variations included ever-use, timing of initiation of treatment from menopause, types of regimen, age at start or stopping treatment, and duration of treatment. The outcomes selected for their clinical importance included coronary artery disease, stroke, peripheral artery disease and breast cancer. We report the findings after 14 years of follow-up.

MATERIALS AND METHODS

The women in this study belonged to a cohort who have been recruited for a multidisciplinary longitudinal study on ageing and to date, follow-up has reached 14 years. The design and recruitment procedures of the overall study have been previously described[9]. Approval was given by the Ethics Committee of the Royal Brisbane and Women's Hospital. Informed consent was also obtained from all women recruited into the overall Study.

The cohort was recruited by random invitation from the electoral roll, based on age stratification into four age-decades: 40-49 years, 50-59 years, 60-69 years and 70-79 years. Each woman on entry to the study was interviewed by a dedicated medical practitioner on an annual basis. In addition to other specific questions

Table 1 Distribution of outcomes and variation of use of menopausal hormone therapy in study cohort

	No. of women			
	Never-users		Ever-users	
No. of events during 14 yr follow-up (% in group)				
Coronary artery disease	33	(11.5%)	29	(14.3%)
Stroke	23	(8.0%)	19	(9.4%)
Peripheral arteria disease	10	(3.5%)	19	(9.4%)
Breast cancer	20	(6.9%)	12	(5.9%)
Timing of initiation of therapy from onset of menopause				
"Early start" (≤ 3 yr)	-		162	(80.2%)
"Late start" (> 3 yr)	-		40	(19.8%)
Type of regimen				
Oestrogen-only	-		30	(14.8%)
Oestrogen + progestogen	-		168	(83.2%)
Tibolone	-		4	
Combination of treatment strategy				
Early start plus oestrogen-only	-		20	(9.9%)
Early start plus oestrogen + progestogen	-		140	(69.3%)
Late start plus oestrogen-only	-		10	(4.9%)
Late start plus oestrogen + progestogen	-		28	(13.9%)
Other			4	
Age of patient when treatment started				
20-39 yr	-		17	(8.4%)
40-49 yr	-		85	(42.0%)
50-59 yr	-		82	(40.6%)
60-69 yr	-		18	(8.9%)
70-79 yr	-		17	(8.4%)
Age of patient when treatment stopped				
20-39 yr	-		0	
40-49 yr	-		20	(9.9%)
50-59 yr	-		85	(42.1%)
60-69 yr	-		69	(34.1%)
70-79 yr	-		28	(13.9%)
Duration of use (yr)				
< 1	-		15	(7.4%)
1-5	-		67	(33.2%)
6-10	-		37	(18.3%)
11-15	-		45	(22.3%)
16-20	-		19	(9.4%)
21-25	-		11	(5.4%)
> 25	-		7	(3.4%)
Total No. of women	287		202	

and assessments required by the other projects, information was obtained on use of MHT to include detailed menstrual and menopausal history and health status. Follow-up was continued by questionnaires on an annual basis to ascertain use of MHT, menopausal status as well as development of serious health outcomes, as determined by the attending medical practitioner. In particular, coronary artery disease, stroke, peripheral artery diseases (including carotid, femoral and popliteal arteries) and breast cancer were specifically ascertained. In women in whom information was uncertain, confirmation was made by direct contact with the attending medical practitioner. If death had occurred, cause of death was checked with the Registry of Births, Deaths, Marriages and Divorce, Department of Justice and Attorney-General, Queensland.

An ever-user of MHT was defined as a woman who had used MHT for 6 mo or more at any time during the study period of 14 years.

A cut-off time of three years was used to define timing of initiation of MHT in relation to onset of menopause. An "early start" user was a woman who had started within three years of onset of menopause; a "late start" user was one who had started MHT more than three years after menopause.

Statistical analysis

The analysis was revised and performed by a Biomedical Statistician (co-author: Lee Tripcony). The generalised linear statistical package (GLIM4) was used to examine the data. Univariate logistic regression models were used to describe the relationship between patient characteristics and a disease outcome. Variations in use of MHT analysed were used by patient (never-user, ever-user), timing of initiation of treatment in relation to onset of menopause (never "early start", "late start"), type of regimen (never, oestrogen-only, oestrogen plus progestogen), age of starting or stopping treatment (in categories), and duration of use (in categories). The dependent variables or outcome events were coronary artery disease, stroke, peripheral artery disease, and breast cancer. Hazard ratios with 95%CIs and *P* values were constructed. A *P* value ≤ 0.05 (two-sided) was taken to represent a significant association. From these results, only factors that had a *P* value < 0.1 were included in the stepwise construction of the multivariate model. Lifestyle and other factors fitted to the model included age at recruitment, body mass index, alcohol use, physical activity, smoking, and existing conditions at entry to study (diabetes mellitus, hypertension and hypercholesterolaemia).

RESULTS

There were 489 women in the cohort. Their ages in the two groups were comparable: 202 ever-users with mean age of 61.0 years (range 44-79; 95%CI: 59.9-62.0) and 287 never-users with mean age of 57.9 years (range 41-80; 95%CI: 56.4-59.3).

As shown in Table 1, the difference in the incidence of the four outcomes between never-users and ever-users was greatest for peripheral artery disease (3.5% *vs* 9.4%) and least for breast cancer (6.9% *vs* 5.9%).

Interestingly, 19.8% of women started MHT more than three years after menopause; 17.3% started MHT when aged 60 years or more, and 48.0% stopped MHT after age 60 years or more. There was a wide range of duration of use; 33.2% had used MHT for 1-5 years, another 50.0% for 6-20 years. Surprisingly, there were 8.9% of women who had used MHT for more than 20 years.

As expected, the majority (80.2%) of ever-users started treatment within 3 years of menopause, but there was still 19.8% who started late (17 women started MHT at age 70 years or more). A sub-group of 30 women had a hysterectomy and removal of both

Table 2 Menopausal hormone treatment and risk of serious outcomes: Ever-use, timing of initiation of treatment and type of regimen

	Relative risk estimate (95%CI)			
	Coronary artery disease	Stroke	Peripheral artery disease	Breast cancer
Ever-use	1.29 (0.76, 2.20) $P = 0.35$	1.14 (0.61, 2.14) $P = 0.69$	2.16 (0.99, 4.71) $P = 0.05$[1]	0.84 (0.40, 1.76) $P = 0.65$
Timing of initiation from onset of menopause				
Early start	1.02 (0.56, 1.86) $P = 0.94$	1.04 (0.52, 2.06) $P = 0.92$	1.65 (0.69, 3.94) $P = 0.26$	0.97 (0.45, 2.08) $P = 0.94$
Late start	2.57 (1.15, 5.72) $P = 0.02$[1]	1.57 (0.56, 4.37) $P = 0.39$	4.42 (1.55, 12.64) $P = 0.0052$	0.34 (0.05, 2.37) $P = 0.28$
Type of regimen				
Oestrogen only	2.34 (0.93, 5.88) $P = 0.07$	0.78 (0.18, 3.49) $P = 0.75$	5.01 (1.62, 15.48) $P = 0.005$	2.05 (0.65, 6.46) $P = 0.22$
Oestrogen + progestogen	1.16 (0.65, 2.06) $P = 0.62$	1.23 (0.64, 2.37) $P = 0.53$	1.59 (0.67, 3.79) $P = 0.30$	0.67 (0.29, 1.55) $P = 0.35$
Combination of treatment strategy				
Early start plus oestrogen-only	2.57 (0.88, 7.52) $P = 0.09$	1.22 (0.27, 5.56) $P = 0.80$	1.32 (0.16, 10.78) $P = 0.80$	2.36 (0.64, 8.72) $P = 0.20$
Early start plus oestrogen + progestogen	0.86 (0.44, 1.66) $P = 0.65$	1.03 (0.50, 2.12) $P = 0.94$	1.72 (0.70, 4.26) $P = 0.24$	0.81 (0.35, 1.89) $P = 0.62$
Late start plus oestrogen-only	1.92 (0.39, 9.45) $P = 0.42$	NC	16.73 (4.12, 67.91) $P < 0.001$[2]	1.48 (0.18, 12.29) $P = 0.71$
Late start plus oestrogen + progestogen	3.08 (1.26, 7.55) $P = 0.01$[1]	2.38 (0.83, 6.83) $P = 0.11$	0.93 (0.12, 7.46) $P = 0.94$	NC

NC: No convergence due to small numbers. By multivariate analysis adjusting for age at recruitment, body mass index, smoking, physical activity and alcohol use, co-morbidities (diabetes mellitus, hypertension, hypercholesterolaemia) shows: [1]P 0.05 – 0.01; [2]$P < 0.01$.

ovaries (for benign pathology) before the expected menopausal age (45-55 years). They were treated by an oestrogen-only regimen; treatment was started usually after the surgery, 17 of them were aged 20-39 years, and the other 13 women were aged less than 45 years. At that period 2000-2002, only oral oestrogen only regimen was available.

Ever-use, timing of initiation of treatment and type of regimen

As shown in Table 2, there was no significant increase in risk estimate for coronary artery disease, stroke and breast cancer in ever-users, compared with never-users. However, in ever-users, the overall risk of peripheral artery disease was significantly increased by two-fold (RR = 2.15; 0.99, 4.71; $P = 0.05$). This association was confirmed after adjusting for age, body mass index, alcohol use, physical activity, smoking and co-morbidities.

Whilst "early start" of MHT was not significantly increased for all four outcomes, "late start" was associated with a significant risk increase for coronary artery disease by 2½-fold (RR = 2.57; 1.15, 5.72; $P = 0.02$) and for peripheral artery disease by more than 4-fold (RR = 4.42; 1.55, 12.64; $P = 0.005$). A "late start" was confirmed after adjusting for other factors to be an adverse independent factor for coronary artery disease and peripheral artery disease.

Essentially, MHT was given as an oestrogen-only regimen (oral or transdermal) or oestrogen plus progestogen continuous regimen (oral or transdermal). For the oestrogen-only regimen, the risk was only significantly increased for peripheral artery disease by 5-fold (RR = 5.01; 1.62, 15.48; $P = 0.005$). The combined oestrogen plus progestogen regimen did not have an effect on any outcome. When the type of regimen was paired with the timing of initiating treatment, the risk for coronary artery disease was significantly increased by three-fold with "late start" together with the combination treatment (RR = 3.08; 1.26, 7.55; $P = 0.01$), and the risk for peripheral artery disease was significantly increased by 16-fold with "late start" together with the oestrogen-only regimen (RR = 16.73; 4.12, 67.91; $P < 0.001$). The significant independent adverse association between "late start" plus oestrogen + progestogen combination and coronary artery disease was confirmed by multivariate analysis, as was the association between "late start" plus oestrogen-only regimen and peripheral artery disease.

There were no other significant effects of MHT on other outcomes. Notably, the risk for breast cancer was not significantly affected by any variation in use.

Age of patient at start and stopping of MHT and duration of use

As shown in Table 3, there was no significant effect of age when treatment was started on risk of the outcomes until the late age group of 60-79 years (35 women in the cohort). For these women, the risk for coronary artery disease and peripheral artery disease was significantly increased (RR = 7.70; 2.85, 20.76; $P < 0.001$, and RR = 5.01; 1.27, 19.80; $P = 0.02$, respectively). The age when treatment was stopped had a similar effect on these two outcomes, when the risk was significantly increased in women aged 60-79 years.

There was a wide range of duration of use - from less than one year (but more than six months to be included) to 21-25 years (11 women) and more than 25 years (seven women, one woman used MHT for a record 31 years). There was no significant impact of duration of use on the outcomes, except for peripheral artery disease where the risk was significantly increased when use continued for 21-25 years (RR = 5.57; 1.07, 28.92; $P = 0.04$) and for more than 25 years (RR = 10.03; 1.75, 57.58; $P = 0.01$).

Notably, duration of use had no effect on risk of

Table 3 Variation of use of menopausal hormone treatment and risk of serious outcomes : Age of patient and duration of use

	Relative risk estimate (95%CI)			
	Coronary artery disease	Stroke	Peripheral artery disease	Breast cancer
Age of patient when treatment started (yr)				
20-39	0.48	0.68	1.57	0.83
	(0.07, 3.35)	(0.09, 5.35)	(0.19, 12.79)	(0.11, 6.57)
	P = 0.47	*P* = 0.72	*P* = 0.67	*P* = 0.87
40-49	1.14	0.98	2.25	0.66
	(0.55, 2.37)	(0.41, 2.37)	(0.85, 5.96)	(0.22, 1.97)
	P = 0.72	*P* = 0.97	*P* = 0.10	*P* = 0.45
50-59	0.83	1.18	1.63	0.87
	(0.37, 1.87)	(0.51, 2.75)	(0.55, 4.79)	(0.32, 2.38)
	P = 0.65	*P* = 0.69	*P* = 0.37	*P* = 0.78
60-79	7.7	2.19	5.01	1.67
	(2.85, 20.76)	(0.59, 8.11)	(1.27, 19.80)	(0.36, 7.77)
	P < 0.001	*P* = 0.24	*P* = 0.02	*P* = 0.52
Age of patient when treatment stopped (yr)				
20-39	1.36	1.22	2.79	1.48
	(0.38, 4.88)	(0.27, 5.56)	(0.57, 13.53)	(0.32, 6.85)
	P = 0.64	*P* = 0.80	*P* = 0.20	*P* = 0.61
40-49	0.48	0.54	0.92	0.32
	(0.18, 1.27)	(0.18, 1.59)	(0.25, 3.37)	(0.97, 1.41)
	P = 0.50	*P* = 0.26	*P* = 0.90	*P* = 0.13
50-59	1.3	1.24	2.83	1.75
	(0.61, 2.80)	(0.51, 3.00)	(1.06, 7.60)	(0.74, 4.16)
	P = 0.50	*P* = 0.64	*P* = 0.04	*P* = 0.20
60-79	4.98	2.99	4.18	NC
	(2.19, 11.55)	(1.11, 8.08)	(1.24, 14.14)	
	P < 0.001	*P* = 0.03	*P* = 0.02	
Duration of use (yr)				
< 1	1.1	NC	3.59	1.91
	(0.24, 5.04)		(0.72, 17.75)	(0.40, 8.98)
	P = 0.90		*P* = 0.12	*P* = 0.41
1-5	0.76	1.28	1.18	0.85
	(0.31, 1.87)	(0.53, 3.11)	(0.32, 4.34)	(0.28, 2.57)
	P = 0.55	*P* = 0.59	*P* = 0.81	*P* = 0.77
6-10	2.12	0.3	1.43	0.37
	(0.90, 5.03)	(0.04, 2.32)	(0.31, 6.74)	(0.40, 2.85)
	P = 0.09	*P* = 0.25	*P* = 0.65	*P* = 0.34
11-15	0.75	1.69	3.14	0.95
	(0.26, 2.20)	(0.65, 4.38)	(1.04, 9.50)	(0.27, 3.35)
	P = 0.60	*P* = 0.28	*P* = 0.04	*P* = 0.94
16-20	2.75	1.29	NC	0.74
	(0.93, 8.11)	(0.28, 5.92)		(0.09, 5.84)
	P = 0.07	*P* = 0.74		*P* = 0.78
21-25	2.89	2.44	5.57	1.33
	(0.73, 11.41)	(0.50, 11.92)	(1.07, 28.92)	(0.16, 10.96)
	P = 0.13	*P* = 0.27	*P* = 0.04	*P* = 0.79
26+	1.28	1.83	10.03	NC
	(0.15, 10.97)	(0.21, 15.80)	(1.75, 57.58)	
	P = 0.82	*P* = 0.58	*P* = 0.01	

NC: No convergence due to small numbers.

coronary artery disease, stroke and breast cancer.

DISCUSSION

When our LAW study was planned some 14 years ago, it was considered an opportunity to test the effects of the variables in the use of MHT because there was a suitable group of women aged 40-79 years at recruitment who were randomly invited from the population to join the study. The findings of WHI study gave an impetus to investigate the impact of variation in use such as early and late initiation of treatment and type of hormone regimen on relative risks of major clinical outcomes. For this report, we chose arterial conditions in the heart, brain and periphery (carotid, femoral, popliteal arteries). We included peripheral artery disease because of its association with older women and there were 29 events in our study. This study differed from other large studies in several aspects: the women were given MHT by their individual medical practitioners because of clinical indications, not to asymptomatic volunteers; the study was longitudinal and non-interventional, not randomised placebo-controlled; the cohort was smaller but closely followed for 14 years with very good outcome information on all the women; and despite the smaller cohort size, there were adequate outcome events to allow the power for analysis, based on relative risks estimated by comparison with an adequate group of never-ever users, as reference.

The present study confirms the general view that MHT is generally safe in healthy women. However, it may have unfavourable effects on the vascular system, both arterial and venous. In particular, risks of cardio-vascular disease have been extensively analysed - with differing results between cohort, retrospective and prospective observational studies and randomised controlled studies. Whereas the observational studies (liable to inherent bias such as the "healthy user effect") demonstrated a significant 40%-60% reduction in risk of disease and of mortality[10-12], the controlled studies (susceptible to faulty matching, loss to follow-up) showed no significant decrease, or even an increase as found by the WHI study with a risk increase with the oestrogen plus progestogen regimen[2] and a decrease with the oestrogen-only regimen[1]. We found in our prospective and observational study a significant increase in risk of clinically-reported coronary artery disease only with "late start" to initiate treatment more than three years from onset of menopause, and when treatment was started when the woman was much older, aged 60-79 years. This age-group is certainly considered contra-indicated now to start MHT but some of these women had been treated more than 20-30 years ago, based on a different set of evidence. Notably, ever-use of MHT generally had no significant effect on coronary artery disease, a common health outcome in ageing. We believe the increased risk associated with "late start" reflected old practice when MHT was considered safe and beneficial to the heart; since WHI, the older and less healthy women were advised to stop MHT by their medical practitioner. Therefore, our findings on risk of coronary artery disease support use in women within three years from menopause and started before age 60 years; duration of use did not appear to have a significant impact on the disease.

Risk of stroke with MHT was variably estimated by many studies, with the view that risk was increased with ischaemic stroke but not with haemorrhagic stroke[13,14]. Although the risks were increased in WHI studies (RR =

1.31; 1.02, 1.68 ischaemic stroke), they were not found in the post intervention phase of the study[15-16]. There appears to be general agreement that the risks for all types of stroke were increased, as in total stroke, non-fatal but stroke leading to disability; and no significant heterogeneity with any subgroups[17]. However, an association with oestrogen dose has been reported[18], but not with age or time since menopause, or with low dose transdermal patch. We found no effect of MHT on risk of stroke, regardless of current or past use, timing of initiation of treatment from menopause or hormone content in the 42 events recorded in our cohort. The younger user effect may explain the finding, or more follow-up time is required to show this effect in light of the increasing incidence of hypertension with time in the cohort.

There is one vascular outcome seldom analysed with MHT - that is, the effect on peripheral artery disease. Because the disease is a component of the spectrum of arterial diseases, we decided to investigate its risk as a clinical condition which is less studied in epidemiological studies because it occurs less frequently - 29 events in our cohort of 489 women. We included clinically-proven diseases in the carotid, femoral and popliteal systems. As shown in the results, MHT had a strong impact on the risk of peripheral artery disease during follow-up of 14 years. The risk was significantly increased with "ever-use" and "late start", oestrogen-only regimen, and older age of women when treatment was started or stopped, and confirmed as a significant adverse association by multivariate analysis after adjusting for lifestyle factors and co-morbities. This is the only outcome where duration of use had a significant impact; from a duration of 11 years onward, the risk was significantly increased. We believe that the effect relates more to older women who already have existing atherosclerosis of the peripheral arteries.

Breast cancer was the cancer outcome selected for analysis because of its frequency of occurrence and the known influence of hormones on the breast. Consideration has been given to contributing factors such as type of MHT, duration of use, body mass, interval between menopause and initiation of therapy, previous MHT, mammographic density[5]. Generally, the studies suggest an increase in risk. The Collaborative Group found the risk of breast cancer increased by 2.3% (RR = 1.023; 1.011, 1.036) per year of use, reaching 35% (RR = 1.35; 1.21, 1.49) after five years[19]. The Million Women Study also found an increased risk for oestrogen plus progestogen regimen by 100% (RR = 2.00; 1.88, 2.13) and less with oestrogen-only regimen by 30% (RR = 1.30; 1.21, 1.45), with no differences between routes of administration[3]. Also, an increased risk of breast cancer was found with increasing body weight - with a 3.1% increase per kg/m^2 of body mass index[20]. However, the increased risk was significantly greater in thin women using MHT, than overweight and obese women. The importance of timing of initiation of MHT from onset of menopause, the so-called "gap time" or defined in our study as "early start" and "late start" has been highlighted in more recent studies. The WHI and MWS studies found an increased risk when the cut-off was 10 years after menopause, with the risks greater when MHT was given late (RR = 2.04 *vs* 1.53) for oestrogen plus progestogen regimens. For oestrogen-only regimens, the risk estimate fell from 1.43 to an insignificant 1.05. The reasoning behind these findings is based on the premise that exogenous hormones accelerate growth of pre-existing occult breast cancer (mitogenic and not carcinogenic) and differential sensitivity of breast tissue to hormones is related to age and menopausal status. We found no significant effect of MHT on risk of breast cancer, regardless of the use variation analysed. It is possible the small number of events (32 events) of breast cancer in our study, did not allow an appropriate calculation of risk estimate in the cohort. However, it is reassuring that there was no apparent significant risk increase.

In conclusion, the study found the use of MHT was associated with no overall increased risk of coronary artery disease, stroke and breast cancer, and an increased risk of peripheral artery disease. However, variation in use may have an adverse impact on some outcomes, for example, when MHT was started long after onset of menopause in much older women over the age of 60 years, there was a significant increase in risk for coronary artery disease and peripheral artery disease.

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COMMENTS

Background

The study examined the effects of hormones (estrogen and progestogen) given in different ways on long-term serious outcomes in post-menopausal women. This field of clinical remains controversial because of mixed reports.

Research frontiers

The area of women's health continues to be argued about whether hormone treatment is beneficial or harmful.

Peer-review

The availability of long-term use data is consistent with previous experience and might have been more directly related to the women on ET for post-oophorectomy life.

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SYSTEMATIC REVIEWS

Postpartum intrauterine device contraception: A review

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Abstract

AIM: To review the safety (infection, perforation) and efficacy (expulsion, continuation rates, pregnancy) of intrauterine device (IUD) insertion in the postpartum period.

METHODS: MEDLINE, PubMed and Google Scholar were searched for randomized controlled trials and prospective cohort studies of IUD insertions at different times during the postpartum period. Time of insertion during the postpartum period was documented specifically, immediate post placenta period (within 10 min), early post placenta period (10 min to 72 h), and delayed/interval period (greater than 6 wk). Other study variables included mode of delivery, vaginal *vs* cesarean, manual *vs* use of ring forceps to insert the IUD.

RESULTS: IUD insertion in the immediate postpartum (within 10 min of placental delivery), early postpartum (10 min up to 72 h) and Interval/Delayed (6 wk onward) were found to be safe and efficacious. Expulsion rates were found to be highest in the immediate postpartum groups ranging from 14% to 27%. Immediate post placental insertion found to have expulsion rates that ranged from 3.6% to 16.2%. Expulsion rate was significantly higher after insertion following vaginal *vs* cesarean delivery. The rates of infection, perforation and unplanned pregnancy following postpartum IUD insertion are low. Method of insertion such as with ring forceps, by hand, or another placement method unique to the type of IUD did not show any significant difference in expulsion rates. Uterine perforations are highest in the delayed/interval IUD insertion groups. Breastfeeding duration and infant development are not affected by delayed/interval insertion of the non-hormonal (copper) IUD or the Levonorgestrel IUD. Timing of the Levonorgestrel IUD insertion may affect breastfeeding.

CONCLUSION: IUD insertion is safe and efficacious during the immediate postpartum, early postpartum and delayed postpartum periods. Expulsion rates are highest after vaginal delivery and when inserted during the immediate postpartum period. IUD associated infection rates were not increased by insertion during the postpartum period over interval insertion rates.

There is no evidence that breastfeeding is negatively affected by postpartum insertion of copper or hormone-secreting IUD. Although perforation rates were higher when inserted after lactation was initiated. Randomized controlled trials are needed to further elucidate the consequence of lactation on postpartum insertion. Despite the concerns regarding expulsion, perforation and breastfeeding, current evidence indicates that a favorable risk benefit ratio in support of postpartum IUD insertion. This may be particularly relevant for women for whom barriers exist in achieving desired pregnancy spacing.

Key words: Access to intrauterine devices; Contraception; Expulsion; Intrauterine device; Long acting reversible contraception; Postpartum contraception; Postpartum intrauterine device; Postpartum intrauterine device placement; Post-placental insertion

Core tip: Intrauterine device (IUD) insertion is safe and efficacious during the immediate postpartum, early postpartum and delayed postpartum periods. Expulsion rates are highest after vaginal delivery and when inserted during the immediate postpartum period. IUD associated infection rates were not increased by insertion during the postpartum period over interval insertion rates. Despite the concerns regarding expulsion, perforation and breastfeeding, current evidence indicates that a favorable risk benefit ratio in support of postpartum IUD insertion.

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INTRODUCTION

The intrauterine device (IUD), a form of long acting reversible contraception (LARC), is one of the most effective contraceptive methods available as well as one of the safest. Its effectiveness can largely be attributed to its low rate of unintended pregnancy that is likely due to its use being patient-independent[1]. Its safety is supported by recent literature, which documents low rates of infection, perforation, and expulsion that should not deter a clinician from offering it as a contraception option. Furthermore, IUD insertion is not only a viable option for the nulliparous, but should also be considered as an option during the postpartum period[1].

An inter-pregnancy interval of less than 24 mo has been shown to be associated with increased maternal/infant morbidity and mortality when compared to longer inter pregnancy intervals[2,3]. Postpartum IUD insertion is a useful way to achieve recommended pregnancy spacing. Safe and efficacious postpartum insertion of the IUD may occur up to 48 h after delivery of the placenta, and 4-6 wk postpartum[4,5]. Although the immediate placement has been associated with a higher expulsion rate, the benefits of immediate insertion for preventing unplanned pregnancy in select populations may outweigh the risk of expulsion[4,5].

Postpartum IUD insertion has no affect on breastfeeding. It is particularly important that recent studies have documented that even the levonorgestrel-releasing devices have no affect on breastfeeding[6-9]. The levonorgestrel containing IUD has only a local effect on the endometrium with minimal transfer to the serum and even lower levels detected in the breast milk. Women can therefore be reassured that the use of an IUD during the postpartum period will not prevent them from providing their infant with breast milk that is of adequate quantity and quality.

Although IUD insertion in the postpartum period is highly favored by the current literature, many barriers of insertion still exist such as failure to return for postpartum follow up, lack of access to IUD[10], lack of knowledge by provider, and inadvertent early pregnancy[11]. Some of these barriers can be overcome with immediate postpartum insertion[12] as women are more motivated to obtain contraception and counseling services are readily available at this time[11,13].

The objective of this review is to evaluate the safety and efficacy of IUD insertion in the postpartum, with special attention to specific time frames for insertion that are associated with the best clinical outcomes. Additionally, data supporting the safety of IUDs in breastfeeding is used to further reinforce support for the insertion of IUDs in the postpartum period[14].

MATERIALS AND METHODS

Selection criteria

Inclusion criteria: Randomized controlled trials and prospective cohort studies.

Participants: Postpartum women off with no contraindications to IUD insertion.

Intervention time: IUD insertions during any time during the postpartum period, Immediate post placenta period (within 10 min), early post placenta period (10 min to 72 h), and delayed/interval period (greater than 6 wk). Other comparators included vaginal or cesarean deliveries, manual or ring forceps placement and IUD type.

Types of outcomes: Expulsion, pregnancy, continuation, infection and perforation rates. Measurements of quantity and quality of milk production were assessed by breastfeeding duration and infant growth.

Exclusion criteria: Retrospective studies, no time of

postpartum insertion, no cases of immediate or delayed postpartum IUD insertion.

RESULTS

Fifteen studies were included. Ten evaluated the safety (infections, perforations) and efficacy (expulsion, pregnancy, continuation of use) of postpartum IUD insertion. Five studies evaluated the effect on breast-feeding of both non-secreting and hormone secreting IUDs.

Evaluation of safety and efficacy by comparing insertion at different time periods

The safety and efficacy of IUD insertion within 10 min of placenta delivery, early postpartum (10 min to 48 h) and interval/delayed insertion (4-6 wk postpartum) were supported by 4 studies. Expulsion rates were highest when the insertion occurred in the early and immediate postpartum periods. Low complication rates and no significant difference between groups was found for infection, uterine perforation, and unplanned pregnancy[5,15-18].

Two of the four studies compared the three post-partum periods of insertion finding a statistically significant increase in expulsion rate during the immediate postpartum and early postpartum period when compared to interval insertion[16,17]. Two randomized controlled trials compared only two time periods; immediate post-partum and interval period, finding[15,18]. Vaginal deliveries were shown to have higher rates of expulsion across the included studies[15-18].

Evaluation of safety and efficacy for immediate postpartum period insertion

Results of 6 prospective observational studies on IUD insertion only during the immediate postpartum period (within 10 min post placenta) have shown that immediate insertion is safe and efficacious whether after cesarean or vaginal birth[2,13,19-21].

Immediate postpartum IUD insertion is a common practice in countries such as India and China. Two large multicenter studies, one with 300 women, and the other with 2733 women showed lower than expected expulsion rates than the rest of the studies included in this review. However, these studies conducted in India and China reported rates of complications such as infection, perforations and unplanned pregnancy similar to other studies that we reviewed[2,22]. A more thorough study with multiple follow up evaluations and acknowledgement of those lost to follow-up showed expulsion rates that were more representative of the current literature[21].

A possible explanation for the exceptionally lower expulsion rates in the two studies mentioned above is that Singal *et al*[22] did not take into account patients who were lost to follow up. Kumar *et al*[2] had a follow up period of only six weeks, which is not adequate for evaluating safety and efficacy of postpartum IUD insertion. Despite this, these studies provide insight on the use of IUDs in countries where immediate postpartum insertion may be most valuable.

These two studies from India and China demonstrated that regardless of insertion method there is no significant difference in expulsion rates. Although method of insertion has not been shown to affect the safety and efficacy, complication rates are affected by method of delivery. Expulsion rates have been shown to be significantly higher after vaginal delivery *vs* cesarean section[21].

Effect of the copper or levonorgestrel IUD on lactation

Further motivation for a woman to choose an IUD in the postpartum is the fact that both the copper IUD and Levonorgestrel operate locally on the endometrium. Therefore, there is no affection on lactation. Four studies, demonstrated the safety of IUD use during breast-feeding[7,9,23,24].

Two randomized controlled trials compared hormone-secreting to copper IUD demonstrated no significant differences in breast-feeding performance (quantity, duration) and infant growth/development (quality) when inserted in the delayed/interval period to evaluate the evaluate the effect of hormonal contraception on breast feeding success, a double-blind randomized controlled trial found that breast feeding continuation rates and infant growth were not affected by progestin only or combined oral contraception[7,9].

The only study that examined the timing of IUD insertion and its effect on breastfeeding, while not reaching significance found that women in the delayed insertion group continued to breast feed at the 6 mo follow-up and had longer breastfeeding duration compared to the immediate post placental group[15]. These findings certainly support further studies to determine what if any role timing of postpartum insertion has on breastfeeding.

DISCUSSION

Reports we reviewed found that IUD expulsion rates are higher in immediate and early postpartum insertion when compared to delayed insertion at the postpartum follow up visit. In general, reported IUD expulsion rates range from 2%-10%[25]. In contrast, immediate postpartum expulsion can range from 4%-27% within one year of insertion[5].

The reason for higher expulsion rates in immediate postplacental and early postpartum insertion is unclear but is most likely multifactorial. Incorrect insertion by health care providers due to inexperience and/or lack of skill in achieving high fundal placement may play a role[2,3,14,22]. Another explanation for the increased expulsion rate may be due the type of delivery since insertion immediately after a vaginal delivery has been shown to have a higher expulsion rate than after

cesarean[5,21,26]. One study went as far as recommending that immediate IUD insertion be contraindicated after vaginal delivery due to such high expulsion rates in comparison to cesarean: (after a vaginal birth, 50% (ultrasound only) + 27.8% (clinical examination); and post-cesarean section, 0% [$P < 0.001$; odds ratio (OR) = 5.75, 95%CI: 2.36-14.01][26]. Method of insertion, whether by hand or by forceps after a vaginal delivery, demonstrate similar expulsion rates, thus is unlikely to be a contributing factor[20].

Despite higher expulsion rate, the immediate postpartum period provides an opportunity for patient counseling on the possibility of expulsion. Although rates of expulsion are higher in the immediate postpartum, the convenience of insertion after delivery of the placenta may outweigh the expulsion potential by increasing access to effective contraception. This may be especially relevant for select populations that may not return for follow up and be come at risk for an unplanned pregnancy.

Infection and perforations were examined to assess factors that affect the safety of postpartum IUD insertion. For the non-postpartum patient, perforations occur at a rate of about 1/1000 or less insertions[27]. Where as in the postpartum, the studies used for this review demonstrated a range from no perforations to slightly increased rates over that for interval insertion. The most important determinant of uterine perforation based on these studies is the time of insertion. In one study, when compared to interval insertion, there was an increased perforation rate at 0-3 mo postpartum insertion (OR = 11.7, 95%CI: 2.8-49.2) and an even higher increase at 3-6 mo postpartum insertion (OR = 13.2, 95%CI: 2.8-62). However the rates did not increase in the immediate postpartum insertion or after 6 mo[28]. Another study not included in the results section, showed that women had a 10 fold risk of uterine perforation if the IUD was inserted during lactation[29]. With these results, it seems that the puerperium period poses an increased risk of uterine perforation with IUD insertion especially if the woman is lactating. It is possible that the associated hormonal and structural changes that take place during the puerperium period, such as thinning of endometrial wall, make the uterus more prone to perforation[14]. The Mirena is especially implicated in perforations with insertion during lactation, likely due to the compounded effects from progestins and other hormonal changes during lactation that cause endometrial wall thinning[6].

The infection rate (PID) with most of the studies used in this review either demonstrated no infection or very low rates that were not any different than the rates of non-postpartum women. All women in these studies were screened for STIs before insertion, and excluded if positive. These results agree with the current accepted rate of PID after IUD insertion of 0%-2% when no infection was present previously and 0%-5% when insertion occurs with an undetected infection[30]. Furthermore, studies indicate that with prompt treatment with positive chlamydia cultures after IUD insertion are unlikely to develop PID even with retention of the IUD[31]. The Mirena may even decrease the risk of PID due to the thickening of the cervical mucus and thinning of the endometrium[32]. Overall, we found that that IUD insertion at anytime does not significantly increase the risk of PID.

Studies on the effect of IUDs on lactation generally show no detrimental effect on the duration, quantity, and quality of lactation[6]. There may be a theoretical negative effect of progestins on breastfeeding, (which is why it is rated Category 2 by United States Medical Eligibility Criteria for Contraception) but more trials need to be completed to evaluate this proposed lactation risk. Also, an evaluation of the timing of levonorgestrel-IUD insertion and lactation indicated that women who received immediate postpartum insertion had a shorter duration of breastfeeding and were less exclusive with breastfeeding than women who received delayed insertion[23]. The author's suggests that because withdrawal from progesterone helps to initiate lactation, the progestins (from the IUD) placed in the immediate postpartum period, may act to inhibit lactogenesis. However, progestin-only contraception is recommended by the United States Medical Eligibility Criteria for Contraceptive (Category 2) with or without breastfeeding in the immediate postpartum.

Postpartum (including while breastfeeding) insertion of IUD is recommended by ACOG as a safety and effective method of contraception. It is safe and effective to insert an IUD (copper or levonorgestrel-releasing) in the immediate postpartum period (within 10 min of placental delivery)[33,34] despite higher expulsion rates since the benefits may outweigh the risks in select populations. Insertion of the copper or levonorgestrel IUD 10 min to 4 wk and at or after 4 wk has also shown to be safe and effective. Overall, LARC methods have very few contraindications and most women are eligible for its use during the postpartum period[35].

IUD insertion is efficacious and safe during the immediate postpartum, early postpartum and delayed postpartum periods[36]. Expulsion rates are highest in when inserted during the immediate postpartum period after vaginal deliveries. In addition, infection and perforation rates following postpartum IUD insertion are low. Delayed insertion of the copper and levonorgestrel IUDs was found to have no affect breastfeeding initiation. However, immediate postpartum insertion was associated with a decrease breastfeeding duration and exclusivity. Adequately powered randomized controlled trials are needed to further elucidate the effect of timing of postpartum IUD insertion has on lactation[37]. In spite of expulsion, perforations and breastfeeding duration concerns, IUD insertion in the postpartum should remain a viable family planning option for many women[24]. This may be particularly important for select populations where the benefits may outweigh the risk

of failing to achieve desired pregnancy spacing.

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COMMENTS

Background

The intrauterine device (IUD), a form of long acting reversible contraception (LARC), is one of the most effective contraceptive methods available as well as one of the safest. Its safety is supported by recent literature, which documents low rates of infection, perforation, and expulsion that should not deter a clinician from offering it as a contraception option.

Research frontiers

Despite the concerns regarding expulsion, perforation and breastfeeding, current evidence indicates that a favorable risk benefit ratio in support of postpartum IUD insertion. This may be particularly relevant for women for whom barriers exist in achieving desired pregnancy spacing.

Innovations and breakthroughs

IUD insertion is not only a viable option for the nulliparous, but should also be considered as an option during the postpartum period.

Applications

Despite the concerns regarding expulsion, perforation and breastfeeding, current evidence indicates that a favorable risk benefit ratio in support of postpartum IUD insertion. This may be particularly relevant for women for whom barriers exist in achieving desired pregnancy spacing.

Terminology

LARC: Methods include the IUD and the birth control implant. Both methods are highly effective in preventing pregnancy, last for several years, and are easy to use; Postpartum: A postpartum period or postnatal period is the period beginning immediately after the birth of a child and extending for about 6 wk. Less frequently used are the terms puerperium or puerperal period.

Peer-review

This is a good article.

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