OPEN LETTER

Cervical screening: A new way forward (tests of risk and tests of disease) [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract
Cervical screening has been a highly successful health promotion initiative. The way cervical screening is delivered is about to change dramatically, with the introduction of 'tests of risk' and 'tests of disease' based on primary HPV testing and the use of cell host response biomarkers. This article addresses the fundamental basis of this change in clinical practice and offers insights into how the future of cervical screening will look.

Keywords
cervical screening, HPV, biomarkers
Introduction
Cervical cancer is now the fourth most common cancer in women worldwide\(^1\). In Ireland, approximately 300 women are diagnosed with cervical cancer each year, with over 90 deaths. In addition, around 6,500 women require treatment for cervical intraepithelial neoplasia (CIN)\(^2\). Approximately 250,000 to 300,000 women are screened every year in Ireland, making it one of the most important health prevention strategies for our health services.

Infection with human papillomavirus (HPV) is the single most important aetiological factor in the pathogenesis of cervical cancer and pre-cancer. It is now increasingly recognised that HPV is also causally implicated in other cancers, including head and neck, vulval, penile and anal cancer\(^4\). Indeed the World Health Organisation (WHO) has recently stated that there is an epidemic of HPV related head and neck cancer, which needs urgent attention.

There are over 150 different types of HPV, 40 of which are found to infect the male and female genital tract. A number of these are known as ‘oncogenic HPV types’ [16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68] based on their potential to cause cervical precancerous abnormalities and cervical cancer\(^5\). Of these oncogenic HPV types, HPV 16 and HPV 18 are associated with approximately 70% of all cervical cancer cases\(^6\).

Epidemiologically, in relation to HPV there are “three ages of women”:

Age 1: before sexual activity begins (12–14 years), where the risk of acquisition of HPV is negligible. The majority in this age group will be offered prophylactic HPV vaccination.

Age 2: 12–25 years, as sexual activity begins, where girls and young women are exposed to HPV, but the majority of infections are transient and are cleared. A small number of women will develop persistent infection.

Age 3: women 25 years+, where persistent HPV infection is a feature. These women are at risk of developing cervical pre-cancer and cancer.

Infection with HPV is necessary, but not sufficient for the development of cervical cancer. Mild cellular changes and mild dysplasia (CIN 1) may occur after an acute HPV infection, but approximately 90% of these will regress without any treatment\(^7\). However, persistent HPV infection may lead to precancerous cellular changes (CIN 2 and CIN 3), a proportion of which will progress, if not treated, to invasive cervical cancer over a period of 10 to 20 years (Figure 1).

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**Figure 1. Cervical pre-cancer and cancer progression.** CIN1 [cervical intra-epithelial neoplasia grade 1], now called LSIL [low grade squamous intra-epithelial lesion]. CIN2 and CIN3 now called HSIL [high grade squamous intra-epithelial lesion].
Cervical cancer prevention strategies
Optimal cervical cancer prevention requires both effective HPV vaccination and screening. Because HPV vaccines offered in school-based programmes prevent most but not all high-risk HPV (hrHPV) infections, HPV immunised women should continue to participate in screening.

Population based cervical screening programmes operate in many European countries, including Ireland where a programme, CervicalCheck, has been offering free smear tests to all women aged 25–60 years since September 2008. In addition, HPV vaccination programmes are established in several countries worldwide including Ireland, where a national school based HPV immunisation programme began in 2010, using the quadrivalent Gardasil® HPV vaccine, which protects against HPV types 16, 18, 6 and 11. Vaccination is offered to girls (~12yrs) with catch-up immunisation (~18yrs) provided from 2011 onwards. For the first 4 years of the programme, uptake was high (85% amongst 12 year olds, and 71% for catch-up cohorts). However, this has fallen to a low of 50% in 2016/2017 cohorts, with the most recent figures indicating uptake rates of 56%[7]. This decline in uptake is attributed to un-substantiated claims regarding the safety of the vaccine. The vaccine is considered safe and well tolerated. In March 2015 the US CDC reported that ‘HPV vaccines are safe and effective vaccines’. In November 2015, the European Medicines Agency (EMA) reported a review of HPV vaccines. This report found no evidence the vaccine was linked to chronic fatigue-like conditions[8].

The decline in uptake leads to challenges in relation to the management of cervical disease in this context, as the population becomes stratified into distinct biological and epidemiological risk groups: vaccinated/screened; vaccinated/unscreened; unvaccinated/screened; unvaccinated / unscreened.

The changing face of cervical screening
Cervical screening aims to detect women with high grade pre-cancerous changes, which can then be treated, reducing the risk of cervical cancer, before cancer actually occurs. Cervical exfoliative cytology is the traditional technique for screening cervical smears to detect and treat cervical pre-cancer. Introduced in the 1940s by Papanicolaou, it is considered to be one of the most effective disease prevention strategies ever undertaken.

Like any screening test, it can have false negatives and false positives and its use has to balance sensitivity (the ability of a test to detect disease) and specificity (the likelihood of a positive test identifying underlying disease).

Over the last 5 years, HPV testing has been introduced into the management of cervical pre-cancer. HPV testing is used to triage women with low grade cytological abnormalities, and to manage women following treatment for CIN. It is likely that the use of HPV testing in screening will assume even more importance as increasing proportions of women who have been vaccinated against HPV enter the screening population. The utility of HPV testing has been shown in several international trials with a high negative predictive value (i.e. excluding the likelihood of disease)[11–14].

More recently, the use of HPV testing for primary cervical screening has been strongly advocated, as it is significantly more sensitive and has a higher negative predictive value than cytology alone based primary screening.

HPV testing for primary screening is essentially thena “test of risk” rather than a “test of disease”.

In Ireland, a health technology assessment has recently been published byHiQA (Health Information and Quality Authority, Ireland) at the request of the National Screening Service, to examine the clinical and cost effectiveness of using HPV testing as the primary screening method instead of cytology. This report recommends the introduction of HPV primary based screening for cervical cancer prevention.

However, using HPV as a test of risk is not without its problems. While HPV DNA testing has a very high negative predictive value, it has a low specificity or high incidence of false positives[15]. Appropriate protocols to stratify HPV positive women are essential to avoid over-referral and over-treatment and thus it will be necessary going forward to develop “tests of disease”, which reflect the cellular response of women’s cells to transforming HPV infection, essentially giving a biological signature of HPV-related disease and disease progression.

Molecular biomarkers as “tests of disease”
HPV DNA testing as a primary screening test is more sensitive than cytology for identifying women who have CIN2+, but the specificity is lower[11–14]. While high sensitivity is important, many CIN2 and some CIN3 lesions will spontaneously regress as suggested by the natural history of the disease, thus, it is therefore possible that tests with lower sensitivity will still identify those lesions that progress to cancer.

Finding a balance between sensitivity and specificity is hugely important in the context of primary screening to avoid large numbers of unnecessary testing and follow-up of HPV-positive women, which will increase anxiety for women and significantly increase health service-related costs. This could be achieved by avoiding screening in younger women (e.g. <30 years), using more specific HPV tests and using appropriate triage algorithms, based on “tests of disease”.

The majority of evidence from HPV primary screening randomised control trials suggests that reflex cytology is an appropriate option for triage of HPV positive women[12,13,16]. However, challenges remain on how to manage women who are HPV positive with a negative cytology result. An alternative approach is to triage with some form of secondary biomarker(s). Several biomarker options exist for this, including [1] detection of HPV E6/E7 mRNA, [2] genotyping for HPV16/18, [3] co-expression of p16INK4a/Ki-67 (Figure 2)[17,18], [4] detection of a panel of methylation biomarkers (e.g. CADM1, MAL, miR124)[19] and [5] using
extended panels of biomarkers derived from empirical research (Figure 3).

Alternative biomarkers as “tests of disease” have the potential to offer more specific triage of HPV positive women. It is known that HPV subtypes 16 and 18 are associated with over 70% of cervical cancer and consequently induce a higher risk of malignancy. Over expression of viral oncogenes E6 and E7 are necessary for malignant transformation. Detection of these oncogenes by the presence of their mRNA transcripts allows for better distinction between transient HPV infections and those persistent or active transforming infections that are likely to progress to a pre-cancerous or cancerous lesion(s).

The presence of active HPV infections can also be identified through over expression of p16, which plays a major role in cell cycle regulation and Ki-67 a proliferation marker. Over expression of p16 signals E7 mediated deregulation of the cell cycle and thus acts a surrogate marker for active HPV infection (Figure 2).

**Figure 2.** p16/ki-67: “test of disease” biomarker in cervical pre-cancer and cancer. p16 [brown]; Ki-67 [red]. Top Left panel: model of hrHPV E7 protein interaction with cellular transcription factor E2F and its effect on cell division. Top Middle panel: p16/ki-67 staining in CIN 3 [HSIL] (x200). Top Right panel: p16/ki-67 staining in cGIN (x400). Bottom left panel: p16/ki-67 staining in a moderately dyskaryotic [HSIL] squamous epithelial cell (x400). Bottom right panel left: p16/ki-67 staining in a microbiopsy of CIN 3 (x200).

**Figure 3.** Novel “tests of disease” in cervical pre-cancer and cancer. Left panel: Minichromosome maintenance protein 5 [MCM5] in cervical glandular intra-epithelial neoplasia [cGIN] (x200). Middle panel: Geminin expression in cGIN (x200). Right panel: Nuf-2 [replisome associated protein in CIN 3 [HSIL] (x400).
Recently, methylation of particular genes has been found to be linked to high grade pre-cancer and cervical cancer. Methylation of human genes is strongly associated with CIN and cancer. Several candidate genes are shown to be consistently hyper-methylated in cervical cancer and high-grade CIN, most prominently CADM1, EPB41L3, FAM19A4, MAL, miR124, PAX1 and SOX119-21. These markers show promise as triage markers for managing HPV-positive women, although published studies have been largely cross-sectional with short-term follow-up, in predominantly non-screening populations and conducted on cervical scrapes or self-collected samples11,22. Other novel promising methylation markers include GHSR, SST and ZIC, which are associated with a 3q gain21.

**CERVIVA**, through its Health Research Board (HRB) funded CARG [CARG29/2012] programme, is evaluating the range of triage options to optimally stratify women with a HPV positive primary screening smear result.

**CERVIVA/CervicalCheck HPV primary screening pilot study: Molecular triage strategies for HPV-positive women**

CERVIVA is a multi-institutional, international research ecosystem of excellence working in the area of HPV related diseases. It has spearheaded the development of novel biomarkers as “tests of disease”, based on fundamental biological discovery in relation to HPVs ability to subvert cell cycle machinery in the woman’s cells. It has also developed novel RNA interference and proteosomal degradation drug approaches to alter the fundamental activity of hrHPV transforming genes and proteins.

CERVIVA, in partnership with CervicalCheck, are currently undertaking a HPV primary screening study, which is evaluating and comparing different strategies for the triage of women with a HPV positive primary screening test (www.cerviva.ie). This study, funded by the HRB, is an observational cohort study recruiting >13000 women attending primary care for their routine CervicalCheck smear test (Figure 4).

Women attending their routine CervicalCheck smear tests are invited to participate in the study and give written informed consent. Residual smear samples following routine cytological diagnosis are retained and tested for HPV DNA (Cobas HPV DNA test [Roche]) and HPV mRNA (The Aptima HPV test [Hologic]). Women who test positive for HPV DNA are then tested with a series of triage tests, including cytology, HPV16/18 genotyping, HPV mRNA, p16ink4a/ki67, and a panel of methylation biomarkers. The women will be followed longitudinally through CervicalCheck for up to 10 years through several screening rounds to assess the performance of the different triage approaches for stratifying HPV positive women into different risk categories.

**Figure 4. CERVIVA primary screening trial.**
This is the first study of its kind internationally that examines all markers in combination, which is embedded within a national screening programme.

Recruitment to the study is now almost completed. Within the population of the first 6,000 women analysed, 15% tested positive for HPV DNA. Overall women under the age of 30 were significantly more likely to test positive for HPV, while those aged 30–39 were at a higher risk of testing positive compared to those aged 50 years and over. The rate of abnormal cytology among the first set of women enrolled is 6.2%.

Of those that tested positive for HPV; 32% were positive for HPV subtypes 16 and 18 the particular subtypes of HPV that are associated with 70% of cervical cancers, and are targeted specifically by the HPV vaccine. In those women that were positive for HPV16/18, 16% had a high grade cytology, compared to only 2% of women who had an infection with another HR HPV subtype. Overall, 43% of those that tested positive for HPV 16 and 18 had an abnormality detected on cytology compared to only 26% of those that tested positive for non-HPV 16/18 HPV subtype.

This early data provides important information to policy makers on the potential impact of changes to cervical screening tests in Ireland. As more information becomes available, it will directly inform decisions around the management of women with a positive screen on HPV-based primary screening tests.

The data will also be helpful in predicting the potential impact HPV vaccination will have on HPV prevalence rates in Ireland. It is likely that significant changes will be implemented to cervical screening as the picture evolves. The challenge will be to build on the achievements of CervicalCheck to date by integrating new and improved tests to prevent more cervical cancers in the future.

**HPV primary screening: Advantages**

- Improved detection of CIN2+ and CIN3+, especially in women over 30 years of age.
- Long-term possibility of increasing/changing the routine screening interval from 3-yearly to 5- or 6-yearly in women over 30 years of age, thus reducing health economic costs of screening.
- Likely to be more effective and efficacious, when prevalence of cervical cancer and its precursors declines in vaccinated populations and the performance of current tests will be challenged.

**HPV primary screening: Challenges**

- Appropriate triage methods “tests of disease” will be needed for triage of HPV positive samples to avoid false negatives and false positives.
- Long-term follow up of HPV+ women without CIN2+ may be needed.
- There remains a risk of 5–15% false negative rates in some women. Therefore careful decisions need to be made around who to screen and when to screen in relation to particular groups.

**Disclaimer**

The views expressed in this article are those of the author(s). Publication in HRB Open Research does not imply endorsement by the Health Research Board of Ireland.

**Data availability**

No data is associated with this article.

**Competing interests**

No competing interests were disclosed.

**Grant information**

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Reagents for the Aptima HPV mRNA test are provided by Hologic Inc. CERVIVA wish to acknowledge the enormous input from CervicalCheck and all of smear takers across Ireland that are helping to recruit women to the study. We wish to also thank the women who have agreed to participate.

**References**

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This letter describes the design of a HPV primary screening study presently undertaken by CERVIVA in partnership with CervicalCheck in which different triage strategies for HPV DNA pos women are tested to identify those HPV pos women who have clinically relevant disease. The basic concept of the project description is clear but a few points should be more clarified.

Remarks:

1. It is a project description and only some early results are given. The outcome parameters of the triage testing are unclear and not well described. It looks like it is abnormal cytology, which is subjective and a relatively weak endpoint. I would prefer a more firm histologic endpoint like CIN3, but perhaps a short explanation can be given why one has taken cytology as endpoint. The endpoint should be made more clear in the text.

2. Moreover 13,000 women are tested by HPV but a short description of the statistics how the triage markers are evaluated is lacking. This is important for evaluation of cross-sectional results and the 10 years f-up data. I doubt whether with the numbers as given in Fig 4 statistically significant results can be obtained in a 10 years f-up taken in account loss to f-up.

3. Besides HPV-DNA tested by COBAS (Roche), HPV mRNA is tested by The Aptima HPV test (Hologic). From Fig 4 it is unclear whether this test is used in parallel to HPV-DNA testing or as triage test. According to the text only women with HPV DNA pos. women are tested by the triage tests: Cytology, HPV 16/18 genotyping, p16/Ki-67, and methylation markers.(CADM1/MAL/miR). Please clarify.

4. The manuscript makes sometimes big steps and is therefore sometimes difficult to follow. I have made appropriate annotations in the text. The annotated article PDF can be downloaded here. Examples are:

   a. Page 3 last paragraph in left column where it is not clear whether primary HPV testing or secondary HPV testing after abnormal cytology is discussed. Please clarify.
b. Page 5 first paragraph in left column: here the triage biomarkers should be more explained

5. The legend of Fig 2 should more explain the relation with E7, pRB, E2F etc.

6. Fig 3 shows nice histological stains of biomarkers but these markers are not explained in the text. So either remove the Fig 3 or explain the markers in the text.

7. Fig 4 should be improved. It is unclear how many women are expected to be HPV DNA pos. In addition make clear why cytology is placed above HPV testing (for cross-sectional evaluation?) and why HPV-DNA COBAS is in the same square of HPV mRNA (APTIMA). (parallel testing to compare sensitivity and specificity. If so this should be done with CIN2/3/Cervical carcinoma as endpoint)

Is the rationale for the Open Letter provided in sufficient detail?
Yes

Does the article adequately reference differing views and opinions?
Yes

Are all factual statements correct, and are statements and arguments made adequately supported by citations?
Yes

Is the Open Letter written in accessible language?
Yes

Where applicable, are recommendations and next steps explained clearly for others to follow?
Partly

**Competing Interests:** I have received speakers’ fee from SPMSD/Merck, served occasionally on the scientific advisory board (expert meeting) of Qiagen, SPMSD/Merck. I have been co-investigator on a Sanofi Pasteur MSD sponsored trial, of which the study funding went to my Institute. I am part-time director of – and minority stock holder of Self-Screen b.v., a spin off company of VUMC, which holds patents of methylation markers and makes an HPV test and a triage test based on methylation of host cell genes and I have a very small number of Qiagen shares. Until April 2016 I had minority stock of Diassay b.v.

**Reviewer Expertise:** HPV and cervical cancer, gynaeco-pathology, clinical immunology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
This is a timely and well written piece which outlines the key aspects and challenges of (1) cervical screening based on molecular HPV testing, (2) the challenges of risk stratification or triage of HPV positive women and (3) priorities for research and service developments therein. The arguments presented support the conclusions drawn.

There are some minor issues that are worthy of attention.

The authors state that HPV infection is negligible in the <14; however prevalence of HPV in females < 14 is possible and will vary according to setting. Non sexual transmission of HPV of infants and children is also possible including via vertical and horizontal transmission where abuse is not suspected. As this is an educational piece I think it is important to make the point that while relatively rare, HR-HPV infection of young sexually inactive men and women is possible.

There are a couple of typos - if the authors search for "thena" isan" then these are easily remedied!

The section "HPV Primary Screening - Challenges" contains the following statement "There remains a risk of 5–15% false negative rates in some women. Therefore careful decisions need to be made around who to screen and when to screen in relation to particular groups". This is slightly vague - 5-15% is quite a range and it is not clear what evidence this is derived form particularly as the outcome is not defined... i.e. false negative for what? ..CIN2+?

Is the rationale for the Open Letter provided in sufficient detail?
Yes

Does the article adequately reference differing views and opinions?
Yes

Are all factual statements correct, and are statements and arguments made adequately supported by citations?
Yes

Is the Open Letter written in accessible language?
Yes

Where applicable, are recommendations and next steps explained clearly for others to follow?
Yes
**Competing Interests:** Non personal. KCs institution has received consumables and or research funding from the following commercial organisations in the last 3 years: Cepheid, Euroimmun, Hologic, Qiagen, LifeRiver, Genomica, SelfScreen, GeneFirst.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.