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Authors
Kyrgiou, Maria
Mitra, Anita
Moscicki, Anna-Barbara

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Does the vaginal microbiota play a role in the development of cervical cancer?

Maria Kyrgiou[1,2], **Anita Mitra**[1], and **Anna-Barbara Moscicki**[3]

[1]Institute of Reproductive and Developmental Biology, Department of Surgery & Cancer, Faculty of Medicine, Imperial College, London, UK

[2]Queen Charlotte's & Chelsea – Hammersmith Hospital, Imperial Healthcare NHS Trust, London, UK

[3]Department of Pediatrics, University of California, Los Angeles

Abstract

Persistent infection with oncogenic Human Papillomavirus (HPV) is necessary but not sufficient for the development of cervical cancer. The factors promoting persistence as well those triggering carcinogenetic pathways are incompletely understood. Rapidly evolving evidence indicates that the vaginal microbiome (VM) may play a functional role (both protective and harmful) in the acquisition and persistence of HPV, and subsequent development of cervical cancer. The first studies examining the vaginal microbiome and the presence of an HPV infection using next generation sequencing techniques (NGS) identified higher microbial diversity in HPV-positive as opposed to HPV-negative women. Furthermore, there appears to be a temporal relationship between the VM and HPV infection in that specific community state types (CSTs) may be correlated with a higher chance of progression or regression of the infection. Studies describing the VM in women with pre-invasive disease (squamous intra-epithelial neoplasia – SIL) consistently demonstrate a dysbiosis in women with the more severe disease. Although it is plausible that the composition of the VM may influence the host innate immune response, susceptibility to infection and the development of cervical disease, the studies to date do not prove causality. Future studies should explore the causal link between the VM and the clinical outcome in longitudinal samples from existing biobanks.

Corresponding author: Anna-Barbara Moscicki, Chief, Division of Adolescent and Young Adult Medicine, Ronald Reagan UCLA Medical Center, UCLA Medical Center, 10833 Le Conte Ave MD 22-432, Los Angeles, California 90095, Phone: 310-825-0867; Fax: 310-206-5146; AMoscicki@mednet.ucla.edu.

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Introduction

Although cervical cancer is largely preventable through detection and treatment of the pre-invasive precursor, high-grade squamous intra-epithelial lesions (HSIL), it remains the commonest female malignancy in virtually all low-resource countries and the seventh more frequent malignancy in females worldwide [1]. It is estimated that, globally, almost 530,000 women develop cervical invasive disease annually and more than 265,000 die from the disease [2]. The comparatively low incidence of cervical cancer in affluent societies is largely related to the presence of population–based screening programmes and education that led to a dramatic decrease in the incidence and mortality from the disease [3]. Although the HPV vaccine has the potential to dramatically decrease these rates, the introduction into most developing countries has been slow so these changes are not expected to impact cervical cancer rates for at least one to two decades.

HPV and Cervical Carcinogenesis

There is strong evidence that infection with Human Papillomavirus (HPV) is a necessary, but not sufficient for the development of cervical pre-invasive and invasive disease. With more than 200 HPV subtypes recognised today, it is only a fraction of these that has been found to have a carcinogenic potential [4]. Of these, subtypes HPV-16 and HPV-18 are most commonly associated with invasive cancers and are thought to cause approximately 65-75% of cases. It is now recognised that it is the persistence of infection by these, and a handful of other high-risk oncogenic HPV subtypes that leads to precancerous lesions.

One of the early observations in most epidemiology studies was the high frequency of HPV DNA detection. The more HPV types tested, not surprisingly the higher frequency of detection. In most studies, but not all, age influenced prevalence with young age being associated with higher rates—some as high as 45% in western societies [5]. This vulnerability is thought to be due to immature or naïve immune responses as well as biologic vulnerability of the immature cervical epithelium inherent in adolescents [6]. There is some evidence to suggest that the microbiome of epithelium predominantly covered by columnar epithelium as seen in immature cervixes differs to that in epithelium predominantly covered by squamous epithelium [7]. It may be reasonable to ask “Does the microbiome of immature squamous epithelia contribute to the vulnerability to HPV infections? “.

Interestingly, lifetime risk of acquiring any HPV infection likely exceeds 80%. With more sensitive testing available, studies show that HPV infection is more commonly the rule, not the exception. HPV types associated with the alpha species predominate in the anogenital area but other HPV types such as the beta and gamma HPV types, once thought to be predominantly cutaneous only, can also be commonly detected [8-10]. Since the lifetime risk of developing invasive cervical cancer is much lower at 0.6% [11], cervical cancer should be regarded as a rare complication of a very common infection by the human papillomavirus. The commonness of many of these HPV types, begs the question, “Should non-oncogenic HPV be considered commensal organisms and do they play a protective role against the *oncogenic HPV types*?”.

Epidemiology studies have shown that several risk factors have been correlated to heightened or reduced risk of cervical cancer. The most consistent factors are tobacco smoking, oral contraceptive use, and parity. All have biologic plausibility. Nicotine and its carcinogenic metabolites can be detected in cervical mucous [12] and smoking has been associated with a dampening of local immune markers [13]. Both estrogen and progesterone increase cell proliferation and hence vulnerability to DNA damage [1415]. Higher parity may be associated with high levels of hormone exposure and/or repeated trauma [16]. This raises many questions, including “Does smoking, hormonal contraceptives and parity negatively influence microbiota such that HPV carcinogenesis is promoted? “.

Observational data show that the estimated time from the infection to the development of invasive disease is approximately 15 years, although there may be a swift progression in rare cases (Figure 1). Cervical carcinogenesis normally has a lengthy precancerous phase that has been well defined through different grades of SIL, although the continuum of the carcinogenic process has been questioned in some cases [17]. Despite these major advances in our current understanding of the disease, the exact factors that determine infection and / or disease that will persist, progress or, conversely, spontaneously resolve are incompletely understood.

In general, HPV is a nonlytic infection, hence the inflammatory response to HPV is much more subtle than other mucosal infections, such as *C. trachomatis.* The initial immune response to acute HPV infections is likely mediated by the local innate immune system, probably involving mechanisms such as activation of toll-like receptors and natural killers cells to name a few [18]. Persistent infections are likely cleared by the development of adaptive immune responses, which are dependent on antigen–presenting cells. HPV 16 is thought to down regulate both innate and adaptive immune responses. Recent data show that local microbial communities also play significant roles in regulating immune responses [19]. Final pathways to cancer result in interference with telomerase activity and viral integration--although a percent of cancers are found to have episomal HPV DNA only [20]. HPV E6 and E7 are known oncoproteins, which control fundamental carcinogenic events including proliferation, senescence, and apoptosis. Cellular targets include p53, E6AP, CBP, p300, Bak, hTERT, MAGUK, cIAP, survivin, p107, pRB, p130 [21]. One of the important questions is “Whether the microbiome can also manipulate these genes in addition to HPV’s influences or whether the observed changes are actually due to microbiome dysbiosis induced by HPV?”.

More recently, emerging data support the notion that the complex interactions of the host with the commensal bacteria in the vagina (vaginal microbiome, VM) may be involved in the natural history of the disease. This review aims to provide a comprehensive summary of the existing evidence describing the interplay between the host, HPV and the bacteria in the vaginal microbiome.

The vaginal microbiome

Much of the work to date on mucosal microbiota has been focused on the gut including its influence on immune function, behaviour as well as local and systemic inflammatory

diseases [22-24]. The Human Microbiome Project has extensively examined the vaginal microbiota although in contrast to the gut, less is understood about the role of the vaginal microbiome (VM) in human disease. A detailed description of the vaginal microbiome is outside the scope of this article and can be found in several review articles [2526]. In the following, we highlight certain aspects of the VM that are pertinent to our topic.

As any of the mucosal compartments, there are likely to be differences between specific anatomic sites within the vagina. There has been attempts at examining some of the vaginal compartments (cervix vs proximal vagina vs distal vagina), however, other sites remain not well characterized including ectocervical and endocervical or microbacteria associated with immature and mature cervical epithelium. Interpretation of these studies is also difficult because of the close proximity of these sites where contamination can occur during sampling. Defining “healthy” microbiota is challenging since community clustering seems to be a moving target with variability through the women’s menstrual cycle as well her reproductive age.

The healthy, premenopausal vaginal bacterial communities are usually populated by *Lactobacillus* spp. that are commonly regarded to ensure a low pH [27], which is thought to provide the first-line of defence against pathogenic agents. Additionally, these bacteria are able to produce numerous other protective peptides and metabolites capable of inhibiting bacterial growth, adhesion and through disruption of biofilms. All Lactobacillus, however, are not necessarily stable or “healthy”. *L. iners* is present in all women including those with “dysbiosis” whereas *L. crispatus* is mostly seen in ‘healthy” women. For example, in one study, a predominance of *L. iners* predicted the development of bacterial vaginosis (BV), one of the best-studied vaginal “dysbiosis” [28-32]. In comparison, *L. crispatus* predominance appears protective against the development of BV [33].

Ravel *et al.* were the first to classify the VM according to structure, using more recent next generations sequencing platforms (NGS) [34]. They studied 396 healthy women and identified 282 taxa in total, proving the VM is far more complicated that previously appreciated. Based on the presence of a particular *Lactobacillus* species, or their absence they assigned 5 different community state types (CSTs); CST I, II, III and V are dominated by Lactobacillus crispatus, L. gasseri, L. iners and L. jensenii respectively, and CST IV (bacterial vaginosis associated bacteria) conversely, is a heterogeneous group typified by depletion of *Lactobacillus* spp. with presence of strictly anaerobic species such as *Gardnerella, Megasphera, Sneathia* and *Prevotella.* Longitudinal studies show that within-subject variation is lower over time than those between-subjects in the vagina, which also exhibits greater longitudinal stability that most other body compartments [35]. The majority of women have a relatively stable VM with a relatively low diversity in comparison to other mucosal sites, and it is those with the highest diversity VM in whom we observe the greatest instability (i.e moving from one state to another) [36].

Bacterial vaginosis (CST IV) is an enigmatic disorder characterized by increased species diversity. Prevalence of the condition is high ranging from 12% in Australian women [37], up to 50% in sub-Saharan African women [38], making it the most prevalent vaginal disorder of women of reproductive-age. This has important public health implications, because BV has

been associated with serious and significant reproductive morbidity including pelvic inflammatory disease [39], miscarriage [40] and may increase the risk of pre-term birth between 2 and 4-fold [40]. Furthermore, BV is associated with increased rates of bacterial sexually-transmitted infections (STIs) [41] and human immunodeficiency virus (HIV) transmission [42], which demonstrates how important it is to understand the interplay between the microbes and potential pathogens.

Interestingly the gut does appear to be a reservoir for many of the VM species (both healthy and pathogenic) but clearly the vagina is protected from colonization of the majority of gut species. As mentioned, the overall diversity of the VM is several-fold less than the gut. In contrast the diversity of Lactobacillus spp. is much higher in the vagina. Certain bacterial species are also shared with the mouth such as G. vaginalis [43]. Many of the BV associated bacteria can be found in the rectum. In one study, presence of *Megasphaera* and *Sneathia* spp. in the rectum was predictive of clinical BV in the women during follow-up [44].

Longitudinal studies of the VM indicate that bacterial community structure is dynamic and hormonally influenced with a propensity to become less stable during menstruation [36] and conversely more stable and less diverse during normal pregnancy [4546]. The vaginal microbiota are also likely affected by numerous exogenous factors, although most of these have not been well studied specifically in longitudinal samples. Of particular interest are hormonal contraceptives and cigarette smoking since both have been associated with the development of cervical cancer [4748]. Combined oral contraceptive use is associated with increased level of inflammatory cytokines in the cervix [49]. The source of these cytokines is unknown however it is plausible that the microbiota influence this inflammatory environment as is found in the gut [50]. Acute inflammation may be protective against acquisition of STIs including HPV, however, chronic exposure to inflammation is toxic to cells resulting in DNA damage and potentially carcinogenic changes [51].

Although many studies found conflicting data, in general, oral contraception (OC) use is associated with decreased risk of BV [52]. The few studies available using NGS continue to show a protective effect in that hormonal contraceptives are associated with Lactobacillus species dominated microbiomes [53-55]. Most of these studies were conducted over short periods of time, so the long-term effect of OCs on the VM and immune cells remains unknown. Studies on other methods such as the intrauterine device (IUD) and medroxyprogesterone acetate (MPA) are rare and show no major impact [52]. One study by Mitchell et al. [56] showed that MPA users had a decline in vaginal H_2O_2 producing lactobacilli but did not have an increased risk of developing BV.

Tobacco use and VM has also not been well studied. Certainly, tobacco smoking has been associated with altered diversity in the gut, oral cavity and respiratory tract [5758]. Bradshaw et al. [59] showed that BV (using Nugent Score) was increased in women who smoked more than 30 cigarettes per week. Brotman et al. [60] have further used NGS to show that in a small cross-sectional cohort of 20 smokers and 20 non-smokers, smokers had a significantly higher prevalence of CST IV (50% smokers vs 15% non-smokers), and identified *Peptostreptococcus* and *Veillonella* as genera most significantly associated with smoking.

Further studies are required to ascertain whether smoking does indeed have a causal relationship with VM alteration and dysbiosis.

Vaginal microbiome, HPV and immune response

There are no good direct data that show how altered VM influences local immune function. However, it is plausible that the stability and composition of the vaginal microbiome may play an important role in determining host innate immune response and susceptibility to infection as well as playing a role further downstream regarding the development of cervical disease.

Several studies have shown that BV and BV-associated bacteria effect immune parameters within the vagina including cytokines/chemokines, antimicrobial proteins and immune cell populations [61]. BV, as defined by Nugent's has shown relatively consistently higher levels of IL-8 and IL-2 beta [62]. *In vitro* studies of individual bacteria show that several are capable of inducing proinflammatory responses. For example, *Atopobium vaginae*, a BV associated bacteria activates the proinflammatory transcription factor nuclear factor (NF)-κB, tumor necrosis factor (TNF) alpha, interleukin (IL)-6 and IL-8, macrophage inflammatory protein (MIP) 3 alpha and Regulated on Activation, Normal T Expressed and Secreted (RANTES) [6364]. Similarly, the BV associated bacteria *G. vaginalis, P. bivia, M. mulieris, S. amnii* and *S. sanguinegens* have also been shown to induce similar *in vitro* cytokine and chemokine profiles [65-67]. Clinical studies also show that microbiomes with a predominance of BV-associated bacteria and increased diversity have similar proinflammatory profiles with elevated IL-8, IL-1α, IL-1β, INF gamma, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) compared to women normal flora [6667]. Clinical studies have also demonstrated immunosuppressive effects with lower levels of INF gamma-induced protein 10 and soluble leukocyte protease inhibitor (SLPI) in women with BV [6869]. In comparison, certain bacteria, primarily a predominance of specific Lactobacillus species, such as *L. crispatus, L. jensenii, and L. gasseri*, is associated with a relatively non-inflammatory state in the cervicovaginal environment. These pro-inflammatory states result in tissue damage possibly enhancing HPV's oncogenic potential. Despite DNA damage, expression of E6 and E7 result in inhibition of apoptosis and enhance cellular proliferation increasing aneuploidy and chromatin abnormalities leading to the development of cervical dysplasia and cancer. As mentioned, *Lactobacillus* spp. provides many protective substances of which many may be relevant to HPV. *In vitro* studies show that *Lactobacillus spp*. exerts cytotoxic effects on cervical tumor cells [70]. Human normal fibroblast-like cervical (HNCF) and HeLa cervical cancer cell lines were treated with *L.crispatus* and *L.gasseri,* which inhibited cell proliferation and induced cell death to a significantly greater degree in the cancer cell line. This indicates probable interactions amongst cervical cells, the microbiota and metabolites [70].

BV has also been shown to effect immune cell populations within the vaginal mucosa. Findings are not consistent with evidence of suppression as well as enhancement of leukocytes (reviewed in [61]. Conflicting data is likely due to the fact that most of these studies used the Nugent's criteria rather than NGS for defining "dysbiosis".

Of course, there are likely genetic and hormonal factors that play a role in host response to vaginal pathogens. These include cytokine and chemokine polymorphisms and endogenous and exogenous synthetic hormones making these interactions complex and poorly understood [7172]. In addition, cervical epithelial type (columnar, metaplastic and squamous) also influence immune response. In one study women with large areas of cervical columnar epithelium typical of pubertal cervixes, had much higher levels of proinflammmatory cytokines [73].

HPV infection and the vaginal microbiome

Several pieces of evidence suggest that HPV is affected by the vaginal microbiome. In meta-analyses of mostly cross-sectional studies, the presence of BV was associated with higher rates of HPV infection (12 studies; odds ratio (OR) 1.43, 95%CI 1.11 to 1.84) [74] suggesting that a diverse, Lactobacillus-depleted microbiome may contribute to HPV persistence. There is also evidence to suggest that persistence is more likely in those with altered microbiome. In one study, women with persistent high risk (hr) HPV had a prevalence of BV of 11% compared to only 5% in those women who cleared their hrHPV [75]. Similarly, King et al. [76] found that women diagnosed with BV had delayed clearance of HPV (adjusted Hazard ratio (aHR)=0.84, 95%CI: 0.72, 0.97). The major limitation of these studies was that all used the crude measure of BV using Nugent's criteria, which is highly subjective [77].

One of the first studies to examine the vaginal microbiome and HPV using NGS was conducted in Korea and used cervico-vaginal samples collected as part of the Healthy Twin Study within Korean Genome Epidemiology Study of 912 women [78]. A total of 68 women were analysed of which 23 were HPV-positive and 45 HPV-negative. The analysis of 45 premenopausal women with or without HPV infection found that *Fusobacteria*, particularly *Sneathia* spp. could be used as microbiological markers of HPV infection. HPV-positive women exhibited higher microbial diversity with a lower proportion of *Lactobacillus* spp. than HPV-negative women, while there was reduced abundance of *L. iners* in HPV infection positive versus negative women amongst 9 pairs of HPV-discordant monozygotic twins (P = 0.03) (Table 1, Figure 1).

Using culture independent PCR-denaturing gradient gel electrophoresis, Gao et al. [79] examined 70 healthy women (32 HPV negative and 38 HPV positive) with normal cervical cytology. Their group found that HPV positive women had greater biological diversity using the Shannon-Weiner diversity index. When they examined specific species, *L. gasseri* and *G. vaginalis* were significantly higher in HPV infected women.

Both of these studies are cross-sectional and unable to determine whether HPV induces a change in the VM or that the VM influences persistence of HPV. Brotman et al. [80] examined the temporal relationship between the vaginal microbiome and HPV infection. Thirty-two women were to collect serial two-weekly self-samples of vaginal secretions over the course of 16 weeks. The authors identified a significant association of the CST to the chance of remission (P=.008), but this did not seem to affect detection of new HPV infections (P = . 10). They were also able to examine the impact of CST on transition between HPV-negative and – positive states using the adjusted transition rate ratio (aTRR), which is equivalent to

hazard rate ratio, and adjusted for normalized menstrual cycle time. Lactobacillus depleted CST IV-A when compared to *L. crispatus*-dominant CST I had higher transition rates to HPV positivity state, although the differences were not significant (aTRR: 1.86, 95% confidence interval (CI) 0.52–6.74). When compared to CST I, *L. gasseri*-dominant CST II was correlated with the fastest HPV regression, while CST IV-B was associated with the slowest (aTRR, 4.43, 95% CI, 1.11–17.70 and aTRR, 0.33, 95% CI, .12–1.19, respectively) (Table 1, Figure 1).

Pre-invasive and Invasive Cervical Cancer and the vagina microbiome

More recently, we published the first study describing the VM in 169 women with biopsy-proven cervical pre-invasive and invasive disease and compared them to healthy HPV negative controls [81]. We found that the rate of Lactobacillus-depleted high diversity microbiome (CST IV) was increased two-fold in women with low-grade squamous intraepithelial neoplasia (LSIL), three-fold in women with high-grade squamous intraepithelial neoplasia (HSIL) and four-fold in women with invasive disease (normal = 2/20, 10%; LSIL = 11/52, 21%; HSIL = 25/92, 27%; cancer=2/5, 40%, p=0.06). There was a trend association between *Lactobacillus crispatus*-dominant VM (CST I) and increasing disease severity (normal=10/20, 50%; LSIL=22/52, 42%; HSIL=37/92, 40%; cancer=1/5, 20%, p=0.30) suggesting that this may be a microbiome community protective against the development of precancerous and cancerous lesions. Furthermore, *Peptostreptococcus anaerobius* and *Anaerococcus tetradius* were found to be more common in women with HSIL opposed to LSIL disease. That was also noted for *Fusobacteria*-primarily *Sneathia sanguinegens* ($P < 0.01$). These could be used as microbiological markers of clinically significant disease (Table 1, Figure 1). Although this was the first study to describe the VM in women with cervical disease, one of the limitations was the lack of adjustment for risk factors and possible confounders amongst the compared groups. Given the previously inconsistent evidence surrounding an association between BV and SIL/cervical cancer, there is a paucity of studies examining the potential bacterial-induced mechanisms of neoplasia in the cervix. Dysbiosis has been implicated in the carcinogenic pathways in the colorectal mucosa, and members of the *Peptostreptococcus* and *Fusobacteria* genera have been implicated not only in the pathogenesis of such a cancer [8283], but the latter also associated with poorer prognosis [84]. Aberrant WNT pathway signaling is implicated in oncogenesis, and *Fusobacterium nucleatum* expresses a cell surface virulence factor; FadA that is capable of activating the WNT pathway in one of the steps in colorectal carcinogenesis [85]. Furthermore *F.nucleatum* appears capable of downregulating the T-cell-mediating anti-tumor response, with CD3-cell density inversely proportional to *F.nucleatum* DNA levels in human colon tissues [86].

Another report published subsequently by Oh et al. [87] included women with LSIL or HSIL on cytology vs normal controls (defined as normal or Atypical Squamous Cells of Undetermined significance (ASCUS) cytology). The results suggested that microbiome patterns determined by paucity of *L. crispatus* and occupied predominantly by *A. vaginae* and secondarily *by G. vaginalis* and *L. iners* were associated by an almost 6-fold increase in the risk of cervical LSIL/HSIL disease (higher vs lower tertile, Odds Ratio (OR) 5.80, 95%CI 1.73–19.4), and thus the authors defined this as a ‘risky microbial pattern’, and

women were subsequently defined as being in the high, medium or low tertile according to relative abundance of the aforementioned species. The risk of SIL of women that were HPV positive and had a high-risk microbial pattern was significantly higher than HPV negative women with a low risk microbial score (OR 34.1, 95% CI 4.95–284.5). The findings were limited as there was no adjustment for risk factors, while the defined comparison groups merged different grades of disease severity. The authors compared women with both low- and high-grade disease to controls that also included the presence of ASCUS cytology, women that may harbor underlying high-grade disease [88] (Table 1, Figure 1). In spite of this, the study highlights the likely protective qualities of certain *Lactobacillus* species and the probable disease-driving potential of some CST IV-associated species. There are several proposed mechanisms through which *Lactobacillus* may afford protection beyond simple inhibition of CST IV-associated anaerobic growth. CST IV is associated with higher levels of amine production compared to the *Lactobacillus* dominant CST's [89], and these biological amines are not only responsible for the characteristic malodourous discharge [90], but also result in nitrosamine production [91]. These nitrosamines, also produced by tobacco smoking, are known carcinogens [92], and most interestingly, certain species of *Lactobacillus* are known to neutralize these carcinogens *in vivo* [93]. It is plausible that *Lactobacillus* spp. not only prevent colonization of high amine-producing bacterial species, but they may also mop up these potentially carcinogenic amine derivatives, providing an additional layer of anti-carcinogenic protection. Most notably, this study by Oh et al. [87] demonstrates that *L. crispatus* appears most protective, whereas *L.iners* in contrast seems most commonly associated with disease, rather than health. This finding underscores other studies that show not all Lactobacillus species are equally protective. The differences in genetic and metabolic properties of the different *Lactobacilli* requires further investigation.

A more recent report by Piyathilake et al. [94] compared well-defined cytological groups of women with HSIL (n=340) versus LSIL (n=90); all women were high-risk HPV positive. The authors did not used the previously described CST's to classify patients according to VM structure, but used the Dirichlet multinomial mixture model to partition samples into 4 different metacommunities (Partition 1-4). Bacterial communities of predominantly *L. iners* and unclassified Lactobacillus spp. (Partition 3) had higher HSIL+ levels as compared to those with diverse taxa unclassified Lactobacillus, L. iners, Bifidobacteriaceae, Clostridiales, Allobaculum (Partition 1) (OR=3.48, 95% CI: 1.27-9.55) when adjusted for other risk factors for HSIL. The samples of women with HSIL were particularly enriched with *Lactobacillaceae, Lactobacillus, L. reuteri* and several sub-genus level *Lactobacillus* operational taxanomic units (OTUs) (effect size>2.0; $p < 0.05$) (Table 1, Figure 1). These observations in women with HSIL in particular, are somewhat contradictory to what has been shown by the two aforementioned studies, as Partition 4 (CST IV/BV-like VM) was not associated with HSIL or worse. We suggest this may arise as a result of the ethnic differences between the cohorts, as ethnicity is a significant differentiating factor in microbiome composition [34]. However, the observation that *L.iners* is associated with HSIL is interesting. As previously discussed, *L.iners* dominant VM's are most likely to transition to CST IV, which Mitra et al. [81] associated with HSIL. Additionally *L.iners* has been implicated in depletion of reduced glutathione, unlike *L.crispatus* and *L.jensenii,* which are both seen to be associated with increased levels of reduced glutathione [95], indicating *L.iners*

colonisation results in higher levels of oxidative stress, which again fits with the former hypothesis of Piyathilake et al. The authors also explored whether oxidative DNA damage associated with different microbiota influences the natural history of HPV persistence and cervical carcinogenesis. 8-hydroxy-2' - deoxyguanosine (8-OHdG) is a well characterized biomarker of oxidative stress-induced DNA damage, which has previously been shown to be elevated in SIL compared to healthy controls [96]. Whilst oxidative stress has been implicated in the carcinogenic mechanisms of microbial-induced gastric cancers [97], the authors of this study did not find a significant correlation between 8-OHdG levels, SIL status, and the VM. BV-associated oxidative stress [98] may result in the generation of reactive oxygen species (ROS) that then create double-stranded DNA breaks in the host genome, as well as the HPV episome, facilitating HPV integration and ultimately neoplastic transformation, a mechanism also employed by the HPV E6 oncoprotein [99]. It is noted the viral integration results in loss of E1 and E2 genes, which control E6 and E7 transcription. Consequently, transcription of these oncoproteins goes unchecked after viral integration leading to increased cellular proliferation, and decrease apoptosis [100].

Barriers and Limitations of the existing literature

Rapidly evolving evidence suggests the importance of the interactions between the human host, the innate immunity, the microbiome and virome in the genital tract with health and disease. Despite variations amongst the studies, the results consistently demonstrate differences in the microbiota noted in women with cervical disease that appear to correlate with the severity of the disease. Lactobacilli produce hydrogen peroxide and preserve an acid protective environment in the vagina with low pH. *L. crispatus* produces more lactic acid as compared to *L.iners* and *L.iners* is associated with increased risk of transiting from normal to abnormal CSTs [33]. Studies consistently demonstrated lower rates of CSTI (*L.crispatus*) in women cervical disease and higher rates of *L.iners* [87] or dysbiosis [81]. The reported differences across studies may be attributed to different sample collection and analysis techniques, different ethnicity, diet and genetic factors together with temporal shifts throughout the menstrual cycle and use of contraception (Table 1).

The published studies are also limited in interpretation since samples examined are in the vast majority obtained from the vagina and not the cervix. The cervical microbiota have been shown to be similar to vaginal but with lower bacterial loads [101]. Interestingly, cervicitis was associated with changes in the microbiota at the cervical level only whereas in BV, bacterial changes are seen in both cervix and vagina. This finding may explain why BV is associated with premature delivery, which is likely an effect on the cervical integrity [102]. Also, gut studies have shown that examining the microbiota from washes are quite different than those from biopsy samples. Washes or swabs often used in VM studies may not get at biofilms, which are very adherent to cells. One hypothesis regarding the development of dysbiosis is that certain bacteria create biofilms (ie. cohesive bacteria). In doing so, they create a scaffolding for other bacteria. When bacteria are dispersed (ie. not cohesive), there appears to be overall a lower bacterial load [103104]. It may be also that viruses may also co-exist favorably in these biofilms, which inhibit penetration of natural antimicrobial compounds, and possibly anti-viral compounds [105]. In addition, most studies do not observe the 3 dimensional relationships of the microbiome, which are likely representative of

functional communities which one bacteria or virus may live off of others, making these relationships even more complex [106].

Approximately one third of the high-grade premalignant lesions go on to develop invasive cervical disease, if not treated. It is plausible that women with *Lactobacillus*-depleted CST IV or *Lactobacillus iners*-dominant microbiomes are those whose lesions are likely to persist and to progress to clinically significant pre-invasive or invasive lesions. However, it is important to note that the findings of the studies to date only demonstrate a possible association between cervical precancer, persistent HPV infection and the synthesis of the vaginal microbiota; they do not prove causality. It may be that the presence of an 'unhealthy' *Lactobacillus* spp.-depleted microbiome renders some women more susceptible to HPV persistence and the development of CIN and cancer. This idea is also supported by studies suggesting that women with BV have much higher rates of sexually transmitted disease, including HPV [4174]. Conversely, it may be that HPV infection has an impact on the host's immune defences and the mucosal metabolism with an adverse effect on the community structure of the vaginal microbiome. The infection of the basal membrane of the mucosal surfaces by HPV initiates a cascade of mediated mechanism related to inflammation, activation of the mucosal immunity with pro-inflammatory cytokines, interferons, activation of macrophages and NK cells and the integration of the viral DNA. All these inflammatory processes and changes in the immune and mucosal environment may in turn impact on the vaginal microbiome [107-111]. Interestingly, this similar increase in diversity and low lactobacilli has also been associated with HIV acquisition [112]. Two studies found that incident HPV was associated with HIV seroconversion [113114]. It is plausible that the mechanism may be that HPV results in a microbiota change vulnerable to HIV acquisition.

Future directions

There appears to be a complex relationship between the host and the VM and composition of the VM may play a role in host susceptibility to HPV infection, its persistence and subsequent development of dysplastic and ultimately neoplastic lesions. The evaluation of all these interactions can be challenging, as the VM shows significant intra-individual variability, while different HPV subtypes that may be present at a given time may follow a different independent course, and these shifts in variability can be rapid. The clinical correlation of these periods of variability remain unknown, therefore it remains essential to include functional assays of pathogenesis such as proteomics, metabolomics and peptidomes. Cancer conversely, is a slow-growing disease and thus future longitudinal studies must be very precisely designed in order to determine a causal link, and this is reviewed by Thomas et al. [115]. Further mechanistic studies are now required to gain a deeper understanding of this relationship with a view to development of future therapeutic strategies. HPV infection is cleared from the body by a predominantly innate immune response, which remains incompletely understood [18]. It is entirely plausible that the VM is able to signal through the cells of the innate immune system, which reside in the cervix, altering this immune response rendering an individual more susceptible to HPV infection, and negatively affecting a subsequent response to clear the viral infection--this interaction requires closer investigation. Furthermore, the cervical epithelial surfaces are known to secrete small antimicrobial peptides, which are often referred to as the 'natural antibiotics',

and several of these have potent antiviral activity [116]. Presence or absence of particular bacterial species are certainly capable of modulating host cell activity, and thus expression, production and activity of these proteins could also be affected by VM composition. The impact of the VM on host cell function likely arise due to changes in the metabolic environment, due to the production and utilization of numerous compounds by the VM, which can result in suppression or activation of cell signaling and metabolic pathways. Metabonomic studies using analytical chemistry techniques such as nuclear magnetic resonance (NMR) and mass spectroscopy (MS) are ideal methods for identification and quantification of the changes in the metabolic milieu to study the association of VM composition and health and disease states [117].

Many studies of the VM focus on the gross structure of the VM, however it is possible that particular species are more heavily involved in disease initiation and progression than others as suggested by both Mitra et al. [81] and Oh et al. [87]. It is widely accepted that not all strains of a particular species are pathogenic, and *Escherichia coli* is a well-established example of this, with strains such as O157:H7 causing enterohaemorrhagic colitis due to the toxin-coding genes in their genome [118], whereas other strains do not contain these genes, and do not cause significant disease. Therefore in the case of the VM, it may be the case that only certain strains of *L.iners* predispose to HPV acquisition and persistence, or conversely only certain *L.crispatus* strains that are protective. Metagenomics is an exciting new field that can be used to study the impact of strains, and identify particular bacterial genes that are associated with cervical pathogenesis.

To add another dimension to the picture, we must also consider the impact that the virome may play in this interaction. Although we are well aware that HPV causes these dysplastic and cancerous lesions of the cervix, whole genome shotgun sequencing has been used to show that there are many other types of virus in the human vagina [119]. It is conceivable that these viruses also play a role in the dynamics of the cervicovaginal environment and should also be incorporated into longitudinal studies..

Taking this information forward to the bedside, there are several avenues that could be pursued for translational application. Firstly, if a particular bacterial species or strain is found to be implicated in disease it may be possible to develop rapid bedside tests, using either microchip array or metabonomic technologies for identification of patients at highest risk, which could be used to triage those patients in need of more intense observation or treatment. Secondly, it is possible to manipulate the vaginal microbiome using probiotics. This has been successfully demonstrated as a way of both treating BV, and reducing recurrence [120]. There are currently numerous commercially available probiotics, however further studies are required in order to determine exactly which preparation is the most effective and protective with regards to clearing HPV and preventing cervical dysplasia and neoplastic transformation. At present there are a lack of medical therapies for treatment of HPV infection and SIL and the current gold standard treatment is surgical ablation or excision of the dysplastic area of the cervix, which although very effective for reducing the risk of future invasive cancer, is associated with significant obstetric morbidity [121122] as well as neonatal morbidity and mortality [123]. Such a development would be a major breakthrough in the field of gynaecological oncology, particularly in developing countries where the HPV

vaccine is infrequently available and gynaecologic procedures unavailable, and even in the numerous developed countries where vaccine uptake is poor.

Conclusion

There is a wealth of emerging evidence to suggest that the cervico-vaginal bacterial population plays a substantial role in the persistence of the virus and the presence of subsequent cervical pre-invasive disease. The role of the microbiome in other HPV-related cancers such as vulvar, anal and oropharyngeal cancers has not yet been explored but likely play important roles. Future studies assessing the impact of the vaginal microbiome in cervical carcinogenesis should explore the causal link in longitudinal samples from existing biobanks that will allow correlation of the microbiome to the clinical outcome and in particular progression or regression of HPV infection and cervical disease. These studies should seek also incorporate measures of microbiome byproducts (i.e. metabolomics), which may be associated with immune dysfunction and cell dysregulation.

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Abbreviations

8-OHdG	8-hydroxy-2' -deoxyguanosine
ASCUS	Atypical squamous cells of uncertain significance
BV	Bacterial vaginosis
CST	Community state type
HNCF	Human normal fibroblast-like cervical
HPV	Human Papillomavirus
HSIL	High-grade squamous intraepithelial lesion
IUD	intrauterine device
LSIL	Low-grade squamous intraepithelial lesion
MPA	Medroxyprogesterone acetate
NGS	Next-generation sequencing
OC	Oral contraceptive
OTU	Operational taxonomic unit
SIL	Squamous intraepithelial lesion

STI	sexually-transmitted infection
VM	Vaginal Microbiome

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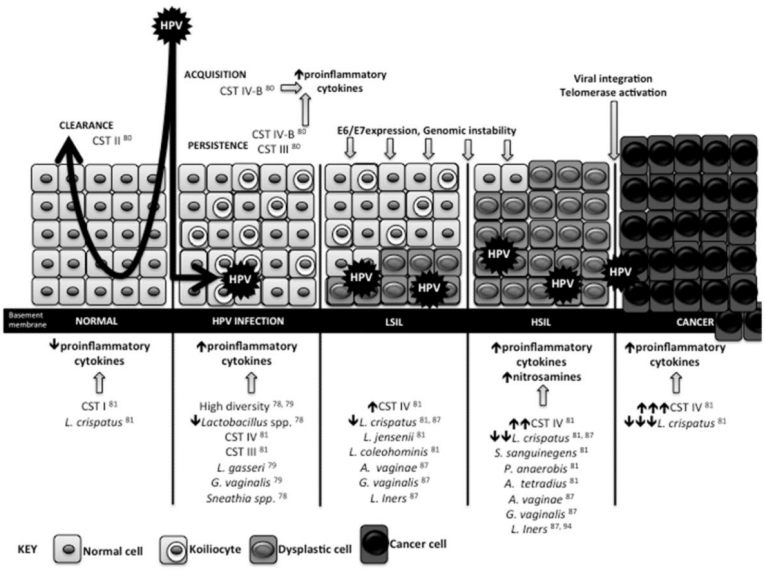

Figure 1. Interactions between the HPV, the vaginal microbiome and the host

CST I and a high relative abundance of *L.crispatus* may be protective against HPV acquisition. Longitudinal studies have shown that transition states such as CST III and a BV-like state, CST IV-B, likely lead to pro-inflammatory states which cause tissue damage and promote E6/E7 expression, genomic instability and viral integration which ultimately promotes development of HSIL. Increasing lack of *L.crispatus* has also been associated with increasing SIL severity, and various other species have been associated with both presence of HPV infection and SIL disease states.

Table 1

Characteristics of studies exploring the association of HPV infection and cervical pre-invasive and invasive cervical disease to the vaginal microbiome using next generation sequence techniques.

Study	Population	Cervical Sample	NGS technique	Key Findings
HPV infection				
Gao 2013	70 healthy women with normal cervical cytology -32 HPV-negative - 38 HPV-positive, infected with a single high-risk HPV subtype	Sterile swab from near the vaginal fornix and Cervix; HPV DNA test: primers MY09/MY11 and GP5+/GP6+ and Hybrid Capture	Nested PCR of 16S rRNA and PCR-denaturing gradient gel electrophoresis; plasmid cloning and sequence identification via NCBI BLAST database	-Increased diversity in HPV-positive (mean=1.64; range=0-3.09) compared to HPV-negative women (mean=0.93; range=0-2.62) (p <0.001) - Lactobacillus: the most predominant genus, detected in all women -L. gasseri & G. vaginalis: isolated more in HPV-positive than HPV-negative women (p=0.005 and p=0.031, respectively)
Lee 2013	68 HPV-infected or uninfected female twins and their families - 9 HPV infection-discordant MZ twin pairs without CIN (N = 18) - pre-menopausal women with or without HPV infection (N=45)	Cervical liquid-based cytology samples: ThinPre and Surepath™); HPV DNA test: primers MY09/MY11 and GP5+/GP6+, PCR amplicons of 450 and 150 bp and HPV typing (high vs. low risk); QIIME	Genomic DNA extraction: DNA/RNA kit (Chemagen, Baesweiler, Germany); V2 and V3 regions of the 16S rRNA genes; 454 Life Sciences FLX Titanium machine (Roche, Indianapolis, IN, USA)	-HPV-positive women higher microbial diversity with a lower proportion of Lactobacillus spp. than HPV-negative women -Fusobacteria, including Sneathia spp., possible microbiological marker associated with HPV infection -HPV-discordant MZ twins: reduced abundance of L. iners in HPV infection positive vs negative (P = 0.03)
Brotman 2014	32 women of reproductive age over 16 weeks twice weekly samples (937 microbiome samples - 930 HPV result) - 5 HPV negative throughout - 2 positive for 1 HPV subtype - 25 were positive for 2 or more HPV subtypes	Cervical samples: mid-vagina self-samples; HPV DNA test: Roche Linear Array HPV Genotyping Test (6, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68)	V1-V2 hypervariable regions of 16S rRNA genes using primers barcoded 27F and 338R	-CST was associated with remission of HPV (P=.008), but not with new detection of HPV (P=.10) -Low Lactobacillus CST IV-A higher transition to HPV positivity compared to CST I (aTRR: 1.86, 95%CI .52–6.74) -L. gasseri (CST II) had the fastest HPV remission and low Lactobacillus community with high proportions of the genera Atopobium (CST IV-B) had the slowest rate compared to L. crispatus (CSTI) (aTRR: 4.43, 95%CI 1.11–17.7; aTRR: 0.33, 95%CI .12–1.19, respectively)
SIL and ICC				
Mitra 2015	169 women: - normal (n=20)	Sample posterior fornix: BBLTM CultureSwabTM containing liquid Amies (Becton Dickinson, Oxford, UK); HPV DNA test: Abbott RealTime HR HPV assay (Abbott M2000 platform)	Whole-Genomic bacterial DNA extraction: QiAmp Mini DNA kit (Qiagen, Venlo, Netherlands); V1-V2 hypervariable regions of 16S rRNA genes; llumina MiSeq SOP Pipeline	- Higher rates of CST IV (L.depleted, high diversity) with increasing disease severity (Normal=10%; LSIL=21%; HSIL=27%; ICC=40%). - Lower rates of CST I (L. crispatus-dominant) with increasing disease severity (Normal=50%; LSIL=42%; HSIL=40%; ICC=20%)

Study	Population	Cervical Sample	NGS technique	Key Findings
HPV infection				
				- HSIL vs LSIL: higher levels Sneathia sanguinegens (P < 0.01), Anaerococcus tetradius (P < 0.05), Peptostreptococcus anaerobius (P<0.05) - lower levels L. jensenii (P<0.01).
Oh 2015	Cases with CIN: - CIN 1 (n=55) -CIN 2 or 3 (n=15) Controls: -Normal cytology (n=25) -ASCUS (n=25)	Cervical Sampler Brush (Digene Co. Gaithersburg, MD, USA); HPV DNA detection: Digene Hybrid capture II DNA Test (Qiagen, Gaithersburg, MD, USA)	DNA extraction: Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA); V1–V3 regions of the 16S rRNA gene; a Roche/454 GS Junior system (Roche, Branford, CT, USA); excluded low-quality reads: average quality score <25 or a read length <300 bp; available in the EMBL SRA database	The CIN risk was higher for the higher vs the lower tertile of: - predominance of A. vaginae, Gardnerella vaginalis, L. iners with a minority of L. crispatus: OR 5.80, 95%CI 1.73−19.4) -A. vaginae: OR 6.63, 95%CI 1.61–27.2). -Risky microbial pattern and oncogenic HPV:OR 34.1, 95% CI 4.95–284.5 (synergistic effect).
Piyathilake 2016	Cases: - CIN2 (n=208) - CIN3 (n=132) Non-cases: - CIN1 (n=90) All were HR-HPV positive	Cervical mucus samples: Merocel ophthalmic sponges (Medtronic Xomed, Inc., Jacksonville, FL); HPV DNA test 13 subtypes: Roche Diagnostics Linear Array; QIIME suite and RDP database	DNA extraction: Fecal DNA isolation kit from Zymo Research; V4 segment of the 16S rDNA gene; Illumina MiSeq;	-CIN2+ higher in community types dominated by L. iners and unclassified Lactobacillus spp. vs those diverse taxa unclassified L., L. iners, Bifidobacteriaceae, Clostridiales, Allobaculum (OR=3.48, 95% CI: 1.27-9.55). -Women with CIN2+ enriched Lactobacillaceae, Lactobacillus, L. reuteri and several sub-genus level Lactobacillus OTUs (effect size>2.0; p< 0.05) - DNA oxidative damage does not mediate the effect of VM on natural history of HPV.

aTRR: adjusted transition rate ratio; A. vaginae: Atopobium vaginae; CI: confidence interval; CIN: cervical intra-epithelial neoplasia; HPV: human papillomavirus; HR-HPV: high-risk HPV; HSIL: high-grade squamous intraepithelial lesion; ICC: invasive cervical cancer; L: Lactobacillus; LSIL: low-grade squamous intraepithelial lesion; MZ: monozygotic twins; NSG: next generation sequencing; OR: odds ratio; OTUs: operational taxonomic units; SIL: squamous intraepithelial lesion; VM: vaginal microbiome