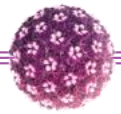
1st EDITION

PRACTICAL TRAINING

HUMAN PAPILLOMAVIRUS

PRACTICAL CASES



MODULE 2.
THE ROLE OF THE MICROBIOME AND IMMUNITY IN PHV

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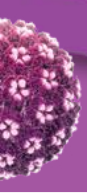
INTRODUCTION

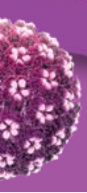
The Human papillomavirus (HPV) causes cervical intraepithelial neoplasia (CIN) and cervical cancer. Despite its high prevalence, only a small number of women experience persistent HPV infection and consequently develop clinically significant disease. The vaginal microbiota (VM) plays an important role in the health and pathologies affecting the female reproductive tract. Next-generation sequencing techniques based on the analysis of bacterial 16S rRNA genes allow in-depth study of the structure of the VM community at a level of detail unattainable with standard culture-based microbiological techniques. There are new indications that an increase in the diversity of VM, combined with a lower relative abundance of *Lactobacillus spp*. is involved in HPV transmission and persistence, as well as in the development of cervical precancerous lesions.



GOAL OF TREATMENT

The objectives of this module are:

- To summarize current knowledge on the role of VM IN HPV infection and the development of CIN and cervical cancer.
- To discuss the potential immune mechanisms involved.
- To develop the concept of manipulation of vaginal bacterial communities through the use of prebiotics and probiotics as a stimulating possibility in the field of cervical pathologies.



1. HPV AND CERVICAL CARCINOGENESIS

Cervical cancer - the most common neoplasm associated with infection - and CIN as its precancerous precursor are caused by HPV strains. There are more than 100 subtypes of HPV, of which 13 have been identified as high-risk and as causative factors of cervical cancer in 100% of cases. HPV-16 and HPV-18 are the most oncogenic and prevalent viruses and cause 70% of cancer cases. The peak incidence of infection in women is around the age of 20. It is estimated that 80% of sexually active women will be infected at some point before the age of 50. More than 90% of cases of HPV infection are transient; that is, within 6 to 18 months, the virus is eliminated by an immune response that is not fully understood, although repeat infections with the same or different HPVs may occur. Virus persistence is essential for the development of high-grade CIN and cervical cancer, and factors correlated with higher persistence rates include age, immunodeficiency, smoking, oral contraceptives, and *Chlamydia trachomatis* infection. There are new indications that the cervicovaginal microbiota plays an important role in the persistence or regression of the virus and in the subsequent appearance of the pathology.

2. THE HUMAN MICROBIOME

Bacteria account for 50% of the cells in the human body and, together with archaea and lower eukaryotes, form what is collectively known as the human microbiome[1].

It is becoming increasingly common to study the composition of the microbiota in multiple body compartments using next-generation sequencing techniques (NGS techniques). These techniques are based on the amplification, sequencing and analysis of specific areas of bacterial 16S rRNA genes. Using different bioinformatics tools and platforms, the resulting sequences are assigned to specific microbial taxa, making it possible to describe the structure of the microbial community.

The relationship between health, disease and the human microbiome is a rapidly advancing and controversial area of research. Likewise, discovering that there are different compositions of the microbiome from person to person allows us to expand our understanding of the underlying pathophysiology of a variety of diseases affecting many organ systems, from colorectal cancer to atopic dermatitis.

3. THE VAGINAL MICROBIOTA

Until recently it was believed that the lower genital tract had a low microbial diversity dominated by one (or a few) species of Lactobacillus[2]. However, there is an important part of it that host comparatively different vaginal bacterial communities[2].

Each woman's vaginal microbial profile is classified on the basis of five "community state types" (CST) which have been used in many studies[3].

CST types I, II, III and V are characterised by a predominance of *Lactobacillus crispatus, L. gasseri, L. iners* and *L. jensenii*, respectively, and tend to show low species diversity and homogeneity.

Normally, CST type IV does not include *Lactobacillus spp.* and is instead rich in strict anaerobes, including *Gardnerella, Megasphera, Sneathia* and *Prevotella.* It is often associated with bacterial vaginosis (BV), a polymicrobial infection characterised by a microbial community structure.

The structure of BV is dynamic and transitions between different types of TSA may occur. types of TSA.

4. FACTORS INFLUENCING THE COMPOSITION OF THE VAGINAL MICROBIOTA

There are numerous factors that influence the composition of the VM:

»"**Ethnicity** is a major intrinsic factor that is significantly associated with the variety of community composition, with Caucasian and Asian women showing a significantly higher prevalence of microbiota dominated by Lactobacillus spp. bacteria, compared to those of Hispanic and black origin[2].

These differences may be due to genetic factors influencing the mucosal immune system or metabolic pathways and which, in turn, would produce preferential conditions for certain species, but may also be due to different intimate hygiene practices.

» **Cultural and social factors** have an important influence on intimate hygiene practices during menstruation; douching, as shown below, is a hygienic practice for almost a quarter of American women[4], doubling the percentage among black women compared to Caucasian women.

» **Female hormones** also significantly influence both the structure and stability of vaginal microbial communities, although the mechanism of this influence has not yet been fully elucidated.

A decrease in estrogen levels during the 3-4 weeks after **birth** leads to a reduction of *Lactobacillus spp.* in the vagina and a greater diversity of species with an increased presence of strict anaerobes and enteric bacteria, which levels are maintained until puberty.

Increased secretion of oestrogen and progesterone prior to the **menarcheal** episode leads to a reduction in VM diversity and an increase in the relative abundance of Lactobacillus spp. relative abundance of Lactobacillus spp.

During a **woman's reproductive age**, fluctuation in the composition of the VM can be associated with the cyclic secretion of oestrogen and progesterone throughout the menstrual cycle. Likewise, maximum diversity and instability is observed during menstruation, when oestrogen and progesterone levels are at their lowest.

After **menopause**, it is believed that low oestrogen production and the resulting vaginal atrophy leads to a decrease in the presence of *Lactobacillus spp.* and an increase in diversity[3].

The widespread use of synthetic hormones for contraceptive purposes also influences the composition of VM. A meta-analysis has shown that **hormonal contraceptives** are associated with a 31% and 32% reduction in recurrent BV episodes and an 18% reduction in the risk of incidence[5].

» Other environmental factors also affect the composition of VM, including smoking and recent sexual intercourse, both of which are associated with a reduction in the relative abundance of *L. crispatus* and an increase in bacterial diversity. Douching - especially after menstruation - has been shown to significantly increase the risk of BV, while cessation of the practice has been shown to reduce the risk

5. THE VAGINAL MICROBIOTA AND ITS RELATIONSHIP WITH HPV, CIN AND CERVICAL CANCER

The composition of MV has been increasingly associated with cervical lesions.

BV has been linked to increased incidence, prevalence and persistence of HPV infection, as well as to the development of CIN6, although other studies have failed to confirm this correlation[7].

Douching has also been associated with an increase in HPV infections, CIN and cervical cancer[8], possibly due to the process resulting in increased bacterial diversity.

Next generation sequencing techniques (NSG techniques) have made it possible to further study the relationship between VM and cervical cancer. Detailed characteristics of the studies and their results are shown in table 1. The grouped findings are summarised below:

» HPV-positive women have a higher vaginal bacterial diversity and significantly lower presence of *Lactobacillus spp*. compared to uninfected women[9].

» "Sexually active and premenopausal women with CST III and IV are more likely to be HPV-positive (71% and 72%, respectively). Also, in women with CST II, with a predominance of *L. gasseri*, it may be associated with a more rapid clearance of acute HPV infection[10]. Such an observation could point to *L. gasseri* as a potentially therapeutic species for maintaining a healthy cervix, although further longitudinal studies are needed. healthy cervix, although more longitudinal studies are needed to confirm this.

» Most studies in women with CIN (and/or invasive cervical cancer)[11-13] show that the more severe the CIN, the higher the diversity of MV and the lower the proportion of *Lactobacilluss spp*. Women with high-grade CIN have much higher levels of *Sneathia sanguinegens, Anaerococcus tetradius* and *Peptostreptococcus anaerobius*, and lower levels of *L. jensenii*, compared to those with low-grade CIN. The presence of *Anaerococcus vaginae, Gardnerella vaginalis* and *L. iners*, together with the absence of L. crispatus, represents the combination of highest risk for developing CIN. Only one study did not show this correlation[14].

All four studies on patients with CIN[11-14] were observational studies and, in the absence of longitudinal data, only longitudinal longitudinal data, we can only demonstrate a relationship with pathology states and not causality.

This represents one of the current limitations of ongoing research on the "oncobiome", that is the microbiota associated with cancer development[15]. Much work remains to be done to understand the sophisticated relationships between host, microbiota and carcinogenesis. However, if a causal link could be established, it would have a huge impact on potential therapeutic strategies involving the manipulation of VM to rule out disease-causing species or structures and identify those associated with health and disease protection.

Table 1. Studies examining the association of HPV infection and cervical cancer with VM using next-generation sequencing techniques. Modified from Mitra *et al.*[16]

Study	Summary of results	Characteristics of the study
Lee *et al.* 2013[9]	• HPV-positive women = higher diversity and lower proportion of *Lactobacillus spp*. compared to HPV-negative women. • *Sneathia spp*. = microbiological marker of HPV infection. • Lower *L. iners* count in HPV positive compared to HPV-negative monozygotic twins with discordant HPV.	**Participants:** 912 women who took part in the Healthy Twin Study as part of an epidemiology study on the Korean genome.
Brotman *et al.* 2014[3]	• CST significantly associated with HPV remission. • CST IV-A: higher transition to HPV-positive compared to CST I. • CST II: Faster remission of HPV infection compared to CST I. • CST IV-B: slower remission of HPV infection compared to CST I.	**Participants:** premenopausal women with sampled twice a week for 16 weeks as part of a study on the discontinuation of douching.
Mitra *et al.* 2015[11]	• CST IV associated with greater severity of the disease. • CST I negatively associated with greater severity of disease. • Higher levels of *S. sanguinegens, A. tetradius, P. anaerobius* associated with HSIL compared to LSIL. • Lower levels of *L. jensenii* associated with HSIL compared to LSIL.	**Participants:** 169 premenopausal women attending the colposcopy clinic.
Oh *et al.* 2015[13]	Higher risk of CIN for the highest tercile compared to the lowest tercile of: • Predominance of *A. vaginae, G. vaginalis, L. iners* with minority of *L. crispatus.* • Predominance of *A. vaginae.* • Microbial risk pattern in the presence of oncogenic HPV.	**Participants:** 120 premenopausal women who attended a gynaecological oncology clinic.

Piyathilake *et al.* 2016[14]	• *L. iners* and unclassified *Lactobacillus spp.* associated with higher rates of CIN2+ compared to different taxa of *Lactobacillus spp, L. iners, Bifidobacteriaceae, Clostridiales* and unclassified *Allobaculum.* • *Lactobacillaceae, Lactobacillus, L.reuteri* and various OTU of *Lactobacillus* subgenera higher in women with CIN2+ compared to women with CIN1. • Oxidative DNA damage not correlated correlated with VM structure.	**Participants:** 430 women with high-risk HPV between 19 and 50 years who attended a colposcopy clinic.
Audirac-Chalifour *et al.* 2016[12]	• VM diversity significantly higher in CIN and compared to normal controls and HPV-negative women. • Predominance of *L. crispatus* and, *L. iners* in normal female controls. • Predominance of *Sneathia spp.* in women with CIN. • *Fusobacterium spp.* in women with ICC. • Higher mean levels of IL-4 and TGF-β1 mRNA in VM with *Fusobacterium spp.*	**Participants:** 32 women between 22 and 61 years old with samples taken from a biobank, collected from the Gynecological Service of the National Cancer Institute.

A. vaginae: Atopobium vaginae; *CIN: cervical intraepithelial neoplasia; HPV: human papillomavirus; HSIL: high-grade squamous intraepithelial lesion; ICC: invasive cervical cancer; L: Lactobacillus; LSIL: low-grade squamous intraepithelial lesion; UTO: operational taxonomic units; MV: microbiota vaginal; CST: community state types.*

6. POSSIBLE MECHANISMS AFFECTING CERVICAL HEALTH AND PATHOLOGIES OF THE CERVIX, AS MEDIATED BY MV

Recent observational transversal studies support the concept that type III and, in particular, type IV TSCs are often associated with the presence of HPV infection and with the development of invasive cervical cancer stages[9-11].

Although microbial diversity is considered to be a sign of good health in many parts of the body, a high diversity of VM is often atypical or in a phase of dysbiosis and is associated with pathological conditions. However, there are insufficient studies detailing the extent to which VM could play a pathological role and therefore further mechanistic studies are needed.

Lactobacillus spp. vaginal prevents the colonisation of bacterial species associated with BV by maintaining a low pH and producing bacteriocins. When BV-associated strict anaerobes colonise the area, they produce enzymes and metabolites, which may compromise this barrier by facilitating the entry of HPV. They also act in several cellular pathways that may facilitate persistent viral infection and, consequently, the development and progression of pathology (Fig. 1).

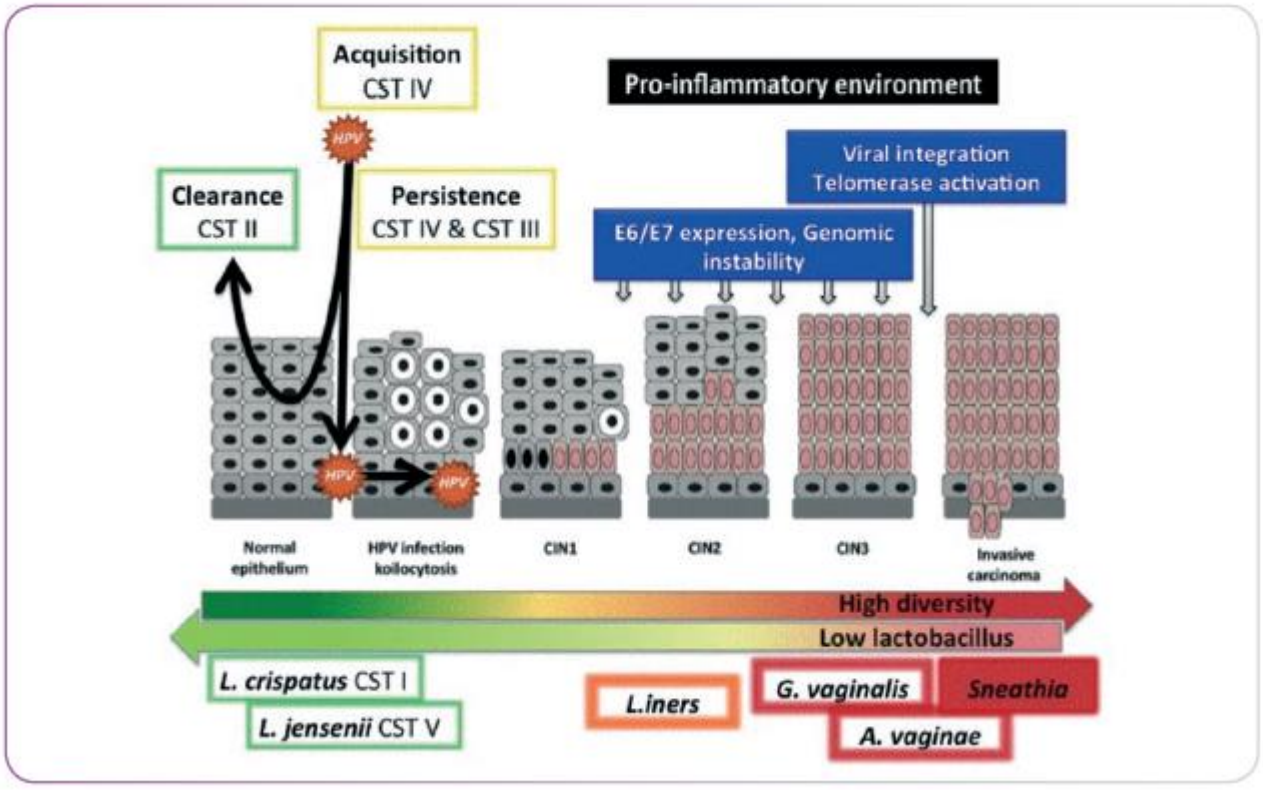


Figure 1. Possible mechanisms linking VM to cervical cancer. Modified from Kyrgiou *et al.*[17]

» **Vaginal pH, lactic acid and hydrogen peroxide.** It is well known that *Lactobacillus spp.* expresses enzymes capable of fermenting glycogen - which is present at high levels in oestrogenised cervical and vaginal epithelium - thus producing large amounts of lactic acid and, as a result, generating a low pH.

- A vaginal pH above 5 has been shown to be significantly associated with a 10-20% increased risk of testing positive for HPV in premenopausal women[18]. Furthermore, the HPV E5 protein responsible for viral transformation is known to be particularly susceptible to low pH. Previous studies have shown higher rates of BV in women with lower vaginal levels of hydrogen peroxide (H_2O_2)-producing bacteria.

- Unlike most *Lactobacillus spp.*, *L. iners* cannot produce H_2O_2, although it has also been shown to have antibacterial and antiviral properties.

- The observation that *L. iners* is often predominant when HPV[10] and CIN[13,14] infection is present could also be related to the relative instability of CST IV, compared to other CSTs predominate by *Lactobacillus spp.*, thus allowing the growth of strict anaerobes that eventually give way to the transition to the transition to CST type IV, which is commonly associated with intracervical neoplasia[11-13].

» **L. crispatus.**

- It is associated with a healthy state and rarely coexists with other bacterial species in an ecosystem, so it tends to be either strongly predominant or, conversely, non-existent.

- It is the species least likely to evolve into type IV TSA. Women with this microbiota structure not only have the lowest pH of the 5 CTS[2], but are also less likely to be infected with sexually transmitted bacterial infections, herpes simplex virus type-2, HIV and HPV.

- Not surprisingly, the presence of *L. crispatus* (CST I) is negatively correlated with CIN[13].

» **Bacteriocin production**

- In addition to influencing pH, species with protective properties can inhibit pathogen proliferation by expressing bactericidal and bacteriostatic proteins such as bacteriocins[19].

- Biosurfactants are another group of peptides excreted by bacteria that can alter surface tension and thus bacterial adhesion, preventing the formation of biofilms, which in turn are related to the proliferation of pathogenic anaerobes and in particular *G. vaginalis*.

- There is insufficient evidence to suggest that *L. iners* produces many of the protective peptides mentioned above, which could explain the high transition rates observed between microbial communities dominated by *L. iners* and *C. vaginalis* and type IV CST.

- Bacteriokines and microbial biosurfactants can disrupt viral infiltration, although further studies are needed to understand the mechanisms and relevance of HPV infection.

» **Disruption of epithelial and mucosal integrity may facilitate viral entry.**

- Impairment of the vaginal epithelial barrier may be an important driving mechanism for infection. Vaginal dysbiosis leads to disruption of key cytoskeletal proteins in the vaginal epithelium, which damages the epithelial cells and causes shedding. It is this change that would facilitate the entry of HPV into the basal epithelial cells of the cervical transformation zone where the virus progresses and ultimately where CIN develops[20].

- The next stage in viral persistence is replication and shedding of viral particles:

  - BV is associated with increased spread of HIV and HSV-2. In addition, *G. vaginalis* has been shown to induce HIV replication in vitro. It is therefore plausible that a similar mechanism may exist for HPV and that dysbiosis, a paucity of *Lactobacillus spp.* or a combination of both factors creates an environment that facilitates the viral life cycle, persistence of infection and, ultimately, the development of intracervical neoplasia.

  - Dysbiosis can also result in decreased mucus production[20] and a consequent decreased virus trapping by antibody capture, as well as increased exposure of the cervical epithelium.

    Sialidases are a family of mucin-degrading enzymes that are produced mainly by *Prevotella* and *Bacteroides spp.* These enzymes are also found at significantly higher levels among women with BV.

  - There is clinical evidence that, in addition, chronic inflammation plays an important role in cervical pathology induced by BV, as there is a higher level of pro-inflammatory cytokines in women with dysbiosis.

» **Oxidative stress**

- Dysbiosis also results in high levels of oxidative stress, which can generate reactive oxygen species that consequently cause breaks in the double-stranded DNA in both the episomal HPV and the host genome, thus assisting HPV integration and ultimately transformation into neoplasia.

- The HPV E6 oncoprotein is also known to employ this mechanism, resulting in the loss of E1 and E2 genes and, consequently, uncontrolled transcription of E6 and E7 that facilitates increased cell proliferation and reduced apoptosis.

- However, Piyathilake *et al.*[14] have not highlighted any significant relationship between VM composition and oxidative stress-induced DNA damage.

» **Cellular targets and a role for specific bacterial species.**

- It is currently unclear whether dysbiotic vaginal microbial communities act synergistically with HPV to manipulate cellular targets such as the p53 gene, pRB, survivin and hTERT, or whether this happens independently.

- However, there are indications that certain species are likely to play a pathological role in HPV transmission and persistence, rather than global dysbiosis:

    - Bacillus *G. vaginalis* is commonly found in CST type IV[2] and is often present in high proportions in the cervix of teenage girls, highly susceptible to HPV.

    - Likewise, the bacterium *Sneathia spp.* has often been identified in association with HPV infection[9] as well as with CIN and cervical cancer[11,12]. *Sneathia spp.* belong to the genus *Fusobacterium* that produces the adhesin FadA, a virulence factor that can activate the WNT, a key cell survival and proliferation pathway that has been found to be dysregulated in cervical cancer.

7. PROBIOTICS, PREBIOTICS AND HPV

The World Health Organization defines **probiotics** as "live micro-organisms which, when administered in sufficient quantities, provide health benefits to the host".

» They have been successfully used as an adjunct to traditional antibiotics (metronidazole and clindamycin) to treat BV and prevent recurrent episodes, due to their ability to restore *Lactobacillus spp.* depletion.

An oral preparation of *Lactobacillus rhamnosus* GR-1 in combination with *L. reuteri* RC-14 has been shown to increase the prevalence of *Lactobacilli* at the vaginal level, as well as prevent the number of BV recurrences, when administered in combination with metronidazole[21]. Since none of the above are endogenous vaginal bacteria, it is indicated that *Lactobacilli* may modulate the vaginal microbial structure through a poorly understood mechanism.

However, as after successful treatment with metronidazole, this special preparation has been associated with a relative increase in *L. iners*, as demonstrated by the use of NGS techniques.

The fact that *L. iners* has sometimes been associated with pathological states should be taken into account in the design of probiotic treatments to ensure that they do not favour the predominance of this particular type of *Lactobacillus*.

» "Probiotics have also been suggested to help eliminate HPV, based on in vitro and in vivo studies.

Treatment of SiHA cells (an HPV-16 infected cell line) with *Bifidobacterium adolescentis* significantly reduced the production of E6 and E7 oncogene mRNA. This suggests that these species may become a novel treatment for virus-transformed cells[22]; however, their efficacy as probiotics has not been tested in humans yet.

L. gasseri has been associated with rapid clearance of those incidentally infected with HPV[10]. This species, along with *L. crispatus*, has also been shown to be cytotoxic to HPV-18 infected cervical cells (HeLa cells), but not to healthy cell lines.

In HPV-infected women with low-grade cervical lesions, oral treatment with *L. caseii* has shown a higher clearance of HPV infections (29% vs. 19%) and a significantly higher likelihood of clearing cervical lesions (60% vs. 31%) compared to the untreated sample[23].

Prebiotics are non-digestible carbohydrates with fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) that promote the multiplication of healthy bacteria already present in the body

- Although they have been extensively studied in the gastrointestinal tract, there are some promising *in vitro* and *in vivo* studies in the vagina. FOS and GOS have been shown to promote the growth of *L. crispatus, L. jensenii* and *L. vaginalis in vitro*, but not *Candida albicans, E. coli* or *G. vaginalis*.

- In human studies, GOS, applied as an intravaginal gel immediately after metronidazole treatment for BV, have shown a significant reduction in the Nugent's test between days 8 and 16 of treatment[24].

- Similarly, konjac glucomannan hydrolysates (GMH) have been shown to promote colonisation

of *Lactobacillus spp*. in women with *C. albicans* infection. When co-administered with probiotics in a symbiotic preparation, they can enhance the growth of probiotic species and the production of their bacteriocins.

Although further studies are needed to understand the mechanisms through which MV plays a role in the pathophysiology of cervical cancer, on the one hand, and, on the other, to identify those species or strains, and their therapeutic doses, that are most protective against intraepithelial injury and cancer due to HPV infection, prebiotics and probiotics allow us to offer a more practical, affordable and safe intervention that in turn and safe intervention that, in turn, has fewer side effects than local treatment for reproductive morbidity[25-27].

8. FUTURE STUDIES

The ability to extract a causal relationship between VM and HPV infection and CIN/cervical cancer is limited by the cross-sectional nature of most studies that have been conducted in this field. This difficulty is compounded in turn by the slow natural progression of the disease, where we see that it can take years or even decades for an acute HPV infection to progress to high-grade CIN. In addition to the above, there are several confounding factors that may influence the results, including smoking and vaginal intercourse without the use of barrier contraception, both of which are associated with decreased presence of *Lactobacillus spp*. These confounding factors should be clearly documented in future evaluations. Therefore, properly stored samples in biobanks represent a valuable asset that can provide us with the opportunity to conduct longitudinal studies to help us answer these types of questions.

Prebiotics and probiotics undoubtedly represent an attractive and novel therapeutic approach to treat cervical cancer, which could also have an impact on other areas of women's health, such as premature births, miscarriages and HIV transmission. Therefore, the importance of investing both time and resources to explore this therapeutic strategy should be emphasised.

Alongside bacterial microbiota, human viroma is already an emerging field of interest. Although for years we have believed that HPV is the aetiological agent associated with precancerous and cancerous pathogenesis of the cervix and lower genital tract, there are other general viruses present in the normal vagina that, together with viruses of the Papillomaviridae family, may be involved in disease progression[28]. We are also aware that there is a symbiotic relationship, we are aware that there is a symbiotic relationship between the bacterial and viral communities that requires further study with a specific focus on HPV and cervical pathologies.

9. CONCLUSIONS

MV appears to play an important role in the transmission and persistence of HPV in the human vagina, as well as in the subsequent development and progression of CIN.

There are data suggesting that the higher the diversity of MV and the lower the proportion of *Lactobacillus spp.* the more severe the pathological situation in the cervix.

It is important to continue longitudinal studies to increase the evidence for this relationship.

The results of these studies may represent an opportunity to develop novel therapeutic agents in the form of probiotics that prevent HPV infection, promote HPV clearance in infected women and eliminate the risk of cervical dysplasia and future fertility problems resulting from its treatment[25,26].

Mechanistic studies are needed to identify the species of bacteria that offer the greatest protection. It is also possible that there are only certain strains of bacteria that may benefit or protect against certain pathological processes.

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RECOMMENDATIONS FOR TAKE HOME

MODULE 2. THE ROLE OF THE MICROBIOME AND IMMUNITY IN HPV

» There is evidence that the vaginal microbiota plays a role in the transmission and persistence of HPV in the human cervix. and persistence of HPV in the human cervix.

» More longitudinal studies are needed to increase the evidence that the more diverse the evidence that the higher the diversity of the VM and the lower the proportion of *Lactobacillus spp.* the more severe the pathological situation in the cervix.

» A better understanding of the mechanism of VC will allow us to identify those species that offer greater protection. Those species that offer the most protection.

» These data may represent an opportunity to develop novel therapeutic agents in the form of probiotics that prevent HPV infection, promote HPV clearance in infected women, and eliminate the risk of cervical dysplasia.