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The Effect of Vitamin D3 on Human Papilloma Virus Patients

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Abstract: The most common sexually transmitted disease in postmenopausal women is human papillomavirus genital infection. Vitamin D deficiency has been associated to HPV infection persistence and the development of cervical pre-invasive lesions. The current study's aims were to detect HPV virus using RT-PCR and show a link between HPV infection and its influence on vitamin D3 levels, BMI, and age groups who are more infected than others. From November 2022 to February 2023, 100 women were recruited from Al-Elwiya Maternity Teaching Hospitals and the Central Health Laboratory in Baghdad. The samples were separated into two groups: those with various gynecologic disorders (patients) and those with normal Pap smear results (control). Cytology, hematology, and hormonal testing were performed on the samples. A study of 50 women with cervical anomalies discovered that 80% were negative for HPV testing, whereas 20% were positive. Also there were nine genotypes discovered among HPV infected patients. Regarding D3, the data suggest that (70%) about 7 of the infected individuals had levels equal to or lower than 30ng/mL. and around 30% (3) of the total ten infected patients were greater than 30 ng/ml. The age distribution of the infected patients was 40% under 30, 60% 30-50, and 30% greater than 30ng/mL. BMI measurements revealed that 80% of patients were overweight, whereas the remaining 20% were obese. The total HPV infection incidence changes modestly between age groups before and after menopause, with the highest rate occurring between the ages of 30 and 50. Vitamin D concentrations Low 25(OH) D levels in blood have been associated to impaired innate immune response and increased vulnerability to HPV infection. Obesity causes a hormonal tangle, reducing immunity and increasing the chance of HPV infection.

Keywords: HPV detection, RT-PCR, women age, D3 level, Body Mass Index.

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Introduction

One of the most frequent sexually transmitted viral illnesses in the world is human papillomavirus genital infection. High-risk HPV genotypes (16, 18, 31, and 45), intermediate-risk (33, 35, 39, 51, 52, 56, 58, 59, and 68), and low-risk (6, 11, 42-44). Clinical observation, Pap smear cytology, electron microscopy, and immunocytochemistry have all been used to diagnose clinical or subclinical HPV infection, but these methods have some limitations, including non-standardization and subjectivity, insufficient sensitivity, and low predictability. The most promising method of HPV diagnosis is the direct detection of DNA from high-risk human papillomaviruses using polymerase

chain reaction. There has been an increase in the identification of human papillomavirus (HPV) infection in the previous decade in mid-adult women, mainly aged 45 to 50 years (1). Despite its convoluted pathophysiology, research have shown that viral infection, sexual behavior, and the number of births are key factors of cervical cancer. Cervical cancer is a serious hazard to women's health, according to recent clinical trials. High risk HPV types are linked to cancer formation and low risk types not (2).

According to scientific and clinical research, adipose tissue frequently shifts from the periphery to the abdomen, hip, and thigh during menopause. Adiposity has been associated with an increased vulnerability to viral infections such as the human papillomavirus, but central obesity has been associated with a proinflammatory state in postmenopausal women. (3).

Postmenopausal women have changed vaginal micro ecology and decreased immunity, increasing their likelihood of catching HPV and impairing the virus's prognosis and capacity to be eliminated from the body. As a result, HPV is more likely to persist and cause cervical cancer (4). Furthermore, as the population ages and menopausal women grow more common, there are more senior patients with cervical cancer. Women over the age of 65 account for 20% of all new cases of cervical cancer. However, women aged 65 to 69 who cervical cancer screening within the first three years had a lower risk of dying from the disease (5).

The steroid hormone calcitriol requires vitamin D as a precursor (1). The fact that vitamin D is a naturally occurring substance makes it a desirable substance for research on cancer prevention and treatment. The "Vitamin D/cancer hypothesis" a link between Vitamin D and the development of cancer has particularly captured the interest of academics in the last two decades. As is widely known, the persistence of the Human Papillomavirus [HPV] is a crucial element in the emergence of cervix premalignant and malignant lesions. The elimination of HPV infection in cervix squamous cells requires cellular immunity. The suppression of tumor suppressor genes as a result of HPV persistence in cervical cells is recognized as a precursor to the development of cancer. These results led us to postulate the idea it has been shown "Vitamin-D deficiency can be a cause of persistence of HPV infection and that this deficiency can cause cervical pre-invasive lesions." Atypical squamous cells cannot exclude high grade lesions (ASC-H), nor can high grade intraepithelial lesions (HSIL).

All patients in this study had HPV DNA testing to determine their HPV status as well as colposcopic examination. Patients with negative HPV DNA results but positive results for any other intraepithelial lesions in their colposcopic biopsy results, patients receiving calcium or vitamin D treatment for any reason, patients whose serum samples cannot be obtained, and patients with HPV positive patients without any signs of high grade intraepithelial lesions were also collected (6). Vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) are the two principal physiological forms in humans (7).

In older women infected with human papilloma virus, found higher

levels of adipokines. Overweight may contribute to However, the increased HPV DNA detection in perimenopausal women is questionable. Conducted a study to determine if obesity is independently associated with incident HPV DNA detection or subsequent loss of detection in perimenopausal women participating in the Natural History of HPV in Perimenopause (HIP) Study. Also examined to see if there were any changes in the relationship between obesity and HPV DNA detection based on whether the infection was recent (within the last six months) or long-term(8).

Materials and methods

Subjects

This study was carried out in Al-Elwiya Maternity Teaching Hospitals and Central health laboratory in Baghdad City, sample collected during period from beginning of November 2022 to the end of February 2023. The study included cervical samples of 100 women who were divided into two groups: i) Sub group I (Patient Group) included 50 patients who visited the Gynecology Clinic with different gynecologic problem, and ii) Sub group II (Healthy Control Group) included 50 healthy individuals with normal Pap smear findings.

Sample collection and processing

The 100 pap smears were taken from women's cervix and put it into a slides were prepared by the physician and kept in (10%) buffered neutral formalin for cytological examination. While the VTM swab (cotton swab) samples were taken from all (100) women and frozen in deep freeze, then subjected to molecular analysis including viral nucleic acid extraction and then real time polymerase chain reaction (RT-PCR). While 3 ml of blood sample were taken for every patient for gel tube for serum prepared to D3 level test, other analysis such as body mass index was prepared by question list filled with healing information from the patient.

Molecular methods

Extraction of DNA to detection of human papillomavirus by real-time PCR

Genomic viral DNA of HPV was extracted from the vtm swab taken from cervix of patient women using a viral nucleic acid extraction kit according to manufacturer's instructions (Geneaid, UKAS).

1. Lysis for cell-free samples (serum, plasma, body fluids), 200 µl sample Transferred to a 1.5 ml microcentrifuge tube. 400 µl of VB Lysis Buffer was added to the sample then mix by vortex. And incubated at room temperature for 10 minutes.
2. Nucleic acid lysis binding. 450 µl of AD Buffer was added to the sample lysate. The tube was shake vigorously to mix. VB Column was placed in a 2 ml Collection Tube. Transferred 600 µl of the lysate mixture to the VB Column and centrifuged at 14-16,000 x g for 1 minute. The flow-through was discard then the VB Column placed back in the 2 ml Collection Tube. The remaining mixture was transferred to the VB Column. Centrifuge at 14-16,000 x g for 1 minute. Discard the 2 ml Collection Tube containing the flow-through binding. The VB Column transferred to a new 2 ml Collection Tube. 400 µl of W1 Buffer was added to the VB Column then centrifuged at 14-16,000 x g for 30 seconds.
3. Wash: The flow-through was discard then placed the VB Column back in

the 2 ml Collection Tube. 600 µl of Wash Buffer was added to the VB Column. Centrifuged at 14-16,000 x g for 30 seconds. The flow-through was discarded and placed the VB Column back in the 2 ml Collection Tube. Centrifuged at 14-16,000 x g for 3 minutes to dry the column matrix.

4. Nucleic acid elution: The dried VB Column was place in a clean 1.5 ml microcentrifuge tube. 50 µl of RNase-free Water was added to the center of the VB Column matrix. And it was let stand for at least 3 minutes to ensure the RNase-free Water is absorbed by the matrix. Centrifuged at 14-16,000 x g for 1 minute to elute the purified nucleic acid.

Genotyping of human papillomavirus by real-time PCR

1- Principle of kit:

HPV Genotypes 14 Real-TM Quant is based on two major processes: isolation of DNA from specimens and multiplex Real Time amplification of 4 PCR tubes for each sample. HPV Genotypes 14 Real-TM Quant detects the most widespread and oncogenic 14 genotypes of human papilloma virus with determination of clinical significance. Since the human papilloma virus is an intracellular agent, there is need to monitor the presence of cellular material in the sample, in order to avoid false-negative results. HPV Genotypes 14 Real-TM Quant kit contains the internal control (human beta-globin gene), which allows to control the presence of cellular material in the sample. If the swab is not correctly prepared (high quantity of mucous or insufficient quantity of epithelial cells) the Internal Control will not be detected. It is known that the parameter of viral load has a prognostic value and the viral load less than 10^5 HPV genomic equivalents in the swab or 10^3 genomic equivalents for 10^5 cells is considered as insignificant and indicates the presence of transitory infection, however such level of load may have a value only in cases of treatment monitoring. Viral load of more than 10^5 genomic equivalents for 10^5 cells is considered to be important with high significance and indicates the existence of dysplastic changes or high risk of their occurrence. Quantitative detection of viral load allows to evaluate the character of the infection and to make a forecast concerning the stage of the disease (9).

2- Preparation of kit:

In biological cabinet. Desktop microcentrifuge for "Eppendorf" type tubes. Vortex mixer. Pipettes with sterile, RNase-free filters tips. 1.5 ml polypropylene sterile tubes. Disposable gloves, powderless. Tube racks. Real Time amplification: Real Time Thermal cycler. PCR Tubes. Workstation. Protocol (total volume: 25 µl, including 10 µl of DNA sample):

1. All the reagent tubes was vortexed and then centrifuged briefly.
2. Required quantity of PCR tubes/strips was prepared according to the number of clinical samples and controls; 4 tubes for each clinical sample (or half strip) and 4 tubes for each included control (or half strip).
3. 4 tubes of Reaction Mix was prepared by adding into each tube the reagents indicate as below:

Reaction Mix 1: 10 µl of PCR-mix HPV1* + 5 µl of PCR-buffer-FRT.

Reaction Mix 2: 10µl of PCR-mix HPV2* + 5 of PCR-buffer-FRT.

Reaction Mix 3: 10 of PCR-mix HPV3* + 5 of PCR-buffer-FRT.

Reaction Mix 4: 10 of PCR-mix HPV4* + 5 of PCR-buffer-FRT.

Signal in a tube in the channel is considered to be positive, if corresponding fluorescence accumulation curves cross the threshold line. The signal is characterized by the cycle (threshold CT) corresponding to the intersection of the fluorescence curve with the threshold line. The software of analysis determines the Ct value (Table 1).

Table (1): Human papillomavirus genotypes detection.

Fluorescence channel	FAM	JOY	ROX	Cy5
Reaction Mix	**Amplification product**			
CR-Mix HPV1	HPV genotype 16	HPV genotype 31	HPV genotype 18	human β- globin gene DNA (IC Glob)
CR-Mix HPV2	HPV genotype 39	HPV genotype 45	HPV genotype 59	human β- globin gene DNA (IC Glob)
CR-Mix HPV3	HPV genotype 33	HPV genotype 35	HPV genotype 68	HPV genotype 56
CR-Mix HPV4	HPV genotype 58	HPV genotype 52	HPV genotype 66	HPV genotype 51

This table explain that:

A. DNA of HPV genotypes 16, 39, 33, 58 is detected if the Ct value determined in the channel FAM did not exceed the boundary Ct value. Moreover, the fluorescence curve of the sample was crossed the threshold line in the area of typical exponential growth of fluorescence.

B. DNA of HPV genotypes 31, 45, 35, 52 is detected if the Ct value determined in the channel JOE did not exceed the boundary Ct value. Moreover, the fluorescence curve of the sample was crossed the threshold line in the area of typical exponential growth of fluorescence.

C. DNA of HPV genotypes 18, 59, 68, 66 is detected if the Ct value determined in the channel ROX did not exceed the boundary Ct value. Moreover, the fluorescence curve of the sample was crossed the threshold line in the area of typical exponential growth of fluorescence.

D. DNA of HPV genotypes 56, 51 is detected if the Ct value determined in the channel Cy5 (in the tubes with PCR-mix HPV 3 and 4, respectively) did not exceed the boundary Ct value. Moreover, the fluorescence curve of the sample was crossed the threshold line in the area of typical exponential growth of fluorescence.

E. DNA of HPV is not detected in a sample if the Ct value is not determined (fluorescence curve did not cross the threshold line or the Ct value exceed the boundary value) in the channels FAM, JOE, ROX, and also Cy5 (in the tubes with PCR-mix HPV 3 and 4), whereas the Ct value determined in the channel Cy5 fluorophore (in the tubes with PCR-mix HPV 1 and 2) did not exceed the boundary value.

F. The result is invalid if the Ct value is not determined (absent) or exceed the boundary value in the channel FAM, JOE, ROX, and also Cy5 (in the tubes with PCR-mix HPV 3 and 4), and the Ct value in the channel Cy5 (in the tubes with PCR-mix HPV 1 and 2) is not determined (absent). In such cases,

the PCR analysis was repeated starting from the DNA extraction stage.

4. Vortex briefly was done for all the prepared Reaction Mixes and spin down shortly.
5. Pipetted the 15 µl of Reaction Mix and 10 µl of DNA sample into each correspondent tube (four tubes for each clinical sample and four tubes for each control) according with (Figure 1) then the run of RT-PCR started as (Table-2).

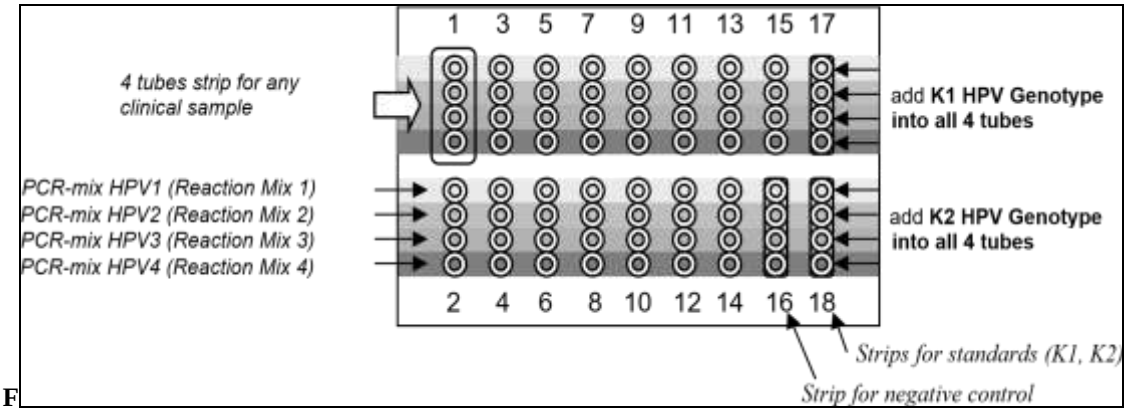

Figure (1): Sample distribution for plate type instruments (9)

Table (2): Amplified PCR program for HPV genotypes.

Steps	Temperature °C	Time	No. of Cycles
Hold	95	15 min	1
Denaturation 1	95	5 sec	5
Annealing 1	60	20 sec	
Extension 1	72	15 sec	
Denaturation 2	95	5sec	40
Annealing 2	60	30 sec fluorescent signal detection	
Extension 2	72	15 sec	

Biochemical method

Vitamin D3 level measurement

For vitamin D3 level measurement quantitative test was use according to manufacturer's instructions (Finecare, china) the test can be performed with serum or plasma following standard phlebotomy procedure:

1. Collect a venipuncture whole blood specimen was done by used a blood collection tube. If collection plasma, use a blood collection tube (gel tube) containing suitable anticoagulant (Heparin lithium, heparin or sodium citrate is recommended).
2. The serum/plasma separated from blood as soon as possible to avoid hemolysis.
3. Test performed immediately after the specimens have been collected. Specimens may be stored at 2°C～8°C for up to 4 days. For long-term storage kept below -20°C.
4. (Reconstituting) 2.5 mL detection buffer C was drawn into the VD Lyophilized Marker Bottle and shake it thoroughly.

5. (Reacting) 75 µl serum or plasma, 75 µl releasing Buffer A and 150 µl Reconstituted Marker sequentially was drawn into a blank centrifuge tube, mixed then incubated at room temperature for 10 minutes.
6. (Loading) 75 µl sample Mixture was drawn and load it into the sample well of Test cartridge.
7. (Testing) The test cartridge was inserted (10).

Body mass index measurement

As for measuring the BMI of patient's women, it was done by measuring the weight and height of each of them, and the mathematical equation from which deduced the universally approved body mass rate was applied, which is as follows: Body Mass Index (BMI) = Body weight in kilograms / Height in meters X Height in meters. It is first done by converting the unit of length from cm to meters. BMI range fit into one of 4 bands: Underweight (<18.5), healthy weight (18.5-24.9), over weight (25-29.9) and obesity (30≤)(11).

Results and discussion

The result of HPV extraction and detection according to techniques and methods used which shown in table (3).From 50 women who diagnosed with cervical abnormalities about 80% of them (40) patients were negative for HPV test. While 20% (10) of them detect with human papilloma virus with different genotypes was shown in table (3) also founded more than one HPV genotype clarified in (Table 4) (12).

Table (3): Distribution of sample according to HPV RT-PCR.

RT-PCR	No. of case	Percentage (%)
Infected	10	20%
Not infected	40	80%
Total	**50**	**100%**

Table (4): Distribution of genotyping of the study sample according to HPV RT-PCR.

Genotype of HPV	No. of case	Percentage (%)
16	4	23.52%
33	1	5.88%
35	2	11.77%
39	2	11.77%
51	2	11.77%
58	1	5.88%
59	1	5.88%
66	1	5.88%
68	3	17.65%
Total	**17**	**100%**
P value	**0.789**	

(Table 4) shown that there is 9 genotype distribute between HPV patients, genotype 16 and 68 was higher distributed between HPV patients with percentage 23.52 and 17.65% respectively (13). While genotype 35, 39 and 51 had the same percentage about 11.77%. Finally about 5.88% of 33, 58, 59 and 66 genotype was the lowest distributed genotype between HPV patients (14). Figure (2, 3) shown the result of HPV genotyping by RT-PCR curves.

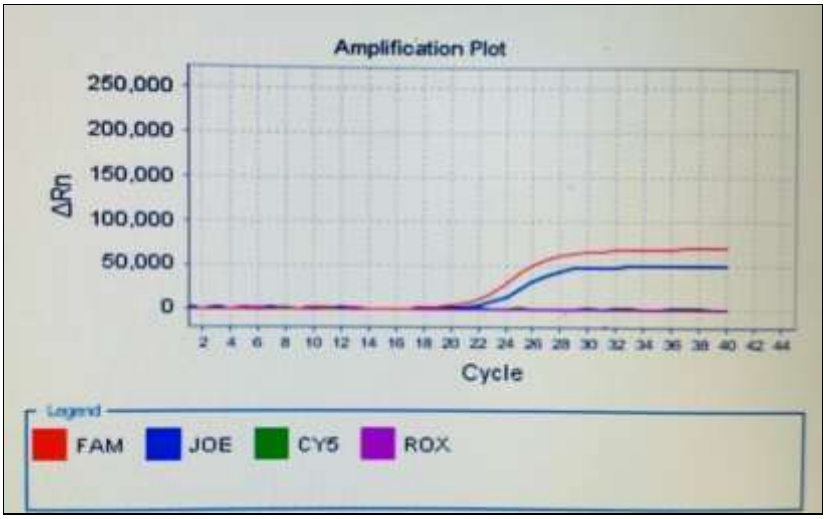

Figure (2): Blue fluorescence of JOE channel indicate genotype 35 and Red fluorescence of FAM channel indicate genotype 33.

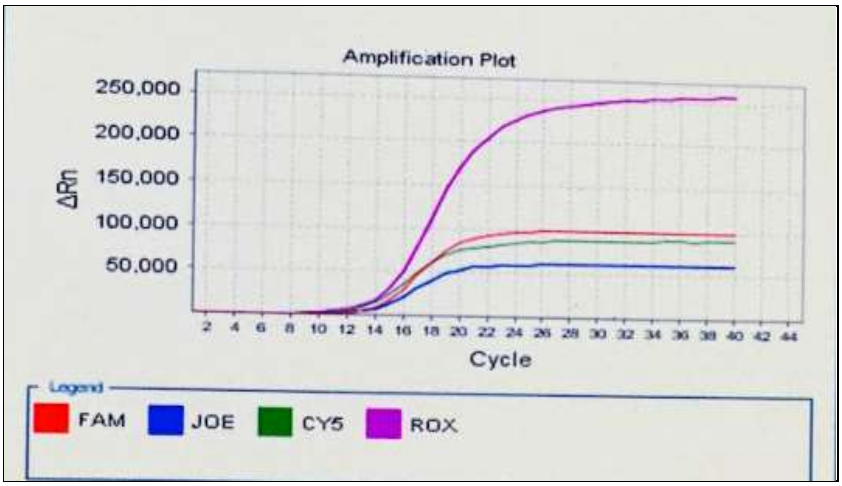

Figure (3): This figure indicate all four channel with different genotypes in high concentration the Ct reaction started in cycle 16: Red fluorescence of FAM channel indicate genotype 58, Blue fluorescence of JOE channel indicate genotype 52, Green fluorescence of CY5 channel indicate genotype 51 and Pink fluorescence of ROX channel indicate genotype 66.

Association between age of women and positive HPV

Table (5): Correlation between women age group and HPV infected patients.

Age group of infected women with HPV	Present Age	
	No. of case	Percentage (%)
Less than 30 years old	4	40%
30-50 years old	6	60%
More than 50 years old	0	0%
Total	**10**	**100%**

The 10 women who diagnosed with positive HPV test. They had different age yet clarify as: (25, 27, 27, 29, 31, 31, 31, 35, 46, and 46) years old. They distribute according to Global divisions: <30 years old, 30-50 years old and >50 years old shown in table (5). These 10 women who diagnosed with positive HPV test the result of their age was: 40% about 4 of them under 30 years old (12). While 60% about 6 of them were between 30-50 years old

(13).. While there are no one infect with HPV in age more than 50 years old there was 0% of total percentage of age group (13). These result clarified in table (5).

Table (6): Effects of vitamin D3 o HPV patients.

D3 level of infected with HPV		
D3 Level	**No. of case**	**Percentage (%)**
≤30	7	70%
>30	3	30%
Total	**10**	**100%**

Vitamin D3 level in HPV patients

The 10 women who diagnosed with positive HPV test the result of D3 measurement of them was: (4.6ng/mL, 6.1ng/mL, 12.08ng/mL, 18.65ng/mL, 23.4ng/mL, 30.42ng/mL, 30.45ng/mL, 43.52ng/mL, 58.72ng/mL and 76.62ng/mL) respectively, table (6) shows the result and percentage of every group (group 1 The normal range of vitamin D is more than 30 ng/mL, group 2 is 30 ng/mL and less than 30 ng/mL, the percentage is considered low, and these divisions belong to some global studies). Table (6) shows that about (70%) 7 of the infected patients were equal to or smaller than 30ng/mL (14). and about 30% (3) out of the total 10 infected patients were greater than 30ng/mL (15).

Body mass index measurement in HPV patients

Table (7): Effects of BMI on HPV infected patients.

BMI state	BMI of infected with HPV	
	No. of case	**(%)**
Under weight	0	0%
Healthy weight	0	0%
Over weight	8	80%
Obesity	2	20%
Total	**10**	**100%**

Women who's diagnosed with positive HPV test had result of BMI measurement was: (25kg/m^2, 25kg/m^2, 25.73kg/m^2, 26.97kg/m^2, 28.12 kg/m^2, 28.21kg/m^2, 28.3kg/m^2, 28.3kg/m^2, 31.11kg/m^2 and 33.2kg/m^2) respectively. Table (7) shows the result and percentage of every division: Underweight (<18.5), healthy weight (18.5-24.9), over weight (25-29.9) and obesity (30≤). Table (7) shows the result and percentage of overweight was the highest in comparison with other states which was 80% of 100% it was mean there were 8 of patient in this stats (16). While the Remaining 20% was obesity (30≤ kg/m^2) (17). As for the other states (underweight and healthy weight), they were 0% of the total percentage (16).

Correlation between HPV infection patients and D3 level with BMI state

Table (8): Correlation of (D3 level and BMI) in HPV patients.

Groups	No. of case	Mean ± SD	P value
D3 level			
≤30	7	17.96±10.76	0.033*
>30	3	59.62±16.57	
BMI state			
Over weight	8	26.95±1.5	0.072 NS
Obesity	2	32.16±1.48	
One sample statics			
Standard	10	27.99± 2.61	0.05

(In Table 8) clarified whether there is a correlation and significant differences between the P-values of both D3 and BMI for 10 patients who infected with human papilloma virus. In the above table the meaning of (one sample statics) was the standard statics that used as a control to distinguish whether there are significant differences or not. The normal P value is 0.05 if the value is smaller than that, it means that there is a significant value and there is a correlation between the two factors. An example of this is the p value between the infected patients (+ve HPV) of the first two groups whose D3 value was smaller or equal to 30 with Mean ± SD 17.96±10.76 and between those who Their percentage was greater than 30 with Mean ± SD59.62±16.57. And because P value of D3 is 0.03 was smaller than 0.05, so there was a significant differences between them (14). While for respect to their BMI values, their P value did not represent the presence of significant differences because p value of it was 0.072 greater than 0.05 and because of low Mean SD there was 26.95±1.5 for overweight and 32.16±1.48 for obesity (16). With 528,000 new instances of cervical abnormalities and cervical cancer recorded in 2012. It is one of the most common cancers in women around the world. Although the vast majority of HPV infections cure on their own, chronic infections with high-risk or oncogenic strains can lead to anogenital and oropharyngeal cancer. Typically, the virus infects the mucocutaneous epithelium, mature epithelial cells produce viral particles, and the consequences are a breakdown of normal cell-cycle control and promotion of unregulated cell division, which results in the accumulation of genetic damage. As of today, there are two effective preventive immunizations against HPV infection: HPV types 16 and 18, as well as virus-like particles of HPV types 6, 11, 16, and 18, have been identified (18). According to previous research on HPV infection in 20,000 women across nine Chinese provinces, the hr-HPV infection rate of over 2000 postmenopausal women (17.2%) is not significantly different from that of non-menopausal women (16.4%). In other words, while the overall HPV infection rate did not change between age groups before and after menopause, the infection rate in one age group after menopause is considerably higher than the infection rate in other age groups. Increased age, increasing loss of ovarian function, vaginal mucosa wear and tear, and a reduction or even full lack of lactobacilli could all be contributing causes to the high HPV prevalence in postmenopausal women (3). Although having adequate vitamin D has been associated to better health outcomes such as a lower risk and severity of respiratory and influenza-like infections and a lower incidence of various cancers, 40% of people in the United States do not get enough. Lower serum 25(OH) D levels have been associated to impaired innate immune activity and an increased susceptibility to infection. Vitamin D has the ability to affect immune responses. Antimicrobial peptides (AMPs) are generated in the vaginal and cervix endothelial cells and protect against sexually transmitted illnesses such as hrHPV. A lack of vitamin D may inhibit AMP production. Because of the virus's shown ability to evade adaptive immune responses, innate immunity-based HPV prevention methods based on optimal AMP synthesis and activity are particularly appealing (19).

Since human papilloma virus has an association to cervical disorders in married women, so this study shows a direct link between overweight and obese women and when infected with this virus, when they gain weight unhealthily, it leads to a mess of hormones, thus reducing immunity and thus increasing the chance of contracting HPV (20). Enumerates the 944,227 women who participated in this study's baseline characteristics. About one-third of the women were obese, and the majority were between the ages of 30 and 49. While the majority was white, compared to normal/underweight women, Women who were overweight or obese were more likely to be black or Hispanic and less likely to be Asian. Women underwent 2.9 screening tests and 4.4 years of follow-up on average; significant disparities by BMI were not seen. 6.3% of women tested positive for HPV, and regardless of age, there was a trend toward a decline in HPV prevalence with rising BMI (P trend <.001) (21).

Conclusion

HPV infects the mucocutaneous epithelium, mature epithelial cells produce viral particles, and so on to reach accumulation of genetic damage. The total HPV infection incidence varies modestly between age groups before and after menopause so, the higher rate in age between 30-50 years old. Vitamin D levels low serum 25(OH) D levels have been linked to decreased innate immune function and a higher susceptibility to HPV infection. There is a clear link between overweight women and this virus it causes a tangle of hormones, lowering immunity and raising the risk of catching HPV.

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