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RESEARCH ARTICLE

A PROSPECTIVE OBSERVATIONAL STUDY TO EVALUATE IMPACT OF ORAL SUPPLEMENTATION WITH AHCC AND *LACTOBACILLUS CRISPATUS M247* ON HPV CLEARANCE AND LOW-GRADE SQUAMOUS INTRAEPITHELIAL LESION REGRESSION

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ABSTRACT: Human papillomavirus (HPV), especially high-risk types like HPV 16 and 18, can progress from low-grade lesions (LSIL) to cancer. While HR-HPV and LSIL often regress naturally, some cases may advance to malignancy. Current treatments vary in efficacy and can have adverse effects. Emerging research on *Lactobacillus Crispatus M247* and Active Hexose Correlated Compound (AHCC) shows potential for enhancing HPV clearance and LSIL regression with minimal side effects. However, the precise impact of these treatments remains under study.

The primary endpoint is to evaluate the effectiveness of AHCC and *L. Crispatus M247* in treating women with chronic cervicitis or low-grade squamous intraepithelial lesions (L-SIL) caused by high-risk HPV. The secondary endpoint is to monitor any side effects and measure patient adherence to the treatment regimen.

This prospective observational cohort study followed 40 women with abnormal cervical cytology up to L-SIL and HR-HPV infection over 6 months. Cohort A (20 patients) underwent AHCC and *L. Crispatus* treatment, while Cohort B (20 patients), received regular follow-up without specific treatment. The study assessed the treatment's impact, controlling for age, BMI, sexual history, contraception use, and smoking habits. Key evaluations included molecular tests, colposcopy, and biopsy at the start and end of the study period, with additional monitoring of dropout and adherence rates and any side effects to determine the treatment's feasibility and safety.

With a 17.5% dropout rate (mostly COVID-related) from the initial 40 patients, no side effects were noted. HR-HPV clearance was achieved by 73.3% in Group A, versus 0% in Group B ($p < 0.001$) at the 6th month. L-SIL regressed to chronic cervicitis in 13% of Group A ($p = 0.048$), while 26.3% of Group B progressed to H-SIL, significantly differing from Group A ($p = 0.042$) at the 6th month. This observational cohort study confirms the feasibility and efficacy of AHCC and *L. Crispatus M247* supplementation for improving HR-HPV clearance and L-SIL regression, with no side effects and good adherence. Results support further investigation through randomized controlled trials and studies on the vaginal microbiota's role in cancer prevention.

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Impact statement: Combining AHCC and *L. Crispatus M247* significantly enhances HR-HPV clearance and LSIL regression, potentially reducing future oncogenesis risks in patients without adverse effects.

Key words: *Human papillomavirus (HPV); Lactobacillus Crispatus M247; Active Hexose Correlated Compound (AHCC); LSIL regression; HPV clearance.*

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INTRODUCTION

Background

HPV is a group of more than 200 related viruses, of which at least 14 are cancer-causing (also known as high-risk types). HPV types 16 and 18 are the most dangerous being responsible for most cervical cancer cases. These viruses infect the epithelial cells of the cervix (1, 2) and induce oncogenesis by two mechanisms: the E6 protein degrades the p53 tumor suppressor, while the E7 protein impairs the retinoblastoma (Rb) protein leading to the accumulation of mutations and ultimately result in malignant cell transformation (3). Low-Grade Squamous Intraepithelial Lesion (LSIL) indicates mild abnormalities in cervical cells, often related to HPV infection. While some HR-HPV infections may spontaneously clear and LSIL can regress without intervention, other cases see the persistence of HPV and progression of LSIL to cancerous states. The contemporary presence of both HR-HPV infections and LSIL reduces the chance to clear the virus infection and the spontaneous regression of LSIL (4-7). At the same time, all LSIL treatments, such as cryotherapy and laser therapy, and excisional methods like Cold Knife Conization (CKC), Loop Electrical Excision Procedure (LEEP), and laser conization, have distinct side effects and may not clear HPV entirely. Ablative methods lack specimen for further study, potentially causing discomfort and requiring multiple sessions while excisional methods vary in side effect rates, with CKC leading to 2-17% major bleeding and a 16% chance of premature birth. While laser conization and LEEP show lower rates of premature birth, it is not 0%. Equipment costs and the need for specialized training can also limit access to these treatments. This uncertainty complicates decision-making regarding treatment, especially considering that no conclusive treatment exists for the complete eradication of HR-HPV infection, which poses a risk of driving malignant transformation in other area of the cervix and genital areas (8, 9). The necessity for a new treatment approach is evident, particularly for patients newly diagnosed with HR-HPV infection, with or without LSIL, where the likelihood but not the certainty of spontaneous resolution argues against immediate, aggressive treatment due to the risk of outweighing potential benefits. The spontaneous clearance rate of high-risk HPV (HR-HPV) without any treatment varies across studies, underscoring the body's innate ability to clear HPV infections and our incomplete understanding of this process. Reported clearance rates vary, ranging from 43% after 6 months to 65% after 18 months (10, 11). Conversely, half of HR-HPV-positive women cleared the virus in a mere 7.5 months (12). Additionally, an observational study involving 1079 women showed that 46.3% cleared HPV, with a notably quicker clearance in the biopsy cohort (68.7%) compared to the baseline. This indicates that conservative management, when excluding high-grade disease via biopsy, is effective for HPV clearance and diagnostic procedures like biopsies do not impair, but rather may correlate with, an increased chance of HPV clearance (13). Existing literature identifies various factors associated with the progression and regression of HR-HPV and LSIL. However, despite the development of numerous nomograms and algorithms, there remains an inability to predict the progression of these conditions with absolute certainty. Persistent HR-HPV infection, particularly types 16 and 18, is a critical factor in the progression from LSIL to HSIL and cervical cancer (14). The immune system's response is central to controlling or eliminating HPV (15), with factors such as nutritional status, particularly levels of vitamins A, C, E, and folate, and zinc, by supporting the growth and normal functioning of lymphocytes, neutrophils, and macrophages, playing supportive roles (16). Lifestyle factors, including smoking, obesity, and physical inactivity, negatively impact immune efficiency and facilitate lesion progression (17, 18). Emerging evidence suggests strongly that HPV vaccination may encourage LSIL regression by enhancing immune response to the virus (19). Other influ-

ential factors include chronic stress, which weakens immune function (20), and specific conditions such as HIV/AID or those undergoing immunosuppressive therapy for any disease which compromise immune surveillance (21). Genetic predispositions (22) and additional cofactors like age, number of childbirths, prolonged oral contraceptive use, and co-infections with other sexually transmitted infections further affect the likelihood of LSIL progression to HSIL and cervical cancer (23). Recent studies have increasingly substantiated the significant role that vaginal microbiota plays in both the risk and progression of HPV infection, leading to HPV-related diseases. It has become apparent that the composition of the vaginal microbiota is a determinant in the trajectory of these diseases; a reduction in the prevalence of Lactobacillus species alongside a rise in microbial diversity correlates with sustained HPV infections and the development of cervical lesions. This association indicates a progression toward more severe HPV-related conditions (24, 25). On the contrary, the predominance of certain Lactobacillus strains, especially *L. Crispatus*, is associated with the regression and clearance of HPV, which suggests a protective effect against the progression of the disease (26). The *L. Crispatus M247* strain, which was isolated from the fecal material of a healthy child, is distinguished by its positive traits related to aggregation, colonization, and the modulation of inflammatory responses. The genome of this strain spans 2.1 Mbp and contains 2187 coding genes that are essential for various cellular processes, including carbohydrate and protein metabolism, DNA and RNA processing, and the biosynthesis of cell walls, capsules, and ribosomes. Remarkably, *L. Crispatus M247* includes genes that encode for Lactobacillus epithelium adhesin (LEA) and fibronectin, which are crucial for vaginal colonization and outcompeting pathogens, such as *Gardnerella vaginalis*. Additionally, this strain synthesizes exopolysaccharides (EPS) that facilitate bacterial adhesion, offer protection from environmental stressors, and display antibiotic properties. The potential of *L. Crispatus M247* for the colonization of vaginal tissues and the production of bacteriocins has been noted, particularly for its antagonistic action against specific strains of uropathogenic *Staphylococcus epidermidis* and *Escherichia coli* (27, 28). Based on current evidence, *L. Crispatus M247* is deemed safe, lacking virulence factors, and is unlikely to carry plasmids. This assessment aligns with the safety criteria established by the European Food Safety Authority (EFSA), confirming its phenotypic safety (29). According to Wan *et al.*, *Lactobacillus Crispatus* can cause cells to undergo apoptosis and inhibit the proliferation of the cervical precancerous cell line Ect1/E6E7 in a time-dependent way (30). *L. Crispatus M247* is a tested probiotic for oral administration shown to have fecal and vaginal colonizing properties (31, 32). Two clinical studies have investigated the effects of *L. Crispatus M247* on HPV clearance. The first, an open, non-controlled trial with 35 HPV-positive women, showed a 70% reduction in HPV positivity after a 90-day oral treatment with the probiotic. Notably, 94% of these women shifted to a healthier Community State Type I status (33). The second study, a randomized controlled trial with 160 women, compared the effects of long-term oral administration of *L. Crispatus M247* against a placebo. It was found that the group receiving *L. Crispatus M247* had a notably higher success rate in eliminating HPV-related cytological anomalies after six months, with 61.5% showing resolution as opposed to 41.3% in the placebo group ($p = 0.041$). Despite this, the rate of complete HPV-DNA clearance by the end of the study did not significantly differ between the two groups (34). Additionally, the study did not account for variables such as the vaccination history of the participants or whether they had single or multiple HPV genotype infections. Cultured *Lentinula edodes Mycelia* Extract also known as, ACTIVE HEXOSE CORRELATED COMPOUND, AHCC, Cultured *Lentinula edodes Mycelia* Extract, Cultured *Lentinula edodes Mycelia* Extract AHCC, ECLM, *Lentinula edodes Mycelia* Extract AHCC, *Lentinula edodes Mycelia* Extract Standardized Extract of Cultured *Lentinula edodes Mycelia* and, Yinuojin Ruanjiaonang is an orally bioavailable capsule-based formulation of a standardized extract of cultured *Lentinula edodes* (Shiitake mushroom) mycelia (ECLM), which is high in the polysaccharides beta- and alpha-glucans, with potential antioxidant, immunomodulating and antineoplastic activities. When ingested, the extract derived from cultured *Lentinula edodes Mycelia* initiates an immune response by interacting with toll-like receptors, particularly TLR-4. This interaction prompts the activation of various immune cells, including dendritic cells, natural killer cells, macrophages, and T-cells, and stimulates the production of cytokines, potentially leading to the elimination of HPV infections (35). AHCC has been the subject of research for its potential immune-modulating effects and its role in supporting cancer treatment,

improving immune responses, and possibly affecting the clearance of viral infections. A recent study by Smith *et al.* suggested that AHCC, a mushroom extract, may effectively clear high-risk HPV infections. The study included laboratory tests and animal studies, which supported the clearance of HPV, followed by two small pilot studies with women having persistent high-risk HPV. In one group, 6 out of 10 participants taking 3 grams of AHCC orally showed clearance after 3 to 6 months, while in another, 4 out of 9 participants taking 1 gram achieved clearance after 7 months. Low levels of Interferon-beta (IFNβ) were observed in those who cleared the virus, suggesting that AHCC might work by modifying IFNβ expression and signaling (36). Despite these encouraging results, the effectiveness of AHCC awaits confirmation from an ongoing phase II clinical trial and another study by Beihua Kong, as AHCC is not yet established as a definitive treatment for high-risk HPV infections (37). AHCC and *L. Crispatus M247*, both recognized for their potential benefits, have found their way into our gynecological practices due to their availability in the free market. The clinical challenge we face involves a specific group of patients: those HR-HPV infection, with or without LSIL. These individuals are not immediately recommended for conventional treatments because of the high likelihood of spontaneous regression. However, relying solely on follow-up care doesn't assure complete HPV clearance, and other treatments might be more harmful than beneficial due to their invasive nature.

Objectives

Our research seeks to address a critical question: can the combined therapy of AHCC and *L. Crispatus M247* enhance HR-HPV clearance and LSIL regression rates beyond what is seen with spontaneous recovery, without inducing side effects?

MATERIALS AND METHODS

Study design

This study is an observational, prospective, single-centre investigation. Our primary goal was to determine the effectiveness of the combined treatment of AHCC and *L. Crispatus M247*, in achieving HR-HPV clearance and in facilitating the regression of LSIL, while also closely monitoring for any progression towards more severe lesions. Additionally, we sought to assess the side effects associated with the treatment and gauge patient adherence to the regimen.

We observed the cohort of patients who decided to follow the combined treatment (Papion plus Crispact) and the matched cohort of patients who completed follow up only. To evaluate the effectiveness of this treatment, both groups underwent HPV-DNA testing, colposcopy, and biopsy after 6 months. Combined treatment included Papion 500mg capsules, derived from *Lentinula edodes*, two capsules in the morning, two after lunch, and two after dinner and Crispact, a sachet containing a minimum of 20 billion colony-forming units of *L. Crispatus M247* (IMG-P-23257), to be taken daily. Dosage and administration guidelines were established based on preliminary research and safety profiles. Written informed consent for observational studies was obtained from all participants; the protocol and other materials were in accord with the Helsinki Declaration of 1975 and approved by institutional review boards.

Setting

This study was conducted in the Eastern Sicily region, within the province of Messina, Southern Italy at a tertiary university hospital. The period of recruitment and data collection extended from July 2020 to July 2023. All procedures, ranging from data collection to diagnostic assessments were conducted at the Department of Gynecology and Obstetrics at the University Hospital "G. Martino" in Messina.

Participants

Participants for this observational prospective cohort study were recruited from women attending the outpatient clinic for cervical cancer prevention and colposcopy at our department. The inclusion criteria were limited to women diagnosed with HR-HPV and concurrent abnormal cervical cytology up to LSIL, who also consented to share their data for the observational study. Exclusion criteria were set for those presenting with HSIL, invasive cervical cancer, a history of surgical treatment for HSIL, immunosuppressive conditions, any oncological diseases, inflammatory disorders, a history of HPV vaccination, or those under 18 years old and pregnancy. To ensure a pure evaluation of the therapy's effectiveness, our study intentionally excludes individuals who have chosen to receive the HPV vaccine from both groups. This decision is based

on two considerations: the vaccine's potential to significantly boost HPV clearance and LSIL regression, and the variability of vaccine efficacy among patients due to age and comorbidities. From the eligible population, we selected two well-balanced cohorts, matched for confounding factors but significantly differing in one variable: the exposure factor, represented by the combined therapy of AHCC and *L. Crispatus M247*.

Variables

The primary endpoints were changes in HR-HPV status and cervical histological status between the two cohorts.

The secondary endpoints focused on the rate of side effects and patient compliance with the combined treatment.

Matching factors for the study consisted of age, BMI, sexual history (age at first intercourse and number of partners), use of contraception, and smoking habits (defined as more than 10 cigarettes per day).

Data Sources/Measurement

The changes in HR-HPV status and cervical histological status were determined using molecular tests, colposcopy, and biopsy. Following the detection of cytological abnormalities on routine PAP tests, HPV-DNA testing was conducted. Women with a single abnormal cytology result (including atypical squamous cells of undetermined significance (ASCUS), atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (ASC-H), or LSIL associated with an HR-HPV type) were referred for colposcopy. Cervical cells from each sample were collected by centrifugation, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Germany). This was followed by a PCR-based HPV-DNA assay and reverse dot blot genotyping (HPV-HS Bio plus HPV-strip or HPV-type, AB Analitica, Padova, Italy), which facilitated the identification of 11 low-risk HPV types (HPV-6, -11, -40, -42, -43, -44, -54, -61, -70, -72, -81) and 18 high-risk or probable high-risk HPV types (HPV-16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -68, -73, -82). Colposcopic examinations were performed by a single experienced colposcopist in accordance with the standards of the Italian Society of Colposcopy and Cervical-Vaginal Pathology (SICPCV) (38). The colposcopic findings were categorized as normal transformation zone (NTZ), abnormal transformation zone of low grade (ATZG1) with minor changes, and abnormal transformation zone of high grade (ATZG2) with major changes. Histological diagnoses were classified into three categories: chronic cervicitis (inflammatory/reactive lesions), LSIL, and HSIL, following the guidelines of the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology (38).

Bias

To mitigate selection bias, we deliberately chose a very specific population within a narrowly defined geographic area. Both the cohorts were matched for cofounding factors. This targeted approach was employed to enhance both the internal validity and the potential external validity of the data, ensuring that our findings could be more accurately generalized to similar contexts. To address measurement bias, we standardized our data collection methods and utilized validated measurement tools across the study. This included comprehensive training for all research staff involved in data collection to ensure consistency and accuracy in the measurements recorded. Regarding the classical observer bias typically associated with this kind of study, in our case, it was effectively nullified since all primary endpoints involved objective assessments. The HR-HPV status was determined using automated processes, eliminating the potential for subjective interpretation by researchers. Similarly, the histological status was assessed by an expert pathologist who conducted evaluations on biopsy samples obtained post-colposcopy. This pathologist was blinded to the study cohorts. To lessen attrition bias resulting from participant dropout, we implemented strategies aimed at maximizing retention, including regular follow-up reminders.

Study size

Considering the very specific and restricted nature of our target population, alongside the exploratory aims of our study, we opted to commence with a sample size of 20 patients in each group. This approach was chosen to ensure a precise and concentrated examination of the research questions within a manageable cohort, facilitating detailed insights into the initial outcomes.

Statistical methods

Numerical data were expressed as mean and standard deviation (S.D.) and categorical variables as absolute frequencies and percentage. Examined variables did not present normal distribution, as verified by the Kolmogorov Smirnov test; consequently, the non-parametric approach was used. Compari-

Table 1. *Descriptive analysis of the clinical characteristics of the 2 groups of patients.*

	GROUP A N.15	GROUP B N.19	P VALUE
Age	35.4 ± 10.7	37.3 ± 10.1	0.603
Body mass index	22.3 ± 2.1	23 ± 1.9	0.365
Smokers n. (%)	5 (33.3)	11 (57.9)	0.154
Age at first intercourse	17.8 ± 2.3	16.5 ± 1.9	0.104
N. of Partners	4.6 ± 3.2	3.6 ± 1.1	0.309
Estroprogestins use (%)	4 (26.7)	5 (26.3)	0.982

P values are typed bold-face if statistically significant (P <0.05 or lower) or bold-face in italics if borderline significant (P = 0.10 to 0.05).

son between group A and B was performed by using the Mann Whitney test for numerical variables and the Chi Square test for categorical variables. To evaluate, for each group examined, the existence of statistically significant differences in two different points (at baseline and after six months), the Mc Nemar test for the binary variables (HR-HPV and histological findings in group B) and the Wilcoxon test (for histological findings in group A) were applied. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 17 (Armonk, NY, IBM Corp.). A p-value smaller than 0.05 was considered statistically significant.

RESULTS

The comparative descriptive analysis, detailed in **Table 1**, reveals the clinical characteristics of the two patient groups, highlighting the lack of significant differences across the cofounding factors including age, Body Mass Index (BMI), smoking habits, age at first intercourse, number of sexual partners, and the use of estroprogestins. The average age in group A was 35 years, compared to 37 years in group B. The BMI was closely matched at 22.3 for group A and 23 for group B. Although the number of smokers in group B was higher compared to group A, this difference was not statistically significant (p-value = 0.154). This suggested that this discrepancy did not materially influence the comparative outcomes of the study. Additionally, the age at first intercourse was comparable (17.8 years for group A vs. 16.5 years for group B), as was the average number of sexual partners (4.6 for group A vs. 3.6 for group B). The use of estroprogestins was also similar, with a 4% usage rate in group A versus 5% in group B. This uniformity across both cohorts underscores

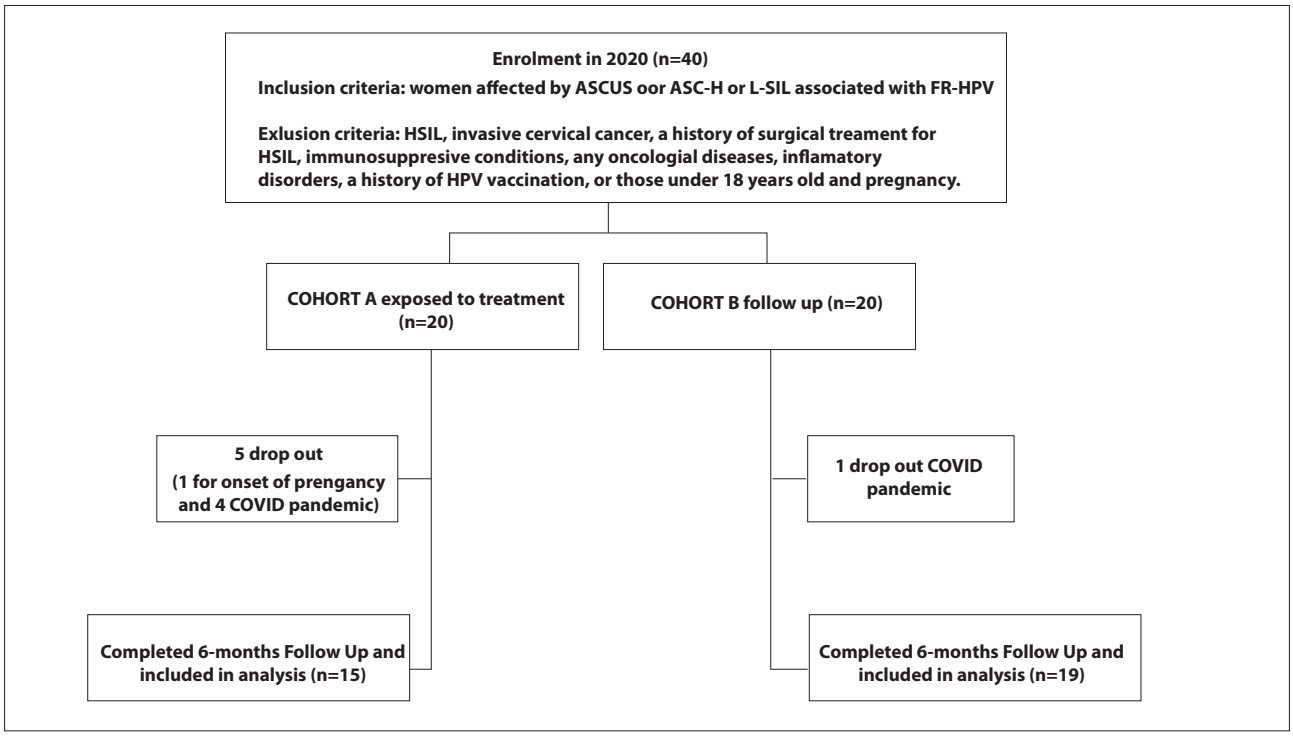

Figure 1. *STROBE flow chart.*

Table 2. *HPV type distribution in cervical samples detected by PCR in groups A and B at time 0 and after 6 months.*

HPV DETECTION	ALL N. (%)	GROUP A N. (%)		GROUP B N. (%)	
	Time 0	Time 0	Time 6	Time 0	Time 6
Single infections	22 (64.7)	9 (60)	0	13 (68.4)	14 (73.6)
Multiple infections	12 (35.3)	6 (40)	4 (26.6)	6 (31.5)	5 (26.3)
HR Type specific					
16	8 (23.5)	0	0	8 (42.1)	8 (42.1)
18	5 (14.7)	3 (20)	0	2 (10.5)	2 (10.5)
31	2 (5.8)	0	0	2 (10.5)	2 (10.5)
39	1 (2.9)	0	0	1 (5.2)	1 (5.2)
45	5 (14.7)	3 (20)	1 (6.6)	2 (10.5)	2 (10.5)
51	5 (14.7)	4 (26.6)	1 (6.6)	1 (5.3)	1 (5.3)
56	2 (5.8)	1 (6.6)	1 (6.6)	1 (5.2)	1 (5.2)
58	3 (8.8)	1 (6.6)	1 (6.6)	1 (5.2)	1 (5.2)
59	1 (2.9)	0	0	1 (5.2)	1 (5.2)
66	9 (26.4)	7 (46.6)	2 (13.3)	2 (10.5)	2 (10.5)
68	1 (2.9)	0	0	1 (5.2)	1 (5.2)
73	1 (2.9)	1 (6.6)	1 (6.6)	0	0
LR type specific					
42	2 (3.3)	0	0	2 (10.5)	2 (10.5)
61	3 (8.8)	3 (20)	3 (20)	0	0
87	1 (2.9)	0	0	1 (5.2)	

HPV: Human Papilloma virus; HR: high risk; LR: low risk. Due to multiple HPV infection, the overall percentage of HPV types exceeds 100%, because it expresses the HPV genotype-specific distribution in the studied population.

the balanced nature of the study's sample, facilitating a more reliable comparison of the treatment outcomes. Out of 40 patients enrolled, 34 completed the study. Among the 20 treated patients (group A), one was excluded because of the onset of pregnancy. Due to the COVID pandemic, 4 patients of group A and one of group B dropped out because of declining to continue follow up (**Figure 1** - **Appendix 1**). HPV type distribution in cervical samples is shown in **Table 2**. In our study the HPV-66 was found as the most common type (26.4.%) followed by HPV-16 (23.5%), -18, -45, -51 (14.7% respectively), -58 (8.8%), -31, -56 (5.8% respectively) and -39, -59, -68, -73 (2.9% respectively). Due to multiple HPV infection, the overall percentage of HPV types exceeds 100%, because it expresses the HPV genotype-specific distribution in the studied population.

The descriptive sub analysis of HR-HPV genotypes within each group revealed a non-uniform distribution across the different HPV types, as illustrated in the heatmap (**Figure 2**). Notably, HPV-16 was absent in group A (0%) but showed a significant presence in group B (42.1%). Conversely, HPV-18 appeared in 20% of cases in group A versus 10.5% in group B. Other types such as HPV-31, HPV-39, and HPV-68 were not found in group A but were present in group B at rates of 10.5%, 5.2%, and 5.2%, respectively. Meanwhile, HPV-45 and HPV-51 were more common in group A, at 20% and 26.6% respectively, compared to 10.5% and 5.3% in group B. HPV-56 and HPV-58 showed similar prevalence across groups, with a slight edge for group A. Significantly, HPV-66 was markedly more prevalent in group A (46.6%) than in group B (10.5%), while HPV-73 was found exclusively in group A (6.6%). The clinical findings after six months are shown in **Table 3**. The clearance of HR-HPV was significantly higher in group A, where 73.3% of the cases (11 out of 15) achieved clearance, compared to group B, which saw no cases of clearance among 19 patients, marking a statistically significant difference between the two groups ($p < 0.001$). While colposcopic findings remained largely unchanged after six months in both groups, the biopsy results revealed a notable contrast. On biopsy, a regression of L-SIL to chronic cervicitis was found in 2/15 (13%) of cases in group A compared to 0/19 (0%) of cases

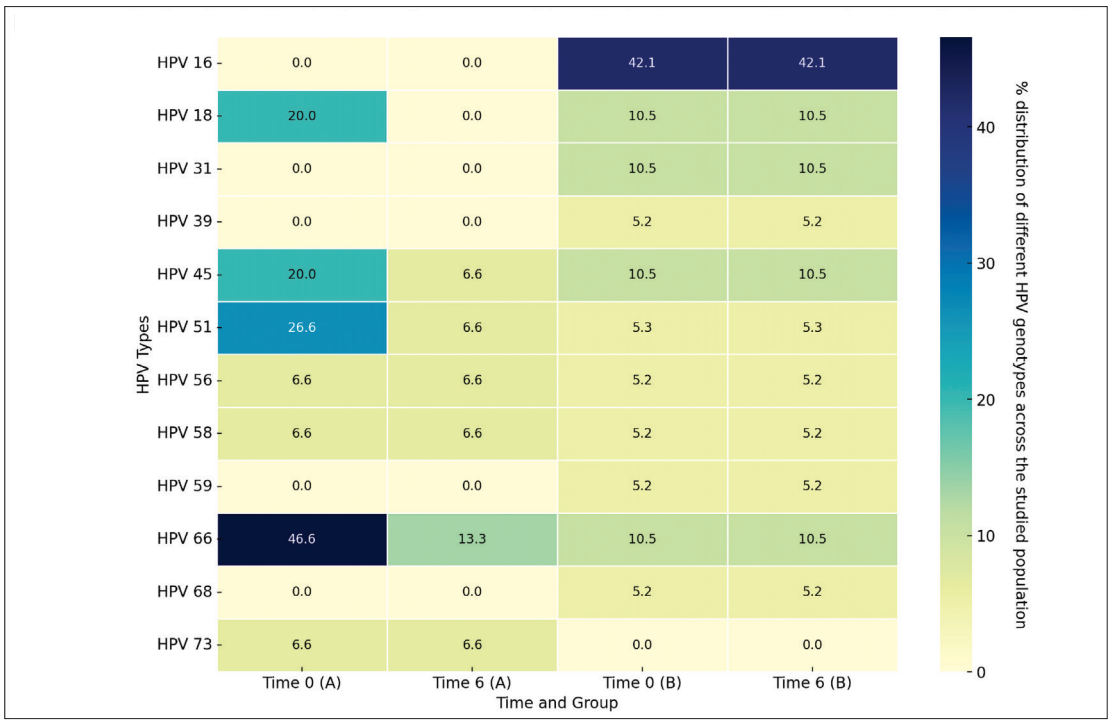

Figure 2. *HPV type distribution in cervical samples detected by PCR in groups A and B at time 0 and after 6 months.*

in group B with significant difference between the 2 groups (p = 0.048); on the other hand, there was no worsening of the histological finding in group A, while it was found a progression from LSIL to HSIL in 5/19 (26.3%) of cases in group B with significant difference between the 2 groups (p = 0.042) (**Figure 3**, **Figure 4**). There were no reported side effects, and patient compliance with the therapy reached 100%. It's important to note that any instances of dropout were attributed to reasons related to the COVID-19 pandemic, rather than any issues associated with the therapy itself. We acknowledge that the differential dropout rates in group A and B pose a limitation that is why our statistical analysis focused solely on participants who completed the study to maintain data integrity.

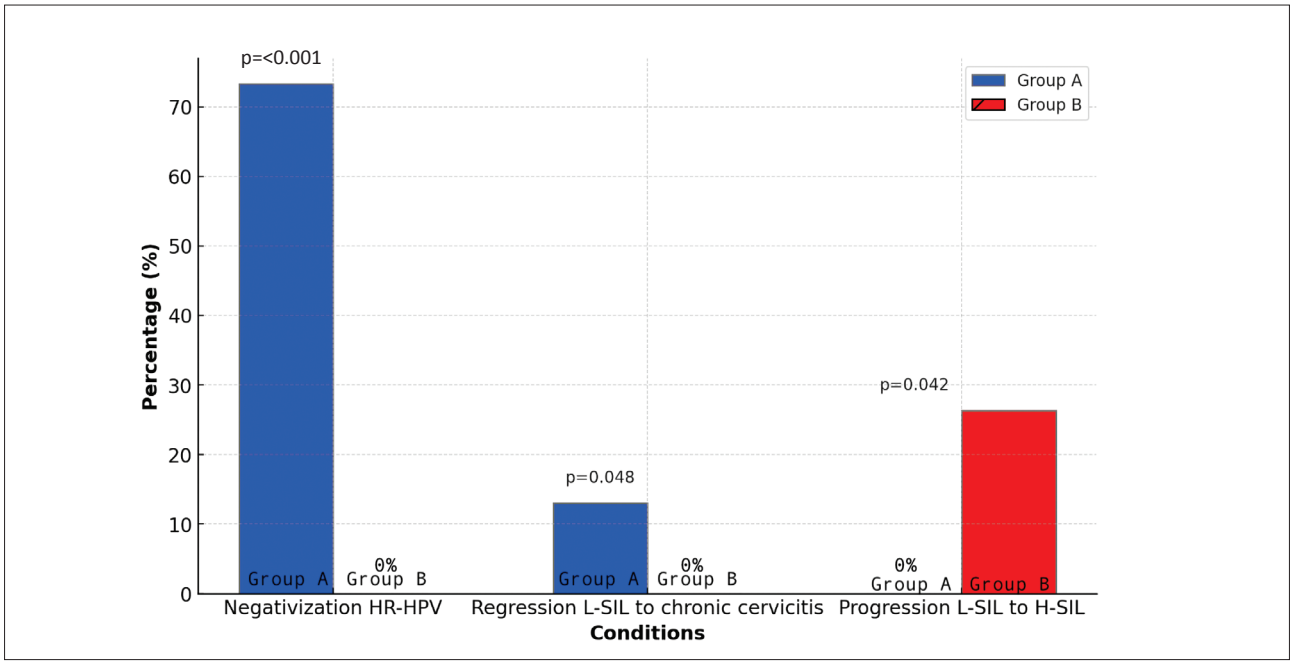

Figure 3. *HR-HPV and histological findings after 6 months in groups A and B (group B zeros highlighted).*

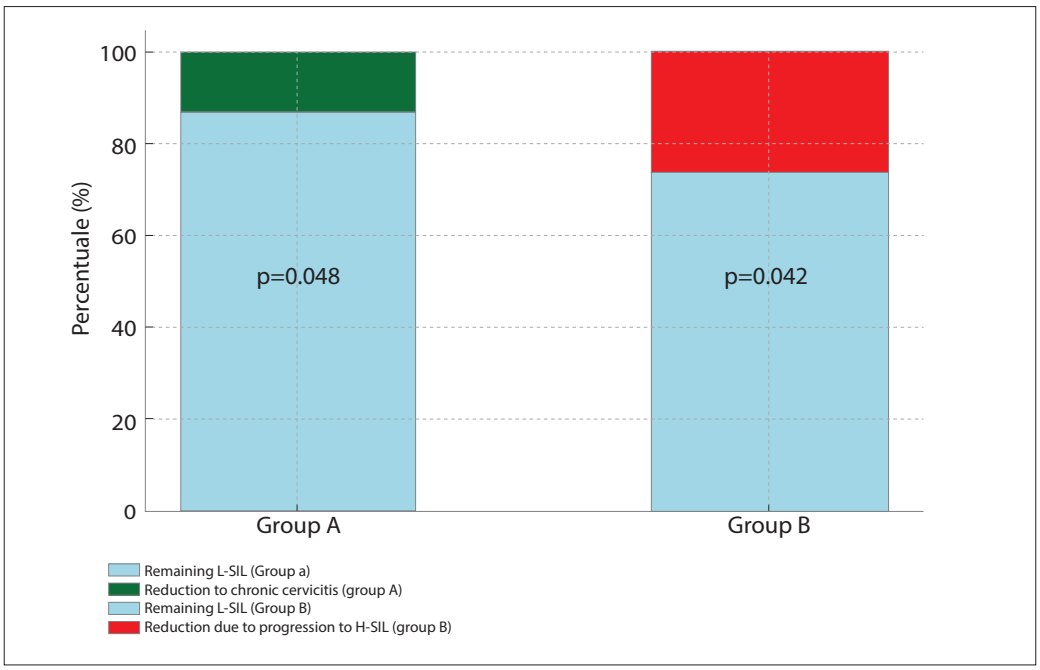

Figure 4. *Histological status changes in groups A and B after 6 months (adjusted).*

Table 3. *HR-HPV and histological findings after 6 months in groups A and B.*

	GROUP A N.15 (%)	GROUP B N.19 (%)	P VALUE
Negativization HR-HPV	11 (73.3)	0 (0)	<0.001
Regression L-SIL to chronic cervicitis	2 (13)	0 (0)	0.048
Progression L-SIL to H-SIL	0 (0)	5/19 (26.31)	0.042

HR-HPV: High Risk Human Papilloma virus; L-SIL: Low Grade Squamous Intraepithelial Lesion; H-SIL: High Grade Squamous Intraepithelial Lesion. P values are typed bold-face if statistically significant (P <0.05 or lower) or bold-face in italics if borderline significant (P = 0.10 to 0.05).

Subgroups analysis:

-differential clearance rates in single and multiple HR-HPV infections: the analysis of HR-HPV clearance rates reveals a marked contrast in the effectiveness of the treatment between patients with single versus multiple infections. Specifically, in group A, which received the combined therapy, patients with a single HR-HPV infection showed a 67% higher likelihood of achieving viral clearance compared to those with multiple infections, a difference that was statistically significant with a p-value of 0.01099. This suggests that the combined therapy was particularly effective for patients with single infections. Conversely, we observed, in group B, that only one patient with multiple infections spontaneously regressed to a single infection, while none of the patients with a single infection regressed to no infection. This light change was not statistically significant (0% clearance rate in "group B single infection" versus 16.6% clearance rate in "group B multiple infections"; p-value of 0.3157) (**Figure 5**). In summary, the findings strongly indicate that within group A, individuals with single HR-HPV infections responded more favorably to the treatment than those with multiple infections. However, it is crucial to interpret these results with an understanding of the limited sample size, which may constrain the extendibility of these conclusions to a wider population;

-dynamics of each HR-HPV genotype clearance: in group A, the therapy led to distinctive responses among various HR-HPV genotypes over the six-month period. Specifically, HPV 18 experienced the highest reduction at 100%, followed by HPV 51 with 75.19%, HPV 66 with 71.46%, and HPV 45 with 67%, while HPV 56, HPV 58, and HPV 73 showed no impact from the treatment. It's important to note that no increases in detection rates were observed for any of the genotypes, and crucially, no new HPV genotypes were identified at the six-month mark (**Figure 6**). In contrast, the

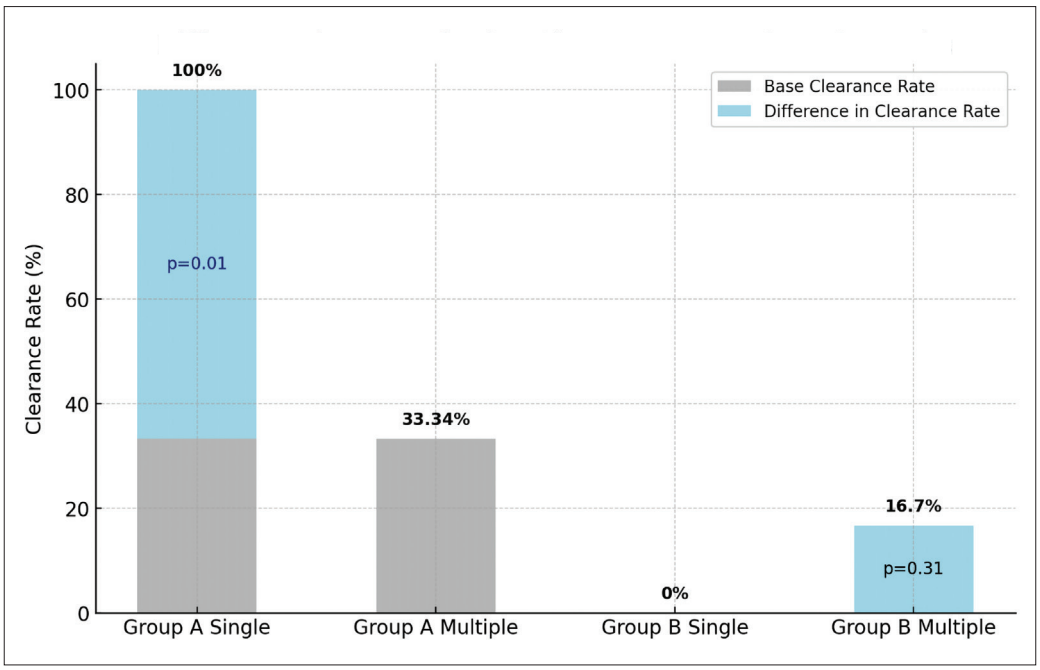

Figure 5. *Differences in HPV infection clearance rates over 6 months.*

HR-HPV genotype distribution in group B showed no change at the six-month mark.

DISCUSSION

In this prospective observational study, the combined therapy of AHCC and *L. Crispatus M 247* was associated for the first time with an improvement in HR-HPV clearance and the regression rate of LSIL, notably without any adverse effects and demonstrating excellent patient compliance. Literature review indicates the best performing spontaneous clearance rates for HR-HPV fluctuate around 65% (10, 11). These variations are influenced by demographic and geographical factors, highlighting the value of targeted studies for gaining insights into the disease's distribution and behavior across dif-

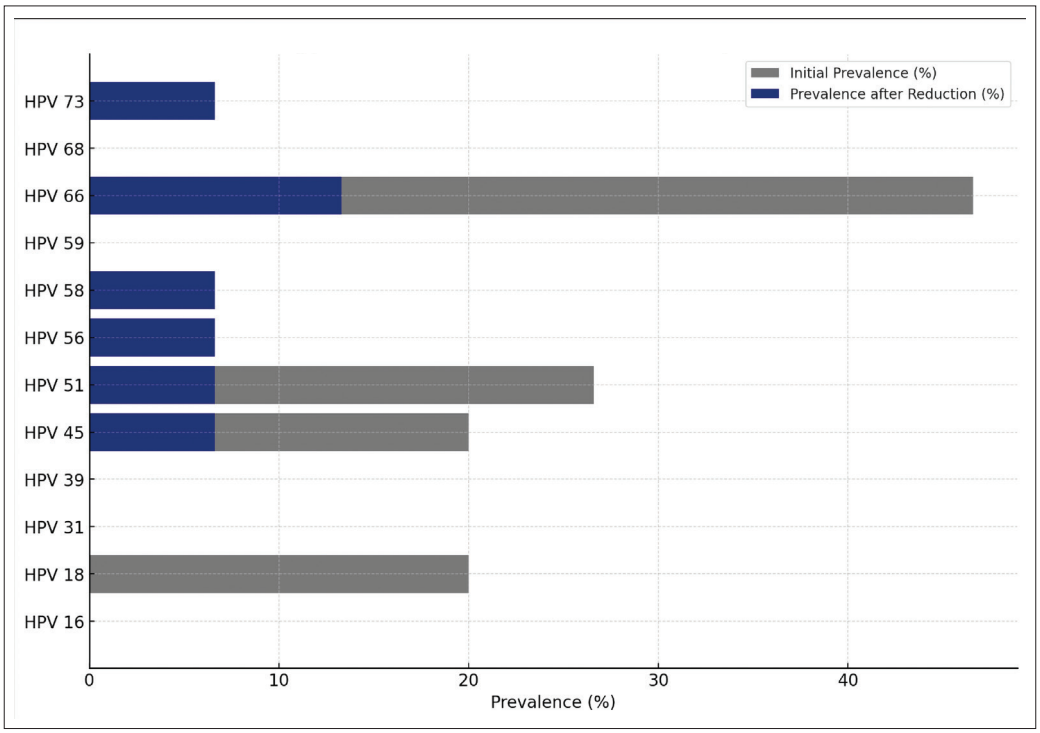

Figure 6. *HPV genotype prevalence and reduction in group A over 6 months.*

ferent populations. Given the high rates of spontaneous HR-HPV clearance, follow-up is often preferred over more invasive treatments due to the potential for adverse effects. However, about 30% of individuals may either retain the virus or experience progression to more severe preneoplastic stages, underlining the urgent need for effective and safe treatments. Observations from our cohort (group B) over six months revealed no instances of spontaneous HR-HPV clearance or LSIL regression; conversely, 19% of cases progressed to HSIL. Our findings from group B contrast sharply with those reported in the literature, often due to the absence of crucial data necessary for normalizing observed populations and drawing definitive conclusions. Factors such as the absence of HPV vaccination, short follow-up durations, and discrepancies in risk factor distribution relative to the general population are believed to explain these differences. Our cohorts, both group A and B, though comparable to each other, represented a high-risk segment for disease progression from HR-HPV infection, indicated by prevalent risk factors such as smoking (33.3%-57.9%), number of sexual partners (4.6-3.6), and age at first intercourse (16.5-17.8 years). Another variable that could influence the disparity between our observed population and the general population, or those in other studies, is the average age of the groups, which ranges from 35.4 to 37.3 years. It is well-documented that spontaneous LSIL regression or HR-HPV clearance is more likely at a younger age, specifically under 25 years old. Despite deviations from the general population, our cohorts were balanced and matched for confounding factors, leading to a significant statistical difference between the combined therapy and follow-up groups in a very short time. And, when comparing our group A, which received the combined therapy, to the observed groups from the literature that underwent only follow-up, still a notable difference emerges. Our group A exhibited a HR-HPV clearance rate of 73.3% within six months, surpassing the best average spontaneous HR-HPV clearance rate reported in the literature of 65%, which was achieved over a period of two years (10, 11). This aligns with other studies that underscore the effectiveness of such treatments. For instance, AHCC supplementation significantly boosted the immune response against persistent HPV infections, with 63.6% of patients (14 out of 22) testing negative for HPV RNA and DNA after six months (39). Oral *L. Crispatus M247* supplementation was linked to a higher resolution rate of HPV-related cytological abnormalities compared to follow-up care alone (60.5% vs. 41.3%, p = 0.05), even though complete HPV clearance was achieved in only 15.3% of treated patients versus 9.3% in untreated cases (34). Furthermore, in our sub-analysis focused on the significant contribution to clearance in group A, when we examine the two subgroups (single infection versus multiple infections, defined by the presence of more than one HPV type), a statistical difference is observed between them: patients with a single HR-HPV infection had a 67% greater likelihood of achieving viral clearance compared to those with multiple infections. This suggests that it is more feasible to reduce a single infection than multiple infections with this therapy within 6 months. One limitation arises from the non-homogeneous distribution of HR-HPV genotypes between the two cohort groups. Given this limitation, further randomized clinical studies are needed to randomize the two arms, ensuring a balance and match in the distribution of this variable that can influence the expected efficacy of the treatment outcome. Despite this limitation, it is noteworthy to report the differential response across all the different HPV genotypes underscores the importance of considering genotype-specific dynamics when assessing the efficacy of HPV treatments, as it highlights significant clearance in certain genotypes, preventing new genotype onset. In contrast, the HR-HPV genotype distribution in Group B remained unchanged at the six-month evaluation. This consistency contrasts with the dynamic changes observed in group A, where specific genotypes showed significant reductions in prevalence. While HPV vaccination and cervical screening have significantly lowered cervical cancer rates and preneoplastic lesions (40-42), thereby mitigating the disease's severe impact on women's survival and quality of life (43-46), global coverage remains low at only 21% in 2022 (47). This gap in prevention accentuates the urgent need to enhance HPV clearance and LSIL regression rates, particularly in populations with high-risk HPV infections. In light of this, our observational study targeted individuals with HR-HPV infection, with abnormal cervical cytology up to LSIL but not beyond. Considering the side effects of invasive treatments and the high rate of spontaneous resolution, current guidelines typically recommend regular follow-up. The overmentioned treatments, however, addressed the lesions without eliminating the HR-HPV infection itself, which could potentially lead to oncogenesis

in other genital areas in the future of the patient's medical history. Since our study demonstrated that the combination therapy of AHCC and *L. Crispatus M247* more effectively enhances HR-HPV clearance and LSIL regression compared to regular follow-up, without causing adverse effects, and potentially reduces oncogenesis risk by directly targeting HR-HPV, there is potential for prescribing this combination to patients with genital HPV infections independently from potential histological lesions of the cervix. This approach could complement existing therapies across various stages of HPV-related diseases, from preneoplastic lesions to cancer.

CONCLUSIONS

The observed HR-HPV clearance rate difference of 73.3% between group A and group B over six months, alongside the notable modification in histological status - with a 13% regression rate in Group A versus a 26.3% progression rate in Group B - highlights the potential of AHCC and *L. Crispatus* supplementation in the immunomodulation of cervical preneoplastic lesions. Despite the challenges in drawing definitive conclusions due to the small cohort size, this observational prospective study involving women at high risk of progression from Sicily, affected by HR-HPV infection with abnormal cervical cytology up to LSIL, suggests a promising avenue for enhancing HR-HPV clearance and preventing cervical preneoplastic lesions through oral supplementation of AHCC and *L. Crispatus*. These findings underscore the importance of conducting further in vivo clinical randomized studies to verify the efficacy of this supplementation in preventing cervical cancer in women with HR-HPV-related LSIL, thereby strengthening the case for such interventions in clinical practice.

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COMPLIANCE WITH ETHICAL STANDARDS

Fundings

No funding was utilized for this study as it comprised observational research incorporating routine clinical practices.

Conflict of interests

The authors declare that there are no conflicts of interest associated with this publication.

Availability of data and materials

The data supporting the findings of this study are available upon reasonable request to the corresponding author.

Authors' contributions

VS, AE, VI and CM: significantly contributed to the conception and design of the work; VS, LP, and RG: played a crucial role in data collection; AA and CM: were instrumental in the analysis and interpretation of the data; ML and LM: dedicated efforts to drafting the manuscript and critically revising it for important intellectual content; VS and AE: provided final approval for the version to be published; FC, FD, and CM: ensured the accuracy and integrity of the work, addressing any related questions thoroughly.

Ethical approval

This research adhered to the ethical standards of the World Medical Association's Declaration of Helsinki and complies with the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals, including the inclusion of diverse human populations in terms of sex, age, and ethnicity.

Human studies and subjects

Written informed consent was obtained from all participants prior to their involvement in the study. The study protocol, along with other relevant materials, adhered to the ethical standards as mentioned above. Approval for all research activities was granted by the institutional review board. As this was an observational study, researchers were limited to observing and analyzing data without interfering with the standard management of the patients. This approach ensured that the observational nature of the study did not impact the man-

agement or clinical care of the patients, who underwent the standard procedures and treatments necessary for their condition and their desire. All patient data were anonymized to protect privacy and confidentiality, maintaining the highest standards of data protection and ethical consideration.

Animal studies

N/A.

Publications ethics

The publication ethics followed by this study align with those outlined by the International Committee of Medical Journal Editors (ICMJE), regarding publishing and editorial issues in medical journals.

Plagiarism

The article provides a comprehensive review of the latest studies in the field, with accurate citations.

Data falsification and fabrication

The writing and contents of the article are entirely original and were developed entirely by the authors.

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APPENDIX 1

STROBE Statement-Checklist of items that should be included in reports of cohort studies

	ITEM NO	RECOMMENDATION	PAGE NO
Title and abstract	1	*(a)* Indicate the study's design with a commonly used term in the title or the abstract	3
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/ rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	6
Methods			
Study design	4	Present key elements of study design early in the paper	6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7
Bias	9	Describe any efforts to address potential sources of bias	7
Study size	10	Explain how the study size was arrived at	7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	
Results			8
Participants	13*	(a) Report numbers of individuals at each stage of study-eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	8
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	8
Outcome data	15*	Report numbers of outcome events or summary measures over time	8
Main results	16	*(a)* Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included *(b)* Report category boundaries when continuous variables were categorized *(c)* If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	8
Other analyses	17	Report other analyses done-eg analyses of subgroups and interactions, and sensitivity analyses	11
DISCUSSION			
Key results	18	Summarise key results with reference to study objectives	12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14
Generalisability	21	Discuss the generalisability (external validity) of the study results	14
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

**Give information separately for exposed and unexposed groups.*

An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at https://journals.plos.org/plosmedicine/, Annals of Internal Medicine at https://www.acpjournals.org/journal/aim, and Epidemiology at https://journals.lww.com/epidem/pages/default.aspx). Information on the STROBE Initiative is available at https://www.strobe-statement.org/).