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ORIGINAL

Effect of a *Coriolus versicolor*-based vaginal gel on cervical epithelialization and vaginal microbiota in HPV-positive women: EPICERVIX pilot study

Eficacia de un gel vaginal basado en Coriolus versicolor sobre la epitelización cervical y la microbiota vaginal en mujeres positivas al VPH: Estudio piloto EPICERVIX

Silvia González[1], Luís Serrano[1], Javier Cortés[2], Teresa Vezza[3,4], José Garrido-Mesa[3], Francesca Algieri[3], Rocío Morón[3,5], Maria Elena Rodríguez Cabezas[3,4], Julio Gálvez[3,4], Alba Rodríguez Nogales[3,4,6]

1. Department of Gynaecology, Policlínico HM Gabinete Velázquez, Madrid, Spain. 2. Cytopathology. Oncological Gynaecology, Private Practice, Palma, Spain. 3. CIBER-EHD, Department of Pharmacology, Centre for Biomedical Research (CIBM), University of Granada, 18071-Granada, Spain. 4. Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA), Granada, Spain. 5. Department of Hospital Pharmacy. Hospital Universitario Clínico San Cecilio. Granada, Spain. 6 Department of Gastrointestinal Medicine, Hospital Universitario Virgen de las Nieves, 18012-Granada, Spain.

Corresponding author
Teresa Vezza PhD
Department of Pharmacology
Centre for Biomedical Research, University of Granada
Avenida del Conocimiento S/N, 18016-Armilla, Granada, Spain
E-mail: teresavezza@hotmail.it

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Abstract

Objectives: A *Coriolus versicolor*-based vaginal gel (Papilocare®) has been shown to speed up the cervicovaginal mucosa epithelialization and a tendency to improve the composition of the microbiota in healthy women. The study aimed to evaluate the effects of this gel on cervical epithelialization and composition of vaginal microbiota in HPV-positive women with normal cytology and colposcopy.

Methods: A total of 21 HPV-positive women with negative Papanicolaou smear and normal colposcopy were once-daily treated with Papilocare® for 21 days. At baseline and the end of treatment, degree of epithelialization of the cervix mucosa was evaluated by colposcopy (and rated by the five-point Likert scale), and vaginal microbiota composition was analysed by next-generation sequencing.

Results: At the end of treatment, cervix epithelialization was improved in 52.6% of women. The treatment produced a statistically significant reduction in phylogenetic diversity. Moreover, abundance in *Proteobacteria* phylum was reduced (38.5% vs 93.6% at baseline) while increased in *Firmicutes phylum* (44.6% vs 2.1%). A significant increase and reduction in the proportion of *Lactobacillus* spp. Beij. (*Lactobacillaceae* family), including *L. crispatus* and *L. iners*, and *Gardnerella vaginalis* was reported, respectively.

Conclusions: The application of the *Coriolus versicolor*-based vaginal gel significantly improved ectocervix epithelialization and the vaginal microbiota composition among HPV-positive women without cervical lesions, which could support its use for preventing HPV-associated cervix lesions.

Keywords: Cervix epithelialization, human papillomavirus, *Lactobacillus*, Papilocare, vaginal microbiota, Coriolus versicolor.

Resumen

Objetivos: Un gel vaginal basado en *Coriolus versicolor* (Papilocare®) ha mostrado acelerar la epitelización de la mucosa cervicovaginal y una tendencia a mejorar la composición de la microbiota en mujeres sanas. El estudio fue diseñado para evaluar los efectos de este gel sobre la mucosa cervicovaginal y la composición de la microbiota de mujeres VPH positivas con citología y colposcopía normales.

Métodos: Un total de 21 mujeres fueron tratados una vez al día con Papilocare® durante 21 días. En el momento basal y al final del tratamiento, se evaluó el grado de epitelización de la mucosa cervical mediante colposcopía (y se cuantificó mediante una escala Likert de cinco puntos) y se analizó la composición de la microbiota vaginal mediante tecnologías nuevas de secuenciación.

Resultados: Al final del tratamiento, la epitelización de la mucosa cervical mejoró en el 52.6% de las mujeres. El tratamiento produjo una reducción estadísticamente significativa en la diversidad filogenética. Además, se redujo la abundancia de *Proteobacteria* phylum (38.5% vs 93.6% en el momento basal), mientras que se incrementó la de *Firmicutes* phylum (44.6% vs 2.1%). Se observó un aumento y disminución significativa de las proporciones de *Lactobacillus* spp. Beij. (familia *Lactobacillaceae*), incluyendo *L. crispatus* and *L. iners*, y de *Gardnerella vaginalis*, respectivamente.

Conclusiones: La aplicación del gel vaginal basado en *Coriolus versicolor* mejoró significativamente la epitelización del ectocérvix y la composición de la microbiota vaginal en mujeres VPH positivas sin lesiones cervicales, lo que podría apoyar su uso para prevenir las lesiones de cérvix asociadas al VPH.

Palabras clave: Epitelización cérvix, papilomavirus humano, *Lactobacillus*, Papilocare, microbiota vaginal, *Coriolus versicolor*.

Introduction

Human papillomavirus (HPV) is the most common viral infection of the reproductive tract and is mainly transmitted through sexual contact. There is clear evidence linking HPV infection and different types of cancers, including the anus, vulva, vagina, penis and oropharynx[1,2]. More than 100 types of HPV have been reported, and at least 14 are considered as cancer-causing or high-risk types. Specifically, HPV 16 and 18 account for 70% of all cervical cancers and pre-cancerous lesions worldwide[3,4]. Despite extensive research, the underlying mechanisms of the HPV life cycle and HPV-induced carcinogenesis have not been fully identified. It is generally accepted that several cofactors, including viral genotype, host immune status, vaginal microbiota and ectocervix histological structure[5-7], may regulate a balance between infection and virus clearance. This is crucial for cervical cancer development in HPV-infected women[8-11]. Although HPV vaccines are available, they do not protect against all HPV types or established infections[1,12], so a more in-depth understanding of the pathological mechanisms of HPV is needed to identify new tools for preventing the infection and development of associated cancers. Regarding vaginal microbiota, several studies have reported an association between abnormalities in its composition (dysbiosis) and the existence of HPV[13-16]. Vaginal dysbiosis has been characterized by a decrease in *Lactobacillus* spp. Beij. (*Lactobacillaceae* family), with a concomitant increase in diversity and anaerobic bacteria abundance, including species of *Gardnerella* Chavan (*Bifidobacteriaceae* family)[17-19].

Considering the histological structure of the ectocervix, it has been reported that a well epithelized cervix with squamous epithelium and limited or non-existent transformation zone with cellular activity prevents integrative colonization of HPV with oncogenic potential[20]. Consequently, promoting ectocervix squamous cell epithelialization, which may function as a barrier, seems a plausible approach to hinder HPV integration and prevent infection. In this sense, a non-hormonal vaginal gel based on *Coriolus versicolor* L. (*Polyporaceae* family), Papilocare® (Procare Health, Castelldefels, Barcelona, Spain), has recently been approved in Spain. Its components possess hydrating properties, act as moisturizer and lubricant, enhance and accelerate the repair of atrophic or injured epithelium of the cervicovaginal mucosa, as well as display immunomodulatory effects and restore the balance of the microbiota[21-26]. Furthermore, *Coriolus versicolor*, traditionally used in Chinese medicine, has been reported to have antimicrobial, antiviral and anti-tumour properties[27]. Remarkably, in an open-label prospective pilot study, 21 asymptomatic healthy women were treated with this vaginal gel for 12 consecutive days, and it significantly improved cervical epithelialization while a tendency to improve the dysbiosis status was observed[28]. These encouraging results prompted us to evaluate the *Coriolus versicolor*-based vaginal gel in HPV-positive women with normal cytology and colposcopy for a longer period (21 days), by assessing its impact on cervical epithelialization and modulation of vaginal microbiota composition.

Methods

Study design

An observational, non-comparative, open-label, prospective pilot EPICERVIX study included HPV-positive women with negative Papanicolaou smear and normal colposcopy who received treatment with Papilocare® between July 2016 and January 2017 in the Department of Gynaecology at the Obstetrics & Gynaecology Institute (Madrid, Spain). The study was performed under conditions of routine daily practice and in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. Approval of the study protocol by the institutional review board was not required since studies with a medical device class 1 (not a drug) already marketed and used within approval indications are exempted, according to Spanish law (http://sid.usal.es/idocs/F3/LYN14832/14832.pdf).

Participants

Inclusion criteria to participate in the study included: sexually active women; aged 25 years or over; attending a routine gynaecological monitoring visit; with diagnosis of HPV by polymerase chain reaction (PCR)-based HPV deoxyribonucleic acid (DNA) detection within three months prior to the consultation; a normal Papanicolaou smear and normal colposcopy findings; and eligible for the prescription of the vaginal gel. Exclusion criteria were: clinically relevant disorders of the immune system or treatment with immunosuppressant agents; abnormal vaginal bleeding (without diagnosis) within the six months prior to the consultation; symptomatic vulvovaginal infection; history of gynaecologic cancer; use of vaginal contraceptives or other vaginal hormonal treatments; scheduled surgery preventing compliance with treatment; participation in another clinical trial; fertile women not using effective contraceptive methods; pregnant or breastfeeding; and contraindication for the use of Papilocare® or known allergies to some of its components.

Study procedures

Women who gave consent to participate in the study and met the inclusion criteria were advised on appropriate use of the vaginal gel Papilocare® according to the patient information leaflet. The gel contains *Coriolus versicolor* as the main component together with niosomes of hyaluronic acid (a moisturizing agent), beta-glucan (an immunomodulator agent), Bioecolia® (a prebiotic agent), phytosomes of *Centella asiatica* L. (*Apiaceae* family) (a tissue regenerating agent), *Azadirachta indica* A. Juss.

(*Meliaceae* family) extract (Neem) (an antioxidant/anti-inflammatory agent), and *Aloe vera* L. (*Asphodelaceae* family) (a re-epithelialization agent). Patients were encouraged to apply Papilocare® once a day before bedtime for 21 consecutive days. The use of douches or vaginal deodorants was not permitted, but sexual activity with or without condom was not limited. Participants found to be eligible, after acceptance for inclusion in the trial, visited the clinic on day 0 (baseline/visit 1) and on day 21 (visit 2). Degree of epithelialization was evaluated colposcopically and samples were obtained to determine the composition of the vaginal microbiota.

Cervical epithelialization

The epithelialization degree of the cervical mucosa was evaluated by the investigator by standard colposcopy and rated using a five-point Likert scale, where five was no ectopy, four: mild (<25% of the external os), three: moderate (25%–50% of the external os), two: severe (>50% of the external os) and one: severe ectopy and bleeding.

Characterization of microbiota

DNA from study samples was isolated as reported elsewhere[29]. Amplicon fragments were PCR-amplified from the total DNA in duplicate with the Phusion high-fidelity DNA polymerase. A single round of PCR was performed using "fusion primers", targeting 16S rRNA V1-4 region with multiplexing on the Illumina MiSeq machine. PCR products were verified visually by running a high-throughput Invitrogen 96-well-E-gel. The PCR reactions from the same samples were pooled in one plate, then cleaned and normalized using the high-throughput Invitrogen SequalPrep 96-well Plate kit. Samples were then pooled to make one library to be quantified fluorometrically before sequencing. For taxonomic analysis, sequences were selected to estimate the total bacterial diversity of the DNA samples in a comparable manner and were trimmed to remove barcodes, primers, chimeras, plasmids, mitochondrial DNA; in addition to any non-16S bacterial reads and sequences < 150 bp. MG-RAST (metagenomics analysis server)[30], with the Ribosomal Database Project (RDP) for analyses of all sequences. The pipeline uses bar coded sequence readings, divides them into individual communities by bar code, makes taxonomic assignments to RDP database with external programmes[31] and predicts phylogenetic diversity, with a minimum e-value of 1e-5, minimum identity of 60% and a minimum alignment length of 15 measured in base pairs for RNA databases. Each value expressed the percentage relative frequency of readings with predicted proteins and rRNA genes defined for the particular taxonomic level. The output file was also evaluated with SPSS 17.0 Software Package (SPSS Inc., Chicago, Ill, USA) and the Statistical Analysis of Metagenomic Profiles (STAMP) software 2.1.3[32].

The composition of bacterial communities was evaluated by calculating three major ecological parameters, including the Chao1 richness index for abundance data (an estimate of a total community)[33], the Pielou's evenness index (to show how evenly individuals in the community were distributed over different operational taxonomic units (OUT)[34], and the Shannon biodiversity index (a combined parameter of richness and evenness)[35]. The Shannon biodiversity index was categorized as less than two (low diversity), two to three (normal), and more than three (high diversity). Furthermore, a two-dimensional scatterplot was generated by principal coordinates analysis (PCoA) to visualize whether the experimental groups in the input phylogenetic tree had significantly different microbial communities. This method enables visualization of dissimilarities of the data in terms of distance[36].

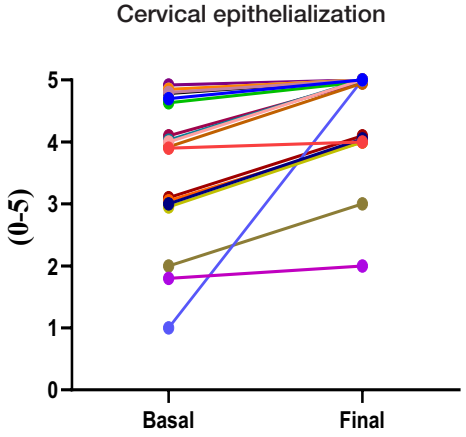
Statistical analysis

Calculation of sample size was unnecessary since the study was exploratory. Quantitative variables were expressed as mean and standard deviation (SD) while categorical variables appeared as frequencies and percentages. Paired samples of continuous data were compared to the Wilcoxon signed-rank test. Data were evaluated using the Power Analysis and Sample Size software programme, version 2011.

Results

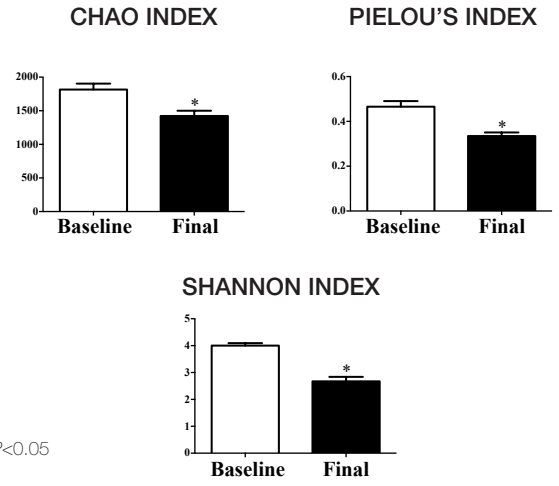
A total of 21 patients participated in the study, with a mean age of 38.9 years (range 25-59 years). Microbiota analysis was performed on all women, but cervical epithelialization was analysed in 19 of them. Treatment with the *Coriolus versicolor*-based vaginal gel revealed a beneficial effect on re-epithelialization of the cervix, with median score of 5 (range: 2-5) at the final visit as compared to 4 (range: 2-5) at the onset of the study (P<0.01) (**Figure 1**) and an overall improvement of 18%. Cervix epithelialization improved in 52.6% of women and a score of 5 (no ectopy) was observed in 66.7%, while only 38.1% of the patients had that value at the beginning of the study.

Figure 1: Degree of epithelialization of the cervix mucosa rated by the 5-point Likert scale.



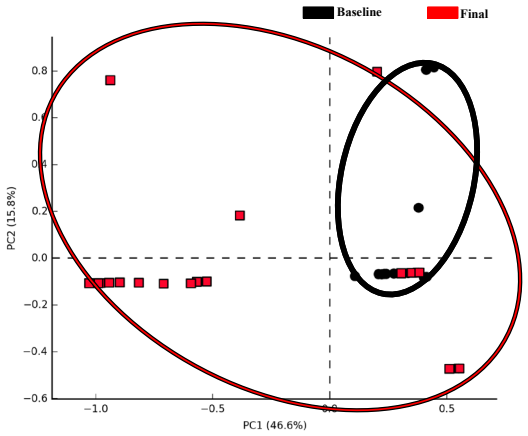
Microbial richness, evenness and diversity, analysed with the Chao, Pielous's and Shannon indices, were significantly decreased at the final visit in comparison with baseline (**Figure 2**).

Figure 2: Estimate of the phylogenetic diversity of the vaginal mcicrobiota at baseline and after 21 days of treatment with Papilocare® using Chao richness, Pielou evenness and Shannon diversity.



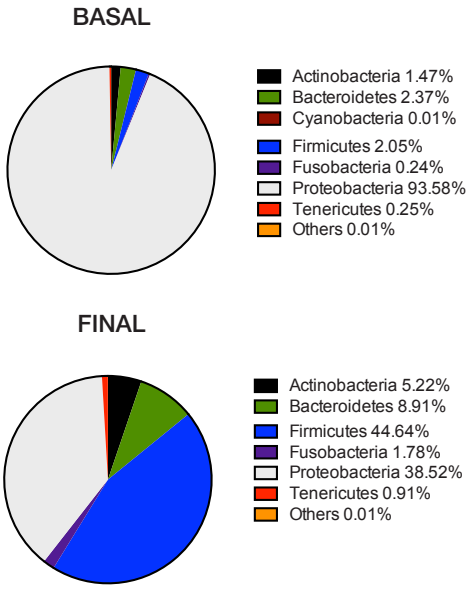
In the PCoA analysis, the composition of vaginal microbial communities clearly differed at the time points evaluated (baseline and end of study) (**Figure 3**).

Figure 3: Principal component analysis plot based on Bray-Curtis distances.



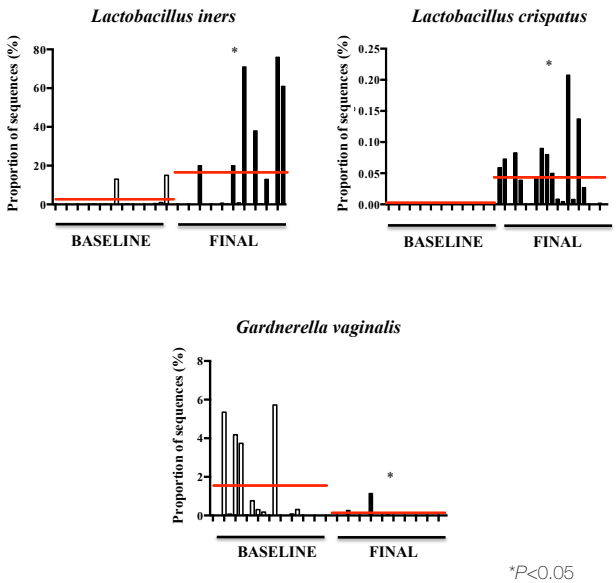
Moreover, there was a notable change of abundance in the most representative phyla. At baseline, the predominant phylum was *Proteobacteria* (93.6%) with a lower proportion of bacteria belonging to the phyla *Actinobacteria, Bacteriodets* and *Firmicutes* (1.5%, 2.4% and 2.1%, respectively). At the end of the treatment, a statistically significantly ($P<0.005$) higher proportion of the phyla *Firmicutes* (44.6%), to which *Lactobacilli* belongs, was observed, together with a decrease in the phylum *Proteobacteria* (38.5%), which includes a large number of potential pathogens (**Figure 4**).

Figure 4: Proportions of the different phyla of bacteria in the ectocervix samples at baseline and at the end of the study after 21 days of treatment with Papilocare®



Finally, at the end of treatment, statistically significant increases ($P<0.05$) in comparison with baseline were documented for *L. iners* and *L. crispatus*. However, other species, such as *Gardnerella vaginalis* decreased significantly (**Figure 5**).

Figure 5: Individual proportions of *Lactobacillus iners* and *crispatus* and *Gardnerella vaginalis* at baseline and after 21 days of treatment with Papilocare®



Discussion

This study performed on HPV-positive women revealed that once-daily vaginal application of Papilocare® for 21 consecutive days had a favourable effect on cervix epithelialization and vaginal microbiota. These findings, although obtained in a small study population, are clinically relevant since the moisturizing, repairing and epithelizing properties of the vaginal gel, as well as its effect restoring the balance of vaginal microbiota could contribute to prevent the clinical course of HPV-associated lesions.

The cervix has a very unstable histological structure with permanent confrontation of the scaly vaginal strata and the cylindrical endocervical glandular epithelium. Below this glandular epithelium there is a lining consisting of 'reserve' cells that retain the ability to grow and differentiate into mature forms of squamous or glandular epithelium. This process has been called metaplasia and generates an area identified in the cervix as a 'transformation zone'. This is a common process in sexually active women, especially in those using hormonal contraceptives or intra-uterine devices and in women who have given birth. The reserve cells in the metaplastic process of re-epithelialization fulfil this condition and are perfect targets for the anchoring of HPV[37,38] since HPV needs to integrate into mitotically active cells[20]. Thus, the preservation of a well epithelized cervical neck and an extended transformation zone would prevent the integrative colonization of HPV. Therefore, the reported effects of Papilocare® in repairing, stimulating squamous epithelialization and remodelling the transformation zone may have an important indirect role in reducing the susceptibility of the cervix to HPV infection. Moreover, and although it has not been explored in this study, *Coriolus versicolor* has been reported to have immunostimulatory properties that mediate the clearance of oral HPV[39] and might control the proliferation of malignancies[40].

Moreover, the vaginal microbiota plays a significant role in the health and disease of the female reproductive tract. Next-generation sequencing techniques based upon analysis of bacterial 16S rRNA genes enable in-depth study of the vaginal microbial community structure, which is not possible with standard culture-based microbiological techniques. There is emerging evidence that increased diversity of vaginal microbiota together with reduced relative abundance of *Lactobacillus* spp. is involved in HPV acquisition and persistence[7]. Actually, a recent study of women with low- and high-grade cervical dysplasia and invasive cervical carcinoma, revealed that elevated vaginal pH and decreased *Lactobacillus* dominance were associated with severity of the cervical neoplasm[41]. Furthermore, a dysbiotic, non-*Lactobacillus*-dominant and highly diverse vaginal microbiota has been linked to the increasing severity of precancerous cervical intraepithelial neoplasia (CIN)[16]. Interestingly, the HPV-positive women recruited to the study presented a dysbiotic vaginal microbiota dominated by *Proteobacteria* and with a reduced abundance of *Firmicutes*. The results in this study revealed that treatment with Papilocare vaginal gel led to a shift towards the vaginal microbiota composition found in healthy women, with a reduction in microbial abundance, evenness and diversity. Moreover, the treatment significantly reduced the abundance of species associated with HPV infection such as *Gardnerella vaginalis*[42] and increased the proportion of *Lactobacillus* spp. Currently, *Lactobacillus* depletion is a well-known feature of vaginal dysbiosis and abnormal vaginal microbiome[43]. These results are in accordance with our previous study in which gel-treatment of asymptomatic healthy women improved vaginal dysbiosis[28].

These results should be interpreted considering the exploratory pilot nature of the study, the single-centre and open label design and especially the short-term use of the vaginal gel product. Nevertheless, HPV-positive women without cytological and colposcopy abnormalities were carefully selected and, to the best of our knowledge, this is the first study to assess cervical re-epithelialization and changes in the vaginal microbiota associated with the use of a natural hydrating and moisturizing gel; whose components have proven anti-inflammatory, epithelizing and immunomodulatory effects, together with the capacity to preserve the vaginal microbiota balance. These encouraging preliminary findings provide the basis for designing an investigational plan involving clinical trials and observational studies, which is currently underway. Subsequently, confirmatory data will support its potential use in HPV-positive patients. In addition, this product will have the advantage of an easy and convenient administration protocol.

In conclusion, Papilocare® significantly improved cervix epithelialization among HPV-positive women without cervical lesions. The higher abundance of specific species of the genus *Lactobacillus* after the treatment suggests a restoration of the altered microbiota composition in these women. These results might account for the mechanisms of action of Papilocare® underlying its positive effects on repairing cervical lesions.

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Authors' Contributions

Conceptualization, S.G., L.S., J.C., A.R..N and J.G.; methodology, T.V., J.G-M., F.A., R.M. and M.E.R.C.; validation, A.R.N. and J.G.; resources, J.C., L.S. and J.G.; writing-original draft preparation, M.E.R.C., J.G. and A.R.N.; writing-review and editing, T.V., J.G. and A.R.N.; supervision and funding acquisition, J.C., J.G. and A.R.N. All authors have read and agree with the published version of the manuscript.

Interests conflict

J.C., S.G. and L.S. have been speakers at Procare Health events in several conferences. The remaining authors have no conflict of interest to declare.

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