




Clinical and laboratory results in vaginal wall restoration using a fractional-pixel-CO₂ laser: histological findings and changes in the Ki67 protein and telomere length

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Received: 9 March 2022 / Accepted: 2 September 2023

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Abstract

Thermal deposition of laser energy in the vaginal epithelium in genitourinary syndrome of menopause (GSM) results in clinical and biological effects, but many cellular and molecular changes indicating cell proliferation or senescence inhibition are unknown. The aim of this study is to evaluate the clinical efficacy of the fractional-pixel-CO₂ laser in the possible improvement of GSM signs and symptoms that can be correlated with histological changes or with cellular or molecular indicators of restoration. A detailed prospective study was designed to assess 17 women diagnosed with GSM who were treated intravaginally with two laser sessions. Seven non-treated women diagnosed with GSM were used as controls. Three validated outcome questionnaires for assessment of quality of sexual life and urinary incontinence were performed. Vaginal biopsies were collected before the first laser treatment and 4 months following the second session. Histological status, elastin, collagen, and hyaluronic acid content of the biopsies were also evaluated. Cell proliferation was assessed by Ki67 staining. Telomere length (TL) was measured by qPCR. The results show an improvement of the clinical symptoms of GSM ($p < 0.05$), vaginal epithelium recovery and enhancement of collagen ($p < 0.05$), elastic fibers ($p < 0.005$), and hyaluronic acid ($p < 0.0005$) content in the lamina propria after fractional-pixel-CO₂ laser treatment. The laser treatment induced a significant rise on the TL of vaginal epithelial cells (VECs), and a positive correlation was found between the improvements of the collagen and hyaluronic acid content and TL changes ($r = 0.82$, $p < 0.05$; $r = 0.38$, $p < 0.05$). The percentage of proliferative Ki67-positive VECs was increased in patients whose vaginal TL lengthened after laser treatment ($p < 0.05$). In conclusion, the results indicate that laser treatment may induce restoration of the vaginal epithelium which is associated to increased TL and proliferation in the VECs. Performing a TL assay could be a suitable tool to evaluate the efficacy of vaginal laser treatment.

Keywords Vaginal wall restoration · Genitourinary syndrome of menopause (GSM) · Fractional CO₂-laser · Telomere length · Ki67 · Vaginal tissue

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Introduction

Genitourinary syndrome of menopause (GSM) is a condition reported in 50% of women who experience different symptoms associated to it such as vulvovaginal atrophy (VVA), atrophic vaginitis, and urinary disorders, which appears as a result from a reduction of estrogen levels in the urogenital tissues [1]. Its definition derives from a number of signs and symptoms that cause several changes not only to the vulva or vagina but also to the urethra and bladder, including dryness due to poor lubrication, altered sexual function, burning and irritation on the area leading to discomfort or pain and in some cases urinary symptoms of urgency, as well as recurrent infection of the urinary

tract or dysuria [1, 2]. Women can be clinically diagnosed with GSM after physical examination and laboratory tests, and they may present some or all distinctive signs and symptoms, which are annoying and are not associated to any other disorder. Some of the typical findings include a very light colored, dry vaginal epithelium that shows a smooth and shiny appearance without tissue rugate.

The use of a fractional CO₂ laser with the purpose of resurfacing the skin has demonstrated after different histological evaluations that there is a direct correlation between energy density and ablative depth of penetration. The subsequent wound healing process showed a post-treatment period of 1–3 days in which granulation tissue appeared followed by progressive neocollagenesis and dermal remodeling up to 30 days after treatment. As observed after standard ablative CO₂ laser resurfacing, the process of neocollagenesis continued for several months thereafter [3]. Moreover, the fractional CO₂ laser proved a micro-ablative effect on the vaginal wall which increased the thickness of the epithelium as well as levels of glycogen in the epithelial cells, and a large number of glycogen-rich cells were shed at the epithelial surface [4, 5]. Increased extracellular matrix content, including collagen and ground substance, and also active fibroblasts have been detected in the connective tissue of the lamina propria. Additionally, after the treatment was performed, it was possible to observe newly-formed connective tissue papillae in addition to undulated epithelium and typical blood capillaries penetrating inside the papillae [6, 7].

Telomeres in humans have a specific nucleotide sequence composed by thousands of repetitions of the TTAGGG hexanucleotide [8]. In order to form the telomere-specific chromatin structure, a protein complex named shelterin associates to this DNA region. Telomeres are essential for the stability of chromosomes and genomes and also protect the chromosomal ends from degradation [9]. However, when in absence, the cell recognizes chromosomal ends as damaged DNA and can be degraded or recombined with other chromosomal ends resulting in the fusion and reorganization of chromosomes [10]. It is therefore of critical importance to maintain telomeres for the genetic stability and proliferative capacity of cells and organisms. The contribution of a specific enzymatic machinery, telomerase complex, is required for the replication of telomeric DNA as it elongates the telomeres by a replication-independent mechanism [11].

During embryonic development, telomerase components, in particular the TERT gene, are expressed to high relative levels allowing high cell proliferation rates; however, TERT expression is repressed in most human adult cells [12] and can only be found in germinal and stem cells especially in those of highly proliferative tissues such as bone marrow, epithelia, and lymphocytes [13]. Very low TERT levels are expressed in the rest of the cells, and their telomeres get progressively shortened after each cell division, turning

them into markers for cellular aging [14]. Several cross-sectional studies document very well a correlation between TL and chronological age, but the rate of telomere attrition was shown to vary throughout a human lifetime [15, 16]. Indeed, some studies indicated that postmenopausal women experienced greater TL decline [17].

The objective of this study is to evaluate the efficacy of the vaginal wall restoration using a fractional micro-ablative CO₂ laser, especially in relation to the improvement of signs and symptoms associated with GSM. In order to establish possible clinical-pathological correlations, histological changes in the treated vaginal areas were analyzed in detail. The proliferative status of the vaginal epithelium was evaluated by quantifying Ki67 protein. Telomere length was measured to assess the degree of cellular aging.

Material and methods

Design, patients, and laser treatment

A detailed prospective study was designed and conducted between September 2017 and December 2018 at the Helicopteros Sanitarios Hospital (Marbella, Spain). The study included 24 non-estrogenized postmenopausal women between the ages of 47 and 80 years, diagnosed with GSM. Seventeen women were treated with laser and 7 served as non-treated controls. Both groups were considered clinically comparable, with all women presenting with at least three GSM symptoms or two symptoms and one GSM sign. Characteristics of the participants included in this study are summarized in Table 1. The study was approved by the local Ethics Committee of the Helicopteros Sanitarios Hospital according to the 1964 Declaration of Helsinki, and all samples were collected after obtaining informed consent (date: 09 June 2017 no. HSH/2017/008).

Before starting the study, each patient was evaluated by a gynecologist in order to rule out any contraindication according to the inclusion/exclusion criteria. Examination included vaginal inspection with speculum and a preliminary documentation with a dedicated camera (FemiCam, Alma Lasers, Inc.). All patients had a Pap smear test and pelvic examination within a month of the procedure. Exclusion criteria included impaired immune system, general or local vaginal bacterial or viral infection, radiation therapy on the pelvic floor, burns in pelvic floor, transvaginal mesh sling, pelvic organ prolapse upper to stage II, and uncontrolled diabetes.

Patients underwent fractional-pixel-CO₂ laser treatment in the vaginal wall with a robotic-controlled handpiece (FemiLift Smart, Alma Lasers Inc.) which provides a uniform treatment of the entire vaginal wall [18]. Three passes with a per-pixel energy of 90 to 110 mJ were performed in

Table 1 Characteristics of the study participants

	Patients (laser treated)	Controls (untreated)
<i>N</i>	17	7
Age		
<i>mean (SD)</i>	55.7 (8.5)	59.8 (7.2)
<i>min–max</i>	47–80	51–72
Age at menopause		
<i>mean (SD)</i>	47.7 (5.4)	47.3 (3.2)
<i>min–max</i>	38–56	41–50
Age at menarche		
<i>mean (SD)</i>	12.3 (1.6)	12.9 (1.7)
<i>min–max</i>	10–16	11–16
Years since menopause		
<i>mean (SD)</i>	8.9 (8.5)	13.7 (7.8)
<i>min–max</i>	1–29	3–24
No. vaginal delivery		
<i>mean (SD)</i>	1.1 (1.2)	0.7 (1)
<i>min–max</i>	0–3	0–2

SD standard deviation

the vagina with the laser in mode-R (repeat 360°) at high speed. The pixelated technology delivers the laser beam through a fractionating lens, splitting the energy into a matrix of 9×9 micro-beams, resulting in 81 micro-ablative “dots” per square centimeter. Immediately after the introitus area was treated with a 7×7 stamp handpiece using 50 mJ/pixel (49 micro-ablative “dots” per square centimeter). Patients were examined after 10 days to check the clinical evolution following procedure.

No adverse effects were observed, except for mild to moderate discomfort during the treatment, which was well tolerated in all patients by avoiding the application of high energies around the introitus. Temporary side effects such as erythema and mild swelling were also observed after the treatment, which, upon examination, resolved within

24–48 h. No complications of any kind occurred in the treated patients.

The laser procedure was repeated after 4–6 weeks with the same protocol. Before the first laser treatment and 4 months following the second session, biopsies of the vaginal wall were obtained from treated patients. A sterilized and disposable biopsy punch (Stiefel) of 4-mm diameter was used to obtain the samples on the vaginal mucosa. Biopsies were taken as follows: 2 cm from the border of the introit to inside the vagina on 9 h of the clock for the first sample (baseline) and 7 h of the clock for the second sample (post-treatment).

Buccal cells were collected by scrubbing the inner cheek with oral swabs (Isohelix, Cell Projects Ltd) [19], and vaginal epithelial cells were obtained with a cervical brush from all study participants.

Clinical assessment

Evaluation of treatment efficacy was performed using three validated outcome questionnaires for the assessment of quality of life in sexual function and urinary incontinence: International Consultation on Incontinence Questionnaire-Short Form (ICIQ-SF) [20], Sandvik severity test [21], and the Pelvic Organ Prolapse/Urinary Incontinence Sexual Questionnaire (PISQ-12) [22]. Scores were obtained from the questionnaires completed by the patients before the first laser treatment and after 4 months from the second laser treatment. The differences in scores allow the quantification of treatment outcomes and the degree of clinical symptoms improvements.

Histological analyses

Vaginal biopsies were fixed in 3.7% formaldehyde solution and embedded in paraffin. Vaginal tissue sections (5 μ) were stained with hematoxylin–eosin for visualization of the vaginal tissue structure; Elastica-van-Gieson staining kit (Merck

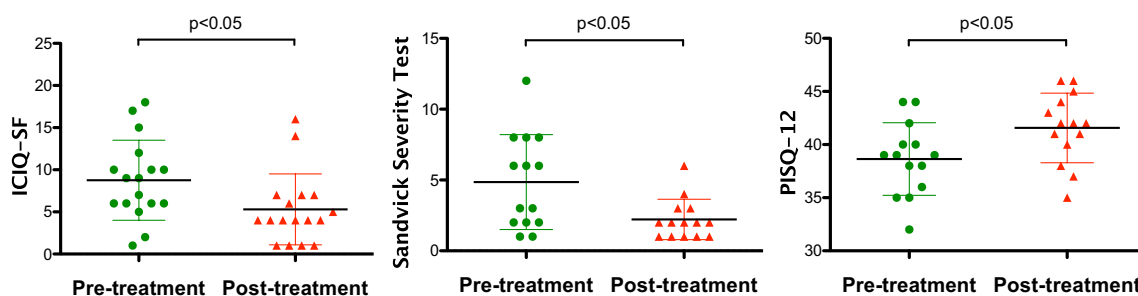
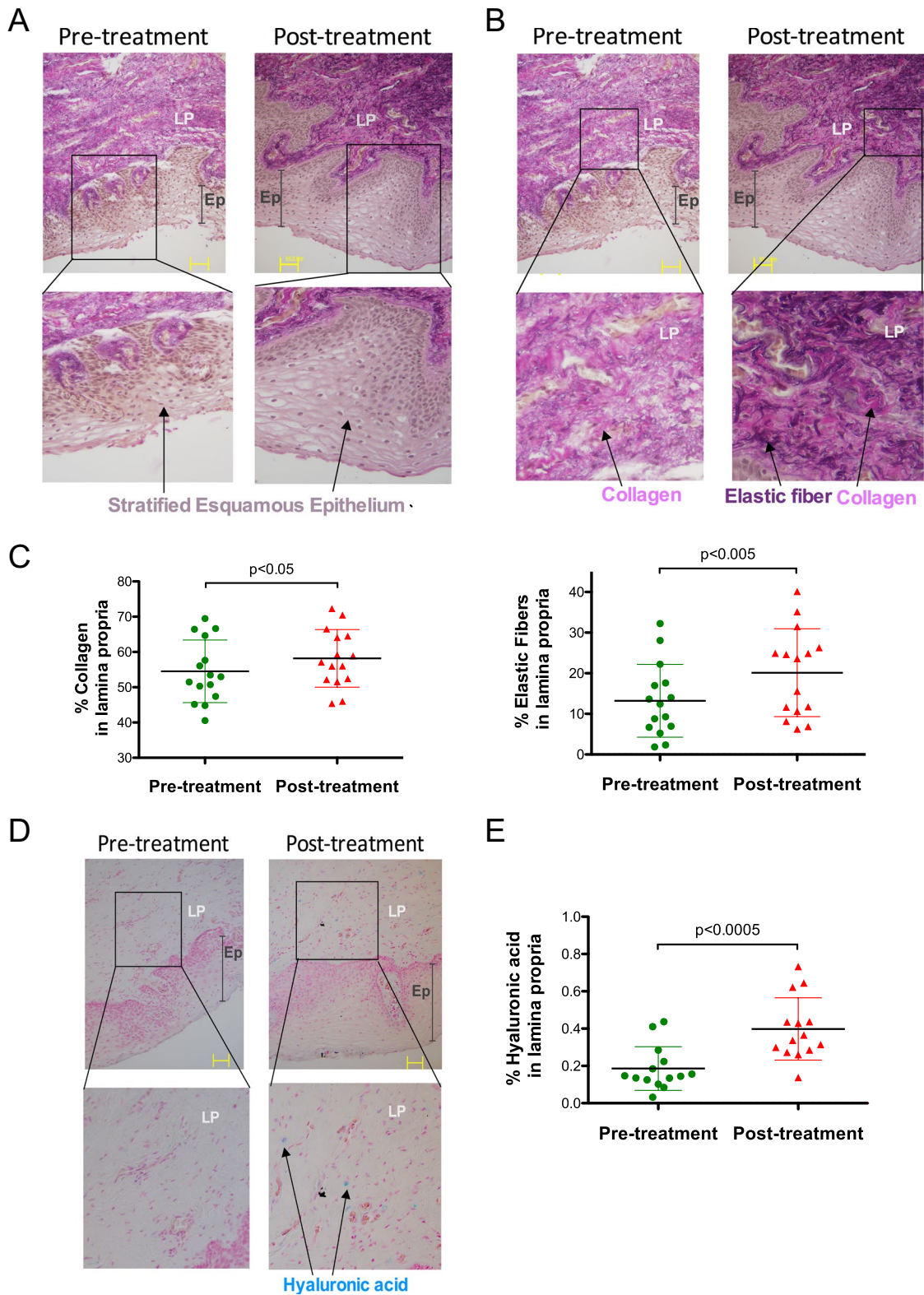


Fig. 1 Efficacy of CO₂ laser in the improvement of clinical symptoms. ICIQ-SF (left), Sandvik severity test (middle), and PISQ-12 (right) scores obtained before treatment (pre-treatment) and after 4 months from a second laser treatment (post-treatment). Each sym-

bol represents the value obtained from a single individual, and mean values ±SD is represented by a black line. Significant differences were obtained using the Wilcoxon comparison test ($p < 0.05$)



Millipore) for the detection of dark blue-stained elastic fibers, magenta-stained collagen, and brown nuclei; and Alcian blue with nuclear fast Red staining for the analysis of the hyaluronic acid (mucopolysaccharides) content of the tissue

[23, 24]. Images were acquired using an axiophot microscope (Zeiss) with a $\times 20$ objective lens. The area occupied by the collagen (magenta-stained), elastic fibers (dark blue-stained), or hyaluronic acid (blue) in each microscopy image

Fig. 2 Histological analysis of the effect of laser treatment on the vaginal wall. **A** representative microscopy images of Elastic-van Gieson-stained vaginal biopsies before (pre-treatment) and after (post-treatment) laser treatment of the vaginal epithelium (Ep) and **B** lamina propria (LP). Amplifications of the framed regions of the images are showed below. **C** percentage of the area of lamina propria occupied by collagen (left panel) or elastic fibers (right panel) in each patient before and after laser treatment. The mean \pm SD is represented with a black line. **D** representative microscopy images of Alcian blue with nuclear fast Red stained vaginal biopsies before and after laser treatment. Yellow scale bar: 50 μ m. **E** Percentage of the area of lamina propria occupied by the hyaluronic acid in each patient before and after laser treatment. Mean \pm SD is represented with a black line

was quantified using the Fiji software program (v. 1.0) with the Colour Deconvolution plugin (v. 3.0.2) for the stain separation [25].

Immunohistochemical analysis of Ki67

Paraffin-embedded vaginal tissue sections (5 μ m) were dewaxed and rehydrated. Antigen retrieval was performed using an unmasking buffer pH 9 (10-mM Tris base, 1-mM EDTA solution, 0.05% TWEEN 20) in a microwave oven for 20 min [26]. Sections were blocked with a Goat Serum blocking solution (5% Goat Serum, 1% BSA, 0.2% Triton X-100 in PBS pH 7.1) and then incubated with a dilution 1:100 of the mouse monoclonal anti-Ki67 (BD-cat# 550609) at 4 °C overnight. Sections were washed and incubated with the secondary antibody Goat anti-rabbit IgG AF488 (Molecular Probes) for 45 min at room temperature. ToPro-3 (Molecular Probes) was used for DNA nuclear staining, and slides were then mounted using ProLong Diamond antifade reagent (Molecular Probes).

All images were acquired using a LSM710 (Zeiss) confocal microscope and analyzed with Fiji software.

Telomere length assay

Genomic DNA from vaginal epithelium and buccal cells was extracted using a commercial DNA isolation kit (Isohelix, Cell Projects Ltd), and the relative TL was measured by a quantitative PCR method, as previously described [27, 28]. Using this TL assay, a T/S ratio was obtained for each DNA sample corresponding to the ratio of telomere repeat (T) copy number to *36B4* single-copy gene (S) copy number. A reference DNA from the human cell line MCF-7 was included in each PCR run to allow the quantification of the samples relative to this reference DNA by a standard curve method. All experiments were performed in triplicate and repeated at least twice.

T/S ratios were transformed to kilobases (kb) using a regression equation ($Kb = (3,86 \times T/S) + 1,89$) calculated with the known absolute TL values in kilobases of several DNA samples previously analyzed by Southern-blots (TeloTAGGG TL Assay, Roche) and qPCR [29].

Statistical analysis

Statistical analysis was performed with Prism-5 (GraphPad Software). Comparisons of unpaired groups were performed with Welch's unpaired *t*-test, and the Wilcoxon test was used for comparisons of paired groups. Correlations were calculated with the Pearson and Spearman tests; $p < 0.05$ was considered significant.

Results

Clinical efficacy of vaginal laser treatment

Scores obtained from the three questionnaires before the laser treatment and after 4 months from the last treatment session showed significant differences. The mean values of the score of urinary incontinence questionnaires, ICIQ-SF (before, mean \pm SD: 8.7 ± 4.7 ; after, mean \pm SD: 5.2 ± 4.2) and Sandvik severity test (before, mean \pm SD: 4.8 ± 3.3 ; after, mean \pm SD: 2.2 ± 1.4), were significantly decreased after treatment ($p < 0.05$, in both cases). Sexual function evaluated with PISQ-12 questionnaire was also significantly improved as shown by the increase of PISQ-12 scores after laser treatment (before, mean \pm SD: 38.6 ± 3.4 ; after, mean \pm SD: 41.5 ± 3.2) ($p < 0.05$) (Fig. 1).

Histological changes of vaginal tissues

Histological analysis of vaginal wall biopsies revealed visible changes such as increased thickness of the epithelium (before, mean \pm SD: 145.5 ± 41.5 μ m; after, mean \pm SD: 285.8 ± 87.9 μ m) ($p < 0.05$) (Fig. 2A) and increased collagen ($p < 0.05$) and elastin fibers ($p < 0.005$) content in the connective tissue of the lamina propria (Fig. 2B and C). Hyaluronic acid content representing the presence of mucopolysaccharides in the vaginal tissue is visible by Alcian blue staining (Fig. 2D) showing a very significant increase ($p < 0.0005$) of the number of vaginal cells with cytoplasmic blue positive hyaluronic acid staining (Fig. 2E).

Effect of laser treatment in the telomere length of vaginal epithelium

In order to analyze the possible effect of laser treatment in the vaginal TL of patients with GSM, TL of buccal and vaginal epithelial cells of 7 untreated controls was initially measured (Fig. 3A) in two follow-up time points ($t = 0$ and $t = 4$ months) by qPCR as described in methods. Buccal and vaginal epithelial TL of control women decreased over time (buccal, mean \pm SD: -137 ± 156 bp; vaginal, mean \pm SD: -263 ± 375 bp) ($p < 0.05$) (Fig. 3A), and a

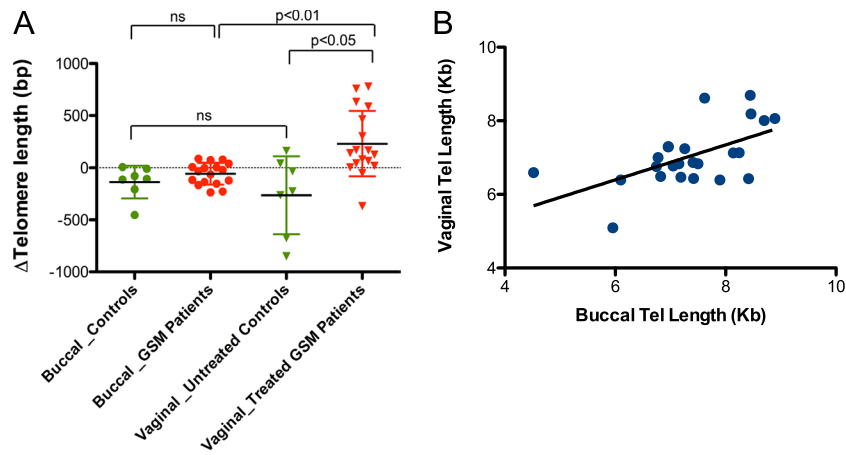


Fig. 3 Effect of laser treatment in the TL of vaginal epithelium. **A** differences of TL values (bp) obtained over the follow-up time points in untreated buccal and vaginal epithelial cells and laser-treated vaginal cells. Each symbol represents one individual, and mean val-

ues \pm SD is represented by a black line. **B** the correlation between the TL of buccal and vaginal epithelial cells from the untreated controls and patients before laser treatment. Significant positive correlation was observed ($r=0.579$, $p<0.005$)

significant correlation was obtained between the TL of the two different epithelial cell samples, buccal and vaginal, collected from each control (Fig. 3B).

In a similar TL analysis with the cohort of 17 patients (Fig. 3A), it was observed that TL of buccal cells (non-laser

treated area) for most patients decreased or was maintained with few changes after 4 months (buccal, mean \pm SD: -57 ± 104 bp). However, in vaginal epithelial cells of the laser treated patients, there is an increased TL of 230 base pairs average value (vaginal treated, mean \pm SD: $+230 \pm 313$ bp) ($p<0.01$) (Fig. 3A).

Fig. 4 Effect of laser treatment on the proliferation levels of vaginal epithelium. Representative images of ToPro-3 nuclear staining (magenta) and Ki67 staining (green) of vaginal biopsies before and after laser treatment. Upper panels: vaginal biopsies from a patient with an increase of the vaginal TL higher than 150 bp after laser treatment. Lower panels: vaginal biopsies from a patient with decrease or slightly increment (<150 bp) of the TL of vaginal epithelium after laser treatment. Percentages of Ki67 positive nuclei are showed. The vaginal epithelium region (Ep) and basal layer (Bl) are indicated

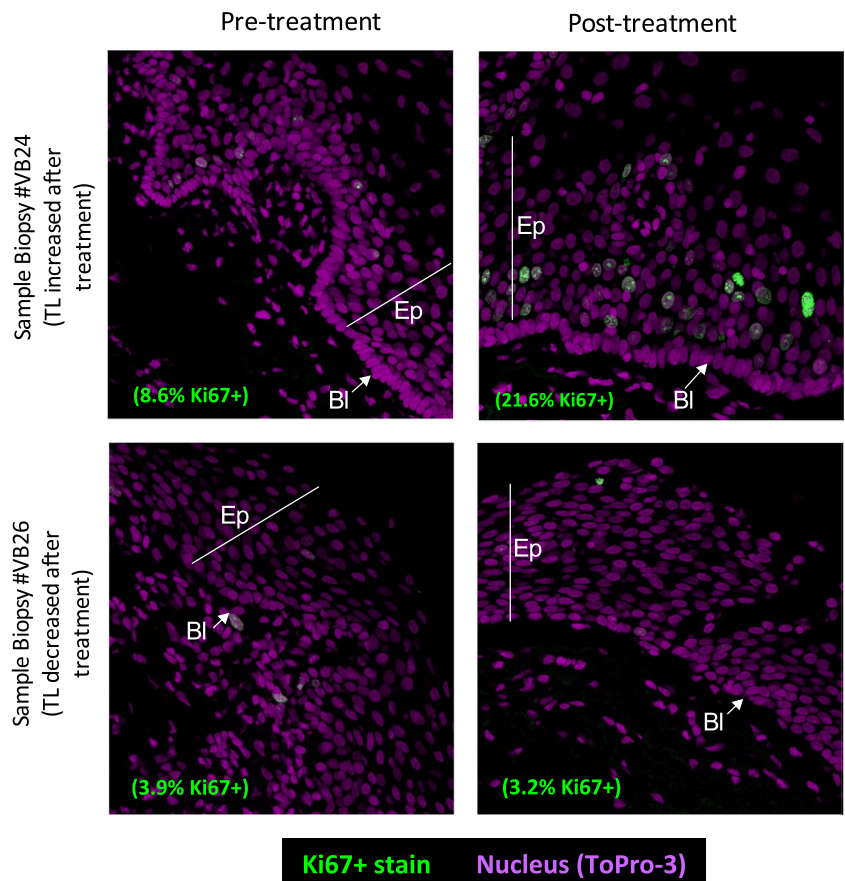


Table 2 Proliferation status of vaginal epithelial cells from extreme representative samples

Sample	Time (month) of sample collection	Vaginal epithelial cells		
		Δ Tel length (bp) changes after laser treatment	%Ki67 positive cells	Δ %Ki67 positive cells after laser treatment
Samples with Tel length increase > 150 bp after laser treatment				
#VB015	Pre-treatment $t=0$	463	9	9
	Post-treatment $t=4$		18	
#VB019	Pre-treatment $t=0$	779	7	17
	Post-treatment $t=4$		24	
#VB024	Pre-treatment $t=0$	585	9	13
	Post-treatment $t=4$		22	
Samples with Tel length increase < 150 bp after laser-treatment				
#VB026	Pre-treatment $t=0$	-51	4	0
	Post-treatment $t=4$		4	
#VB028	Pre-treatment $t=0$	5	25	2
	Post-treatment $t=4$		27	

bp base pair, *t* time, *Tel* Telomere

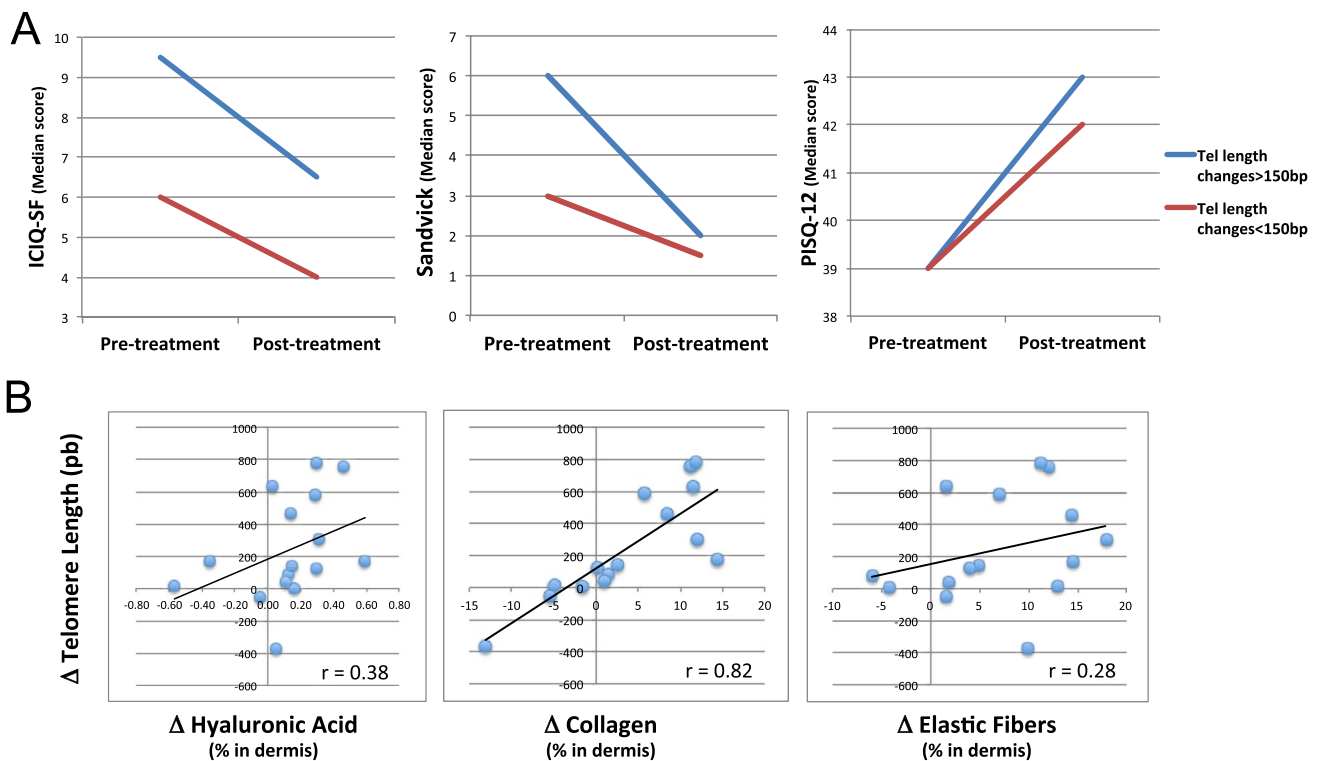


Fig. 5 Relation between the improvements of clinical symptoms and the degree of vaginal TL changes or the histological modifications after laser treatment. **A** lines depict the ICIQ-SF (left panel), Sandvik severity test (middle panel), and PISQ-12 (right panel) median scores obtained before (pre-treatment) and after laser treatment (post-treatment) for the group of patients with an increase of the vaginal TL higher than 150 bp (blue line) and for the patients with a decrease or

slightly increment (< 150 bp) of the vaginal TL (red line) after laser treatment. **B** correlations between the vaginal changes of hyaluronic acid (left panel), collagen (middle panel), and elastic fibers (right panel) content and the TL changes of vaginal epithelial cells after laser treatment. Significant correlation was observed especially with collagen increases

Effect of laser treatment on cell proliferation of vaginal epithelium

To evaluate the proliferative status of the vaginal epithelium, the number of vaginal proliferative cells by quantification of Ki67 immunofluorescence staining of vaginal biopsies before and after laser treatment was compared [30]. A significant increase of the percentage of Ki67-positive epithelial cells was observed in three vaginal biopsies of patients whose vaginal TL increased significantly (> 150 bp increment) after laser treatment (mean \pm SD: $13 \pm 4\%$) (Fig. 4, Table 2). In contrast, no changes in Ki67-expression levels were observed in vaginal epithelial cells whose TL was slightly or not affected (< 150 bp) after laser treatment (mean \pm SD: $1 \pm 1.4\%$).

Correlation between the improvements of clinical symptoms and the degree of vaginal TL changes after laser treatment

In the group of patients with a higher increment of the vaginal TL (> 150 bp) after laser treatment, the median of the urinary incontinence ICIQ-SF and Sandvik test scores were initially higher, indicating worse urinary incontinence symptoms in these patients (Fig. 5A). However, the laser treatment produces a higher decrease of these scores and a better improvement of the urinary clinical symptoms than the group of patients with slight (< 150 bp) vaginal TL changes after treatment.

The median of the sexual function PISQ-12 score was initially similar in both groups of patients, but the improvement of the quality of sexual life after vaginal laser treatment was better in the patient group with a higher increase of the vaginal TL (Fig. 5A).

Correlation between histological changes and TL increase after vaginal laser treatment

A significant positive correlation was obtained between the histological changes in collagen content of the vaginal connective tissues and the level of the TL changes after laser treatment ($r = 0.82$, $p < 0.05$). A less significant correlation was observed with the hyaluronic acid ($r = 0.38$, $p < 0.05$) (Fig. 5B).

Discussion

The primary aim of this study was to evaluate the efficacy of vaginal pixel-CO₂ laser treatment of GSM analyzing several clinical, histological, and age-related molecular parameters. Our results support previous observations about the effects

of various energy-based delivery modes to improve the clinical symptoms of GSM and induce histological changes of vaginal tissues. Additionally, a cellular rejuvenation effect produced by this pixel-CO₂ laser treatment has been observed through the increase of the TL and proliferative status of the vaginal epithelial cells. A positive correlation between clinical symptoms improvement, vaginal tissue remodeling, and telomere length changes induced by laser treatment is documented.

Several studies have shown that vaginal laser therapies stimulate the synthesis of new collagen at the extracellular matrix in the vaginal connective tissue, thickening the vaginal wall epithelium and induce the formation of new papillae, improve vascularization by increasing angiogenesis in the lamina propria, restore the glycogen and acidic pH, and increase hydration of the vaginal epithelium [6, 7]. The effectiveness of this treatment has been demonstrated in previous studies on postmenopausal women who suffer from serious symptoms of vaginal atrophy [31–34], and its clinical efficacy was also confirmed in patients for whom estrogen treatment is contraindicated such as breast cancer survivors [35, 36].

Some molecular mechanisms responsible for restoration of the postmenopausal atrophic vaginal epithelium to a healthy epithelium after fractional CO₂ laser treatment have been described. Controlled thermal deposition of fractional laser pulses induces fibroblast growth stimulation, secretion of heat shock proteins (HSPs), and a local increase of several cytokines stimulating the cell repair process [37, 38].

These observations described by several authors are in agreement with our results. An increase is verified in collagen and elastin fibers content and the vaginal epithelium thickness of most patients after laser treatment. In terms of clinical effectiveness, an improvement in the mean values of urinary incontinence is reported according to the questionnaires, ICIQ-SF and Sandvik test.

Previous reports had approached the study of expression of Ki67-proliferation marker in vaginal biopsies from women with stress urinary incontinence treated with laser therapies and showed that the index of Ki67 labeled nuclei in the epithelium increased significantly after 1–2 months of treatment [30]. Accordingly, with these results, we have also detected an increase in Ki67 expression levels in those patients who presented an improvement in clinical symptoms and biological parameters.

This longitudinal study is the first to assess in vivo changes in TL in the vaginal epithelium of postmenopausal women. Notably, no telomere shortening was observed in most GSM patients after laser treatment in comparison with the telomere attrition detected in the untreated age-matched control group analyzed within the same time period. The novelty of the present study is also based on the correlation observed between the commonly used

indicators for laser treatment and the increase in TL in vaginal cells. This recovery in TL should be associated to the increased proliferation measured by Ki67-positive cells. Some studies conducted previously indicated that as an effect of postmenopausal hormonal deprivation and senescence, vulvovaginal atrophy may occur as the anatomy, and function of urogenital tissues are significantly affected [37]. Our results suggest that heat shock induced by pixel-CO₂ laser treatment is stimulating the increase in the number of Ki67-positive cells. The cells could be derived from activation of quiescent stem cells with longer telomeres that might result in an increase in overall TL of treated tissues. Alternatively, some studies indicate that low power laser treatment increases levels of TRF1 and TRF2, both proteins contribute to telomere protection against DNA damage [39–41]. Additionally, changes in heat shock proteins have been described to be activated after CO₂ laser [42–44]. And since HSP90 facilitates the assembly of telomerase protein, this would also play a role in the increased telomere length in the vaginal epithelia [45, 46]. These two mechanisms could contribute to an activation of telomerase and increase in telomere length. Future experiments are needed to identify the molecular mechanisms involved in the rejuvenation effect observed in the patients treated with pixel-CO₂ laser.

TL would be a useful non-invasive biomarker of treatment efficacy of the fractional CO₂ laser in the GSM. TL analysis by qPCR used in this study has shown a high correlation with histological changes and clinical symptom scores. This assay is a fast, simple, non-invasive, and a cheap one to check the efficacy of vaginal energy-based treatments. Different authors have highlighted the importance of using objective assessment methods of vaginal atrophy compared with subjective methods of clinical improvement [47–49]. Our results may offer an additional quantitative and objective method to assess vaginal wall changes following various treatment modalities. Telomere maintenance could be an end point quantitative biomarker to assess improvement in regeneration of vaginal tissues in GSM after energy-based treatments. More studies are necessary by increasing the number of patients and measurements evaluating by TL analysis to confirm our observations described for the first time in this manuscript.

Some prospective clinical trials report that the fractional CO₂ laser appears to be safe and effective, but conflicting messages still exist about the duration of effects suggesting that maintenance sessions are likely required to extend these results past the 6–12 months [5]. Objective parameters such as non-invasive vaginal wall optical biopsies [49] or measurements of vaginal TL may provide scientific value to the common subjective questionnaires.

Conclusion

The effects of the treatment with the fractional-pixel-CO₂ laser lead to clinical improvements correlated with histological and biopathological indicators. The most notable association in this study is the one found between the increased TL and both clinical and histological indicators of vaginal restoration. This increase in TL can be a readout of vaginal cells proliferation as indicated by the results of Ki67 expression. Telomere length assay may be used as a non-invasive, low cost, reproducible, fast, and quantitative assay suitable for routinely studies and reports for patients treated with vaginal CO₂ laser.

Acknowledgements We gratefully acknowledge the IIB-CSIC and CNB-CSIC histology facilities for the preparation and the histological assays of biological samples. We thank Monica Martin-Belinchón for helping at the IIB-CSIC confocal facility.

Author contribution Virginia Benitez-Roig (VBR), Antoaneta Cosmina-Timircan (ACT), Elena G Arias Salgado (EGAS), and Rosario Perona (RP) design and perform the experimental work. VBR, EGAS, and RP wrote the manuscript. Pedro A. Martínez-Carpio (PAMC) and Mario A. Trelles (MAT) review and gave advice to the manuscript.

Funding This research was funded by the Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III, Spain, grant no. PI20-00335 and co-funded by European Regional Development (FEDER) funds.

Declarations

Conflict of interest The authors declare no competing interests.

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