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ORIGINAL ARTICLE

Ozonides extravirgin olive oil as an alternative to steroids in controlling proliferative behavior in penile lichen sclerosus: a comparative study in pediatric population

Fabiola Cassaro[1,2] · Antonio Ieni[3] · Monica Currò[1] · Cristina Pizzimenti[3] · Natasha Irrera[4] · Federico Vaccaro[1] · Giulia Maisano[2] · Santi D'Antoni[2] · Pietro Impellizzeri[2] · Carmelo Romeo[2] · Salvatore Arena[2]

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Abstract

Purpose Lichen sclerosus (LS) is a chronic inflammatory skin condition affecting genital areas and associated with autoimmune processes. Circumcision is the primary treatment for phimosis in pediatric LS, and corticosteroids are considered the best choice for medical management. Recently, Ozoile, stable ozonides obtained through green technology from extravirgin olive oil (+OIL®), according to a patented process, has emerged as a potential alternative with fewer long-term side effects compared to corticosteroids. The aim of the study was to compare the immune and anti-proliferative effects of the pre-operative topical use of Ozoile and corticosteroids in children with phimosis associated with LS undergoing circumcision.

Methods Thirty-six male patients aged 3–9 years with phimosis linked to LS were divided into three groups: Group A (no pre-operative treatment), Group B (0.1% mometasone furoate), Group C (Ozoile). Post-circumcision foreskin samples were analyzed for immune cell infiltration (CD8 +, CD57 +) and proliferation markers (Ki67,p53). Non-parametric ANOVA (Kruskal–Wallis test) and Dunn tests were used and p value less than 0.05 was considered statistically significant.

Results Histological analysis showed acanthotic epidermis and lymphoid infiltrate in Group A, with reduced lymphocytes and markers in Groups B and C. Groups B and C showed no significant differences in CD8 + and CD57 + lymphocytes ($p = 0.34$, $p = 0.54$, respectively), but Group C had significantly lower proliferation markers Ki-67 ($p = 0.035$) and p53 ($p = 0.039$).

Conclusion Both Ozoile and corticosteroids reduced inflammatory and proliferative activity in pediatric LS patients. Ozoile showed superior suppression of proliferative markers, suggesting potential benefits for patients at risk of steroid-related side effects. Further research is needed to confirm long-term benefits.

Keywords Lichen sclerosus · Ozoile · Corticosteroids · Phimosis · Children · Spinocellular carcinoma

Abbreviations

CD57	Cluster of differentiation 57
CD8	Cluster of differentiation 8
HPV	Human papillomavirus
IFN-γ	Interferon gamma
IL-1β	Interleukin 1 beta
Ki-67	Cellular proliferation marker Ki-67
LS	Lichen sclerosus
Ozoile	Ozonized olive oil
p53	Tumor suppressor protein p53
TG2	Transglutaminase 2
TNF-α	Tumor necrosis factor alpha

Introduction

Lichen sclerosus (LS) was first documented by the dermatologist Hallopeau in 1887 [1]; however, the first recorded case of LS in the pediatric population was only reported by Caterall and Oates in 1962 [2]. Although the exact physiopathology of LS is still not fully understood, there is a growing body of evidence suggesting a link between autoimmunity and genetic predisposition [3]. This connection highlights the multifactorial nature of LS and underscores the importance of further research to elucidate its mechanisms. The hypothesis is further supported by frequent association with other autoimmune disorders, such as thyroid diseases, pernicious anemia, lichen planus, lichen simplex, and vitiligo. Notably, LS shares the DQ7 Human Leukocyte Antigen (HLA) with autoimmune thyroiditis, and additional studies

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also demonstrated the association with other HLAs, such as DRB1-04, DRB1-07, and DRB4 [4].

Histologically, LS is characterized by distinctive features, including epidermal atrophy and hyperkeratosis, alongside a subepidermal layer marked by homogenized collagen and edema. A prominent T-cell lymphocytic infiltrate is often observed in the dermis, predominantly involving CD8- and CD57-positive cells [5, 6]. These histopathological findings are further complemented by increased levels of proliferative markers, such as Ki-67 and p53, in cases of juvenile penile LS, suggesting a possible connection to malignant transformation, specifically spinocellular carcinoma, later in adulthood [7].

Clinically, LS often goes undetected, as it can be asymptomatic, leading to potential underestimation of its prevalence. This silent presentation makes early diagnosis challenging and increases the risk of complications if left untreated. In males, incidence rates are estimated to vary between 0.07 and 0.3%, with a bimodal distribution primarily affecting young boys and adult men. Interestingly, among circumcised boys presenting with phimosis, LS incidence ranges from 5 to 50%, with the highest prevalence observed around ages 7–8 years [8].

The clinical presentation of LS includes grayish or bluish-white discolorations and sclerotic plaques, earning it the historical names "white spot disease" and “lichen sclerosus et atrophicus.” [9]. In males, initial lesions typically appear in the genital and anal areas, affecting the glans, peri-meatal area, and preputial ring, leading to stenosis and tight phimosis due to a dense fibrotic ring [10].

In women, LS lesions mainly appear in the labia majora and in the anal region, with the progressive involvement of the external vulvar structures with the consequent vaginal narrowing [11]. Moreover, patients could complain of intense itching, burning sensation, severe pain, dyspareunia, and bleeding; frequent secondary lesions due to scratching could be observed, such as adherent areas with a fissure-like appearance and post-inflammatory damage [12].

Thin epidermis consequent to basal cell proliferation (epidermal atrophy) reduction as well as hyperkeratosis, flattened epidermal ridges, dermal elastin reduction, and lymphocytic infiltrate represent key features of LS.

Persistent inflammation and chronic tissue damage associated with LS elevate the risk of developing squamous cell carcinoma (SCC) in affected genital areas over time. Thus, vigilant monitoring and timely intervention are essential to manage potential complications and mitigate progression to SCC [13–15].

In LS, there is an increased presence of CD8 + and CD57 + cells in the dermis, and activated T cells, particularly cytotoxic CD8 + cells, are thought to target basal keratinocytes, aided by helper T cells through TH1 cytokine release [16].

Oxidative stress may contribute to the pathogenesis of LS, with lower antioxidant enzyme levels leading to lipid peroxidation, DNA damage, and protein oxidation. Vascular sclerosis in LS also enhances oxidative stress, promoting tumor suppressor gene downregulation and p53 overexpression, which are associated with skin cancer development. The exact mechanisms are still not fully understood [17].

Ki67 is a marker of cell proliferation, expressed during mitosis. Its expression is linked to p53 in several cancers, though the exact relationship between the two is still being studied [18].

For male, circumcision is the recommended treatment of scarring phimosis associated with LS to restore normal penile function [14], especially when conservative treatments are insufficient. The guidelines recommend the use of topical corticosteroids before circumcision to relieve symptoms and prevent further complications, although their long-term use is controversial due to potential side effects and concerns about preventing SCC [18] a similar anti-inflammatory effect was observed with the topical application of Ozoile [19].

Actually, radical excisions of the foreskin remain the only therapeutic option in 96% of cases and the use of topical steroids after circumcision prevents the complications associated with LS.

The aim of the study was to explore the immunohistochemical profile of foreskin tissue in children with LS, particularly focusing on immune responses and cell proliferation markers, and compare the immune and anti-proliferative effects of the pre-operative topical use of Ozoile and corticosteroids in children with phimosis associated with LS undergoing circumcision.

Materials and methods

A total of 36 male patients were enrolled in this study, ages ranging from 3 to 9 years. Inclusion criteria required a confirmed diagnosis of phimosis associated with LS, while exclusion criteria included any prior topical treatment for LS or recent use of immunosuppressive drugs. The cohort was divided into three groups based on the type of treatment they received. Group A consisted of 12 patients who presented with both phimosis and LS but did not receive any prior treatment before undergoing surgery. Group B and Group C comprised 12 patients each, and both received a course of topical treatment for 7 days prior to undergoing circumcision. Group B was treated with 0.1% mometasone furoate, a potent corticosteroid known for its anti-inflammatory properties, while Group C received Ozoile cream. Both treatments were applied once daily in the evening. After the 7-day treatment period, all participants underwent surgical circumcision. During the procedure, foreskin samples were

carefully collected and immediately fixed in formalin to preserve their structural integrity. These samples were then processed for histological analysis to examine any structural changes in the foreskin. Additionally, immunohistochemical staining was performed to evaluate the expression of specific markers (CD8, CD57, p53, Ki-67).

Paraffin-embedded tissue sections were sliced and stained with hematoxylin–eosin (H&E) for histomorphological examination. For immunohistochemistry, 5-µm-thick sections were obtained and deparaffinized using xylene, followed by a series of washes with descending alcohol concentrations. The sections were then treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, followed by additional washing with deionized water. To prevent non-specific binding of serum proteins, the sections were incubated with normal sheep serum for 30 min at room temperature. The following pre-diluted primary antibodies were applied for 30 min at 37 °C: rabbit monoclonal anti-CD8 (clone SP57), mouse monoclonal anti-CD57 (clone NK-1), mouse monoclonal anti-p53 (clone Bp53-11), and rabbit monoclonal anti-Ki-67 (clone 309); all antibodies were sourced from Roche Diagnostics GmbH, Mannheim, Germany. After three washes with PBS, the sections were incubated with a biotinylated goat anti-mouse IgG secondary antibody (1:300; Abcam) for 20 min at room temperature, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 30 min.

The immunostaining was developed using diaminobenzidine tetrahydrochloride and counterstained with Mayer's hematoxylin, employing the ULTRA Staining System (Ventana Medical Systems). Negative controls were prepared by replacing the specific antibodies with PBS. Quantitative immunohistochemical analysis of CD8 + , CD57 + , p53 + , and Ki67 + cells was carried out by two experienced blinded observers (GT and AI). The percentage of positive cells was determined by evaluating 400 consecutive epithelial cells in selected regions, noting the location of the positive cells within the epidermal layers (basal or supra-basal). Only cells with nuclear brown staining were considered positive, regardless of staining intensity. This analysis was performed using digital image analysis of microscopic images captured with a DS-Ri2 color video camera (Nikon Instruments) mounted on a Nikon Eclipse Ci light microscope.

Statistical analysis

A statistical analysis was performed comparing the data for each group. The numerical data were reported as mean, standard deviation (S.D.), median, and range (minimum and maximum values). Since the numerical variables did not follow a normal distribution, as confirmed by the Kolmogorov–Smirnov test, a non-parametric method was used. To determine any significant differences among the three patient groups (ritual operation, phimosis, and phimosis with a histological diagnosis of LS), the non-parametric ANOVA (Kruskal–Wallis test) was applied for all variables (Ki-67, p53, CD8, and CD57). Post hoc pairwise comparisons between the groups were conducted using the Dunn test. Bonferroni's correction was applied for these comparisons, adjusting the alpha significance level by dividing it by the total number of pairwise comparisons ($0.050/3 = 0.017$).

Statistical analyses were carried out using IBM SPSS for Windows, Version 22 (Armonk, NY, USA, IBM Corp.). A p value less than 0.05 was considered statistically significant.

Results

The histological analysis of tissue samples from Group A revealed significant pathological features. The epidermis showed acanthosis, characterized by thickened layers and asymmetrical ridges, accompanied by focal hyperplasia. The sub-epithelial basement membrane was frequently thickened, which is indicative of ongoing inflammation. Additionally, dermal papillae displayed dilated capillary blood vessels (Fig. 1A).

The lymphoid infiltrate was observed as a dermal band, varying in intensity from moderate to dense, indicating a robust immune response. This infiltrate followed a lichenoid distribution, suggesting an immune-mediated attack at the interface between the epidermis and dermis. Focal epidermal lymphocyte exocytosis was also evident, where lymphocytes were found infiltrating the epidermis, further suggesting the active immune process typical of lichen sclerosus.

Histomorphological analysis of Group B and C revealed a normal stratified squamous keratinized epithelium, a slight thickening of sup-epithelial connective tissue, sparse to focally diffuse extent of lymphocytic cells with few dilated capillary blood vessels (Fig. 1B, C).

A significant increase of positive CD8 + and CD57 + lymphocytes was revealed in group A (Fig. 2A, B). Moreover, Ki67-positive cells were diffusely expressed rising in the basal layer (Fig. 2C). Homogeneous and continuous nuclear p53 expression was increased in basal/supra-basal keratinocytes ($24.79\% \pm 14.34\%$) in Group A compared to Group B and C (Fig. 2D). In contrast, in group B, a reduction in CD 8 + CD 57 + positive lymphocytes was observed (Fig. 2E, F) as well as a decrease in the growth fraction evaluated by Ki67 in basal and supra-basal cells and few positive p53 basal cells (Fig. 2G, H), compared to untreated LS patients.

Similarly, a slight increase of positive CD 8 + CD 57 + lymphocytes was revealed in group C (Fig. 2I, J).

Ki67-positive cells were slightly expressed in basal and supra-basal layer and a decreased nuclear p53 expression was observed in basal/supra-basal keratinocytes (Fig. 2K, L) compared to children undergoing circumcision without

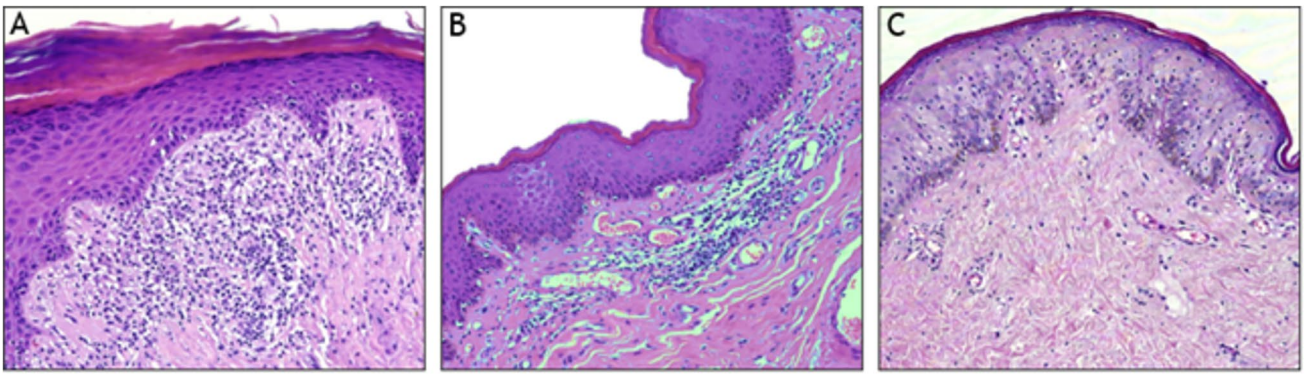


Fig. 1 Hematoxylin & eosin staining (original magnification 100x). **A** Group A. Epidermis with acanthosis, asymmetric ridges, and focal hyperplasia. Thickened sub-epithelial membrane, dermal band lymphocytic infiltrate. **B** Group B. Normal stratified keratinized squamous epithelium; slightly thickened subepidermal connective tissue; lymphocyte cells sparse to focally diffuse. **C** Group C. Normal stratified keratinized squamous epithelium; slightly thickened subepidermal connective tissue; lymphocyte cells sparse to focally diffuse

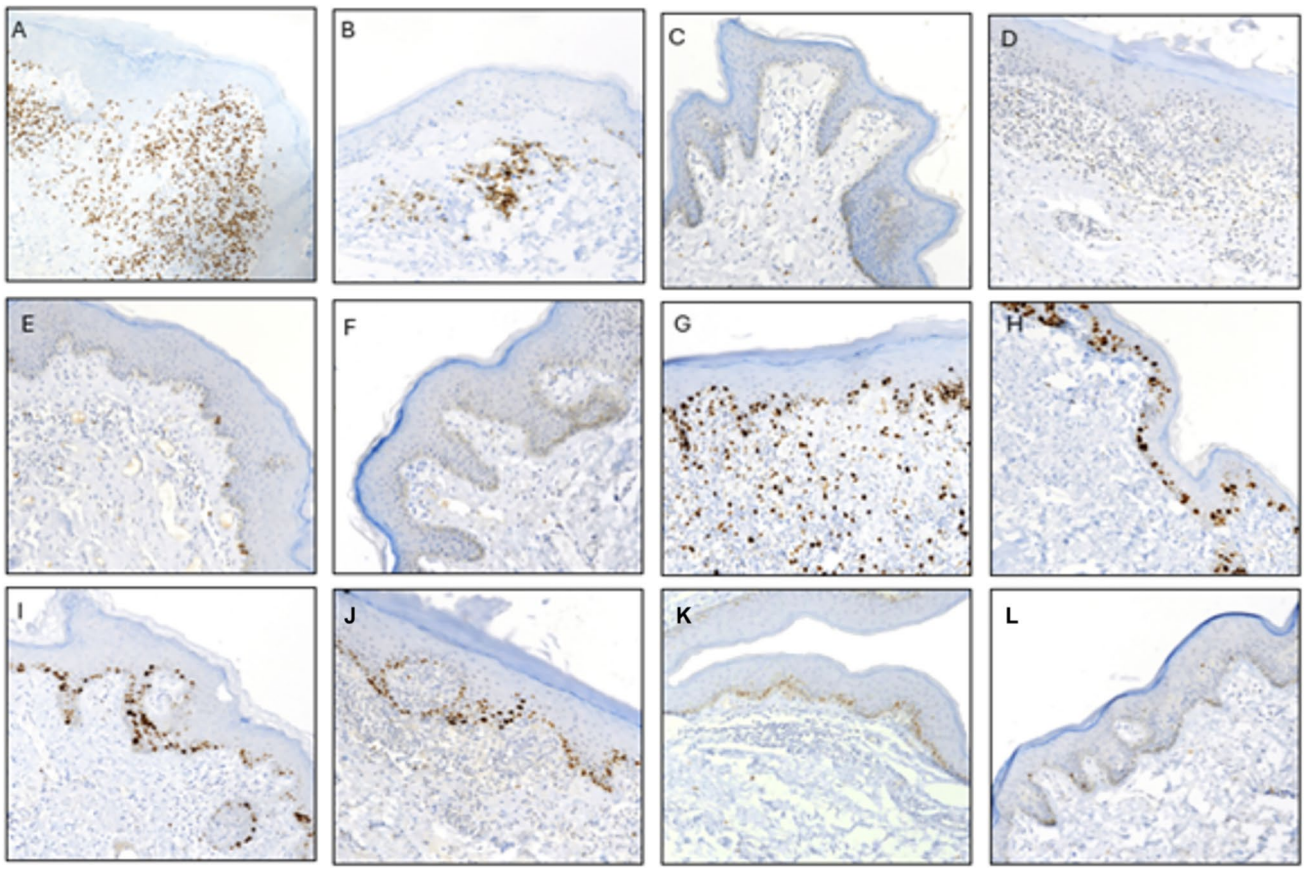


Fig. 2 Immunohistochemical staining (original magnification 100x) **A** CD8 + positive cells of Group A. **B**: D57 + positive cells of Group A. **C**: High density in basal layer of Ki-67 Group A. **D**: High density in basal layer of p53 Group A. **E**: CD8 + cells of Group B. **F**: CD57 + of Group B. **G**: Low density of Ki-67 in basal layer in Group B. **H**: Few positive P53 basal elements in Group B. **I**: CD8 + cells of Group C. **J**: CD57 + cells of Group C. **K**: Ki-67 in basal layer Group C. **L**: P53 basal elements' Group C

pre-operative topical treatment. Statistical analysis data are reported in Tables 1, 2.

Briefly, a significant reduction of tissue immune response (CD8 + and CD57 + lymphocytes) was observed between Group A and Group B ($p < 0.001$ and $p < 0.001$, respectively)

Table 1 The percentage of positive cells in the epidermal layers across groups

Group	CD8 + (%)	CD57 + (%)	Ki67 (%)	p53 (%)
A	8.58 ± 3.36	10.04 ± 5.33	15.27 ± 7.43	24.79 ± 14.34
B	3.73 ± 2.63	4.27 ± 3.32	10.12 ± 5.58	8.08 ± 4.57
C	3.25 ± 2.27	3.92 ± 2.29	7.83 ± 4.93	5.31 ± 3.68

and between Group A and Group C ($p < 0.001$ and $p < 0.001$, respectively). Moreover, p53 and Ki-67 positive cells were significantly reduced between Group A and Group B ($p < 0.001$ and $p < 0{,}001$, respectively), between Group A and Group C ($p < 0{,}001$ and $p < 0{,}001$, respectively). Furthermore, Group B compared to Group C did not show any significant difference in the evaluation of CD8 + and CD57 + cells ($p = 0.34$ and $p = 0.54$, respectively). In Group C, compared to Group B, a significant reduction was observed in Ki67 and p53 expression. ($p = 0.035$ and $p = 0.039$, respectively).

Discussion

The true incidence of LS remains unclear, but it appears to be increasing, likely due to greater clinical awareness among pediatric surgeons and improved diagnostic accuracy through histological examination. While it is difficult to pinpoint an exact figure, LS incidence in pediatrics has been estimated approximately 35% of circumcised patients affected by phimosis [12], with a notable peak of incidence between 7 and 8 years of age, particularly in patients with phimosis [8]. The data obtained in this study showed the involvement of CD8- and CD57-positive cells in LS patients. These immune cells involved in cytotoxic responses play a significant role in the development and progression of the disease. These immune cells contribute to the inflammatory process by promoting chronic inflammation, which, in turn, can alter the tissue environment and foster potential malignant transformations. Interestingly, this mechanism appears to be consistent with what is observed in adult patients with LS, where CD8- and CD57-positive cells are involved in the pathogenesis of the disease, which amplify the inflammatory response and potentially accelerating the disease's progression and increasing the risk of malignancy.

A chronic and unresolved inflammation is reported to dysregulate cell growth, differentiation, and the balance between apoptosis and proliferation. In fact, we observed that the markers of cell proliferation, such as p53 and Ki-67, were augmented in LS patients, consistent with findings in adult. Given these observations, it is crucial to emphasize the importance of prolonged follow-up for patients diagnosed with LS, especially in pediatric populations, to monitor disease progression and intervene early if necessary. Continued monitoring allows clinicians to assess the effectiveness of treatment strategies and address any signs of malignant transformation before it becomes clinically significant [7]. The therapeutic approaches aim at managing the disease and preventing degeneration and/or cancer progression [20].

Currently, surgical circumcision is considered the gold standard treatment for pediatric LS affecting the foreskin [21]. Nevertheless, a non-surgical alternative was also proposed with the topical application of corticosteroids, such as clobetasol propionate and betamethasone valerate, thus resulting in an arrest or delay of LS progression These corticosteroids have been shown to result in the arrest or delay of LS progression by reducing inflammation, controlling symptoms such as itching and discomfort, and promoting healing of the affected tissue [2, 12, 19, 22, 23].

The anti-inflammatory effects of steroids result in immunosuppression and cytokines reduction that would seem to rebalance collagen synthesis and sub-epithelial damage [24, 25]. Although topical administration shows good results in foreskin, solid controversies still remain, in particular regarding the long-term use in terms of recurrence and tissue degeneration, such as skin thinning, the development of striae, and also the risk of rebound symptoms upon discontinuation. These concerns highlight the need for careful management and further research into alternative therapies that may provide similar benefits without the risks associated with prolonged steroid use. Some studies have suggested that these treatment may reduce inflammation, potentially lowering the risk of cancerous changes, while others remain uncertain about their effectiveness in this regard [22].

Ozoile is an innovative formulation that combines ozonated olive oil with vitamin E acetate, known for its potent antioxidant, anti-inflammatory, and tissue-regenerative properties. Ozonated olive oil is produced by infusing ozone gas into olive oil, creating stable ozonides with significant antimicrobial and anti-inflammatory effects that promote wound healing and support tissue regeneration.

Table 2 Statistical analysis of CD8 +, CD57 +, Ki67, and p53 cells among groups A, B, and C, including p values (n.s.: not significant)

	CD8 + (p value)	CD57 + (p value)	Ki67 (p value)	p53 (p value)
Group A vs Group B	< 0.001	< 0.001	< 0.001	< 0.001
Group A vs Group C	< 0.001	< 0.001	< 0.001	< 0.001
Group B vs Group C	0.34 (n.s.)	0.54 (n.s.)	0.035	0.039

Vitamin E acetate, a powerful antioxidant, enhances the skin's healing process and protects tissues from oxidative damage. Studies have demonstrated that Ozoile treatment effectively reduces pro-inflammatory cytokines and chemokines, thereby maintaining a healthy epithelium, promoting the epidermis' barrier integrity, and exhibiting steroid-like effects [20, 26, 27].

Ozoile represents an innovative alternative treatment, and recent findings have proposed a mechanism of action involving the formation of oxidative species at low concentrations. These species act as regulators of key molecular pathways, where the moderate oxidative stress generated by stable ozonides influences the RedOx balance [28]. This, in turn, triggers the activation of cellular antioxidant defense systems and stimulates the production of growth factors, promoting wound healing, tissue repair, and anti-inflammatory responses [29]. Notably, in recent studies, Ozoile has been shown to reduce pro-inflammatory cytokines and chemokines, thereby supporting the maintenance of a healthy epithelium and strengthening the epidermal barrier. Its effects parallel those of corticosteroids; moreover, in studies where patients were treated pre-operatively with a combination of steroids and Ozoile, results demonstrated a significant reduction in immune response and notable anti-proliferative effects. Treatment with Ozoile was associated with a significant reduction in the proliferation marker Ki-67 compared to untreated patients. This reduction in Ki-67 indicates a suppression of cellular proliferation, which is crucial for preventing LS lesions from progressing toward precancerous states. Additionally, p53 expression was reduced in treated patients, suggesting that anti-inflammatory treatments like Ozoile may help mitigate p53 overexpression, which otherwise arises in response to chronic inflammation and oxidative stress in LS and is linked to malignant potential.

Under normal conditions, cells maintain low p53 levels due to the activity of its negative regulator Mdm2. This protein binding to p53 stimulates its degradation. DNA damage disrupts this binding, increasing p53 activity, which helps repair DNA and triggers the expression of WAF1 and p21 to halt the cell cycle Mutations in p53 can inactivate the protein, preventing the DNA repair mechanism or, on the contrary, make the protein hyperactive which, instead of acting as a tumor suppressor, acts as an oncogene, determines the expression of genes involved in cell proliferation. This imbalance in p53 function is crucial in the pathogenesis of LS and its potential progression toward malignancy. The use of Ozoile, through its anti-inflammatory properties, may play a key role in restoring this balance.

In this study, patients treated with Ozoile showed a significant reduction in CD8 and CD57-positive T cells, suggesting a decrease in immune cell infiltration and inflammatory activity. Furthermore, a significant reduction in proliferative markers was observed in treated patients compared to untreated children.

Ozoile treatment for LS of the foreskin led to a strong reduction in the transcription of proteins involved in inflammatory pathways, such as TG2, Il-1β, TNF-α, and IFN-γ, reducing the inflammatory process and stimulating the mechanisms for tissue regeneration [20]. The long-term safety profile of topical corticosteroids continues to be a source of concern due to the lack of long-term adequate studies. Adverse effects, such as skin atrophy, striae formation, rebound reactions, fungal infections, relapse of human papilloma virus (HPV), and herpes simplex virus infection or systemic absorption, are serious and well-documented complications associated with prolonged corticosteroid use [30, 31]. These risks highlight the need for alternative therapies, and Ozoile presents a safer option by eliminating these concerns, providing a more sustainable and safe approach to managing LS. Its suitability is particularly noteworthy for pediatric patients, who are especially vulnerable to the long-term adverse effects associated with steroid treatments, making it an innovative and patient-friendly solution.

Conclusion

This study shows that pre-operative treatment with Ozoile and topical corticosteroids in children with phimosis and lichen sclerosus (LS) significantly reduces the immune response and cell proliferation markers compared to untreated patients, suggesting a marked decrease in local inflammation and cellular activity. Ozoile demonstrated a greater reduction in cell proliferation than corticosteroids, suggesting potential benefits for tissue regeneration and epidermal integrity.

Ozoile appears to be a viable alternative to corticosteroids, especially for patients where long-term steroid use may be contraindicated. Future studies with long-term follow-up will be essential to confirm these benefits and further evaluate the preventive potential of Ozoile in avoiding complications, including malignancy, associated with LS.

Author contributions Methodology, S. A.; validation, A. I.; formal analysis, S. A.; investigation, A. I., C. P., and M. C.; resources, F. C. and N. I.; data curation, F. C., S. D., and F.V.; writing—original draft preparation, F. C., S.A., and G. M.; writing—review and editing, P.I.; supervision, S.A. and C. R.. All authors reviewed the manuscript.

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Data availability https://unimeit-my.sharepoint.com/:f:/g/personal/salarena_unime_it/EgXuCdgytHpHii8ujE4PbVoBnSD1R23OaUWKwBkPfHp-zw?e=ffKo0e.

Declarations

Competing Interests Author Salvatore Arena has received reimbursement for participation in a meeting from Erbagil s.r.l.

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Authors and Affiliations

Fabiola Cassaro[1,2] · Antonio Ieni[3] · Monica Currò[1] · Cristina Pizzimenti[3] · Natasha Irrera[4] · Federico Vaccaro[1] · Giulia Maisano[2] · Santi D'Antoni[2] · Pietro Impellizzeri[2] · Carmelo Romeo[2] · Salvatore Arena[2]

✉ Salvatore Arena
salarena@unime.it

Fabiola Cassaro
fabiola.cassaro@studenti.unime.it

Antonio Ieni
antonio.ieni@unime.it

Monica Currò
monica.curro@unime.it

Cristina Pizzimenti
cristina.pizzimenti@unime.it

Natasha Irrera
nirrera@unime.it

Federico Vaccaro
federico.vaccaro@studenti.unime.it

Giulia Maisano
giulia.maisano@studenti.unime.it

Santi D'Antoni
santi.dantoni@studenti.unime.it

Pietro Impellizzeri
pietro.impellizzeri@unime.it

Carmelo Romeo
romeoc@unime.it

[1] Department of Biomedical and Dental Sciences and Morphological and Functional Imaging, University of Messina, 98121 Messina, Italy

[2] Unit of Pediatric Surgery, Department of Human Pathology of Adult and Childhood "Gaetano Barresi", University of Messina, 98121 Messina, Italy

[3] Unit of Human Pathology, Department of Human Pathology of Adult and Childhood "Gaetano Barresi", University of Messina, 98121 Messina, Italy

[4] Unit of Dermatology, Department of Clinical and Experimental Medicine, University of Messina, 98121 Messina, Italy