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ORIGINAL ARTICLE

# Vitamin C, lactoferrin and elastin—Advancing the science

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## Abstract

**Background:** This study follows an initial scientific validation linking sodium ascorbate (SAC) with elastin conservation and the clinical trial histology observation that the full formulation tested there stimulated elastin development. In an effort to explain the increased elastin response, a candidate was sought that may provide synergy to SAC during elastin stimulation. Lactoferrin was the constituent chosen to explore in this realm.

**Materials and Methods:** Using the previously described ex vivo skin model, freshly collected discarded human skin from 2 donors was used to evaluate the effects of lactoferrin and SAC alone and together, and L-ascorbate CE Ferulic formulation (CEF) on elastogenesis. Four skin explants were topically subjected to the treatments daily for 7 days and one group was left untreated as a negative control. The tissue was fixed and embedded. Sections were evaluated by immunofluorescence using antibodies targeting Tropoelastin and CD44, with DAPI counterstaining to observe nuclei. Images were then analyzed using ImageJ.

**Results:** Treatment with SAC and lactoferrin demonstrated a significant synergistic effect on tropoelastin stimulation compared to the single treatments. In addition, this combination demonstrated intact and increased elastin fibers in contrast to the CEF, which portrayed fragmented elastin fibers. In addition, an additive effect of SAC also contributed to the enhanced CD44, suggesting an increased presence of hyaluronic acid, a new observation for this compound.

**Conclusion:** This study complements a series of studies that have been undertaken to validate the efficacy of a novel antioxidant formulation. Aside from its efficacy in ROS management, the SAC constituent is unique in the different forms of Vitamin C for its ability to conserve elastin. Prior clinical studies demonstrated additive elastin stimulation on histology, not just conservation. From this current study, the combination of SAC with lactoferrin may be responsible for this additive stimulatory effect on elastin. This presents a significant advance in topical antioxidant formulations where the Vitamin C component provides antioxidant and collagen stimulation with additional elastin stimulation rather than degradation.

KEYWORDS
elastin conserving, lactoferrin, reactive oxygen species, Vit C salt

## 1 | INTRODUCTION

Oxidative stress is thought to be involved in initiating multiple pathologic processes and diseases. In the skin, constant exposure to ultraviolet radiation is likely the commonest cause of background reactive oxygen species (ROS) formation necessitating the body's intrinsic formation of antioxidant compounds in response. With time, aging, and "wear and tear," these responses become blunted, and local antioxidants diminish.[1] Thus, multiple topical preparations have been developed to supplement the skins natural supply. One of these products, the sodium salt of ascorbate (SAC), a variation of Vitamin C, has been recently highlighted with particular reference to its elastin-conserving capacity.[2]

Vitamin C (Vit C) is the most recognized antioxidant, concentrated in the epidermis of the skin following uptake from the plasma mediated by specific sodium-dependent vitamin C transporters.[3] As reported in our previous study,[2] a little appreciated fact is that Vit C, a known enhancer of collagen deposition, has also been identified as an inhibitor of elastogenesis, possibly related to ascorbic acid destabilizing tropoelastin mRNA.[4] Tropoelastin is the major protein component of elastin fibers and is thus considered synonymous with elastin fiber identification. SAC, however, has been demonstrated to be elastin conserving.[2,4] In a further clinical study carried out with a formulation including SAC, was shown not only to conserve elastin but to increase and stimulate elastin formation, as demonstrated in 5 of 5 biopsy specimens tested.[5]

Related to that discovery, we set out to determine if another active compound could act synergistically with SAC to bring about elastin stimulation over and above the conservation anticipated by SAC use. After examining all the constituents present in the formulation, lactoferrin was chosen as the most likely candidate because of previous experience with this compound and the extensive literature showing diverse functions and effects of the compound.[6–9]

## 2 | MATERIALS AND METHODS

### 2.1 | Ex vivo model

The ex vivo studies were conducted by 3D Genomics (Carlsbad, CA) as an independent laboratory investigation. Photodamaged skin derived from patients undergoing facelift procedures (study approved under Veritas Institutional Review Board—study ID # 3192) was obtained. The discarded skin was received at the laboratory within 2 h of removal from the patients. All the skin processing was conducted under BSL2 laboratory conditions. The skin was washed in phosphate-buffered saline (PBS) and defatted if necessary. Any visible hairs were removed using a scalpel. The skin was then cut into squares (~5 mm × 5 mm to ~8 mm × 8 mm) and placed into transwells suspended in 12-well plates.

Each lower well was filled with 1.0 mL of Skin Media (composed of DMEM/Ham's F-12 50/50 mix, adenine hydrochloride hydrate, calcium chloride dihydrate, T3 tri-iodothyronine, insulin-transferrin-selenium-ethanolamine (ITS-X), penicillin/streptomycin fetal bovine serum (FBS), glutaGRO, and gentamicin sulfate), and the transwell was filled with about 200–300 μL of Skin Media to surround the skin sample while maintaining an air-exposed epidermal surface. The media was changed daily. The skin samples were acclimated for 3 days before beginning the treatments.

### 2.2 | Ex vivo treatments

For this study, two donors were used both women 56 years (Fitzpatrick3) and 62 years (Fitzpatrick 4) and the skin squares were cut so that each group was in duplicate. As a negative control, one group was left untreated. The treatments were lactoferrin alone, SAC alone, lactoferrin + SAC, and CE Ferulic formulation (CEF–CE Ferulic SkinCeuticals—15% L-Ascorbate C Serum, 0.5% Ferulic acid; 1% alpha tocopherol; Ethoxydiglycol, Hyaluronic Acid, Sodium Hyaluronate, Triethanolamine). About 100 μL to 500 μL of each compound formulation was placed on the surface of a sterile petri plate. The skin was retrieved from the transwell culture plate using forceps and picking up the skin at the edge with minimal forceps compression of the skin. The skin was gently swiped over the formulation to completely cover the epidermis and then returned to the transwell culture plate. The treatment was repeated once daily for 7 days with media changes as well.

### 2.3 | Elastin and CD44 assessment

At the end of the treatment, the skin samples were fixed in formalin and embedded in paraffin following standard procedures. Sections were cut and prepared for staining to detect tropoelastin and CD44 using the following primary antibodies Anti-Tropoelastin (Elastin Products Co.) and Anti-CD44 (Abcam). Goat anti-rabbit IgG Alexa647-conjugated and goat anti-mouse IgG Cy3-conjugated secondary antibodies were used. DAPI was used to identify the nuclei. Scanned images were captured by immunofluorescence microscopy (Zeiss) to obtain a stitched image of the entire section. Tropoelastin and CD44 intensities were evaluated using ImageJ. The color channels were split, and the intensity of the red (tropoelastin) and yellow (CD44) in the dermal-epidermal junction (DEJ) was measured across the entire section. The average values were assessed by a student's *t*-test and $p < 0.05$ was considered significant.

## 3 | RESULTS

### 3.1 | Assessment of elastin and CD44 in the DEJ of the skin explants treated with lactoferrin or SAC alone

Skin explants treated with lactoferrin or SAC alone showed enhanced elastin and CD44 in the DEJ compared to untreated explants. (Figure 1A). Quantification of the intensities of the

fluorescent signals revealed that the increase in elastin was only significant with the skin was treated with lactoferrin (Figure 1B). CD44 was significantly increased for both treatment groups. However, the intensity was significantly greater for SAC compared to lactoferrin (Figure 1C).

### 3.2 | Lactoferrin and SAC synergistic assessment of elastin and CD44 in the DEJ in treated skin explants

Treatment with lactoferrin and SAC in combination showed enhanced elastin and CD44 in the DEJ compared to single treatments (Figure 2A). The quantified data revealed that lactoferrin and SAC together significantly increased elastin compared to the treatments alone. Compared to the data from the untreated group (Figure 1B), the significant increase with both components suggests synergy (Figure 2B). The CD44 assessment revealed that SAC significantly stimulated CD44 compared to lactoferrin alone. The combination of the 2 was also significantly increased. However, the main driver appeared to be SAC (Figure 2C).

### 3.3 | The combined effect of lactoferrin and SAC compared to CEF

Compared to the untreated skin explants the combination of lactoferrin and SAC enhanced the presence of elastin and CD44 in the DEJ (Figure 3A). Quantification of the signal revealed that both were significantly enhanced compared to the untreated group (Figure 3B,C). In contrast, the CEF-treated explants demonstrated little evidence of elastin and CD44 in the DEJ (Figure 3A), and this was verified by the quantification (Figures 3B,C). Consistent with our previous study where the full formulation was used, the CEF-treated samples again showed fragmented elastin fibers.[2]

## 4 | DISCUSSION

Combatting "oxidative stress" is the backbone of many topical formulations in the anti-aging spectrum. The stresses presented by ultraviolet (UV) radiation on the outer stratum corneum layer necessitate a constant production of antioxidants in an attempt to neutralize these ROS. This intrinsic protective mechanism becomes weaker with time, aging, and external stresses, and the antioxidants are depleted, necessitating replacements in the form of topical formulations.[1]

As described in previous publications,[2,4] a little appreciated fact is that Vitamin C, a known enhancer of collagen deposition, has also been identified as an inhibitor of elastogenesis, likely related to the fact that ascorbic acid destabilizes tropoelastin mRNA and causes excess hydroxylation on prolyl/lysyl residues of tropoelastin molecules.[4] SAC, a Vitamin C sodium salt, has been demonstrated to stimulate the production of both collagen and conserve elastic fibers.[4]

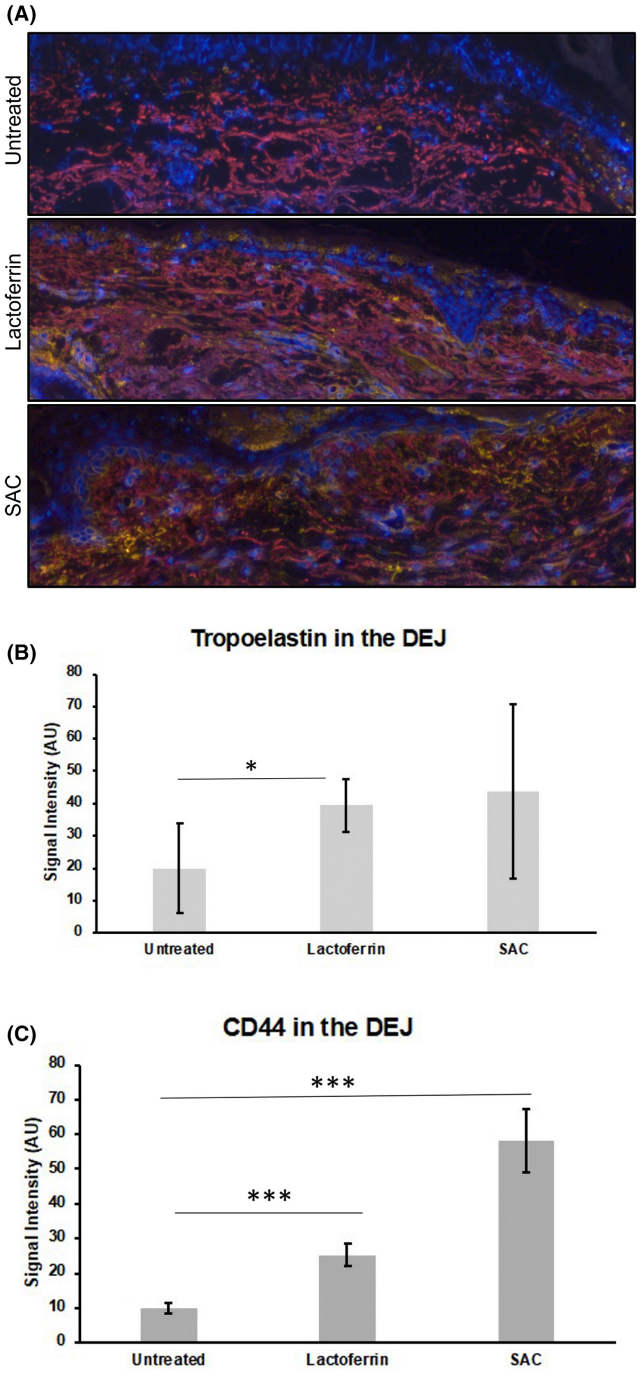


**FIGURE 1** Single Treatment Assessment of Elastin and CD44. (A) Discarded skin was cultured using the established ex vivo model and left untreated or treated with lactoferrin or SAC for 7 days. The tissue was processed, and sections were stained to assess tropoelastin (red) and CD44 (yellow) by immunofluorescence. DAPI was used to detect the nuclei (blue). Representative images are shown. (B) The DEJ was marked, and the intensity of the red was quantified in ImageJ to assess tropoelastin. (C) The DEJ was marked in and the intensity of the yellow was quantified in ImageJ to assess CD44. The data are presented as the mean ± SD. *$p < 0.05$; ***$p < 0.001$.

This study is an extension of this newly developed scientific narrative related to traditional L-ascorbate Vitamin C and elastin.[2,5] After confirming previous studies showing the adverse effects of L-ascorbate

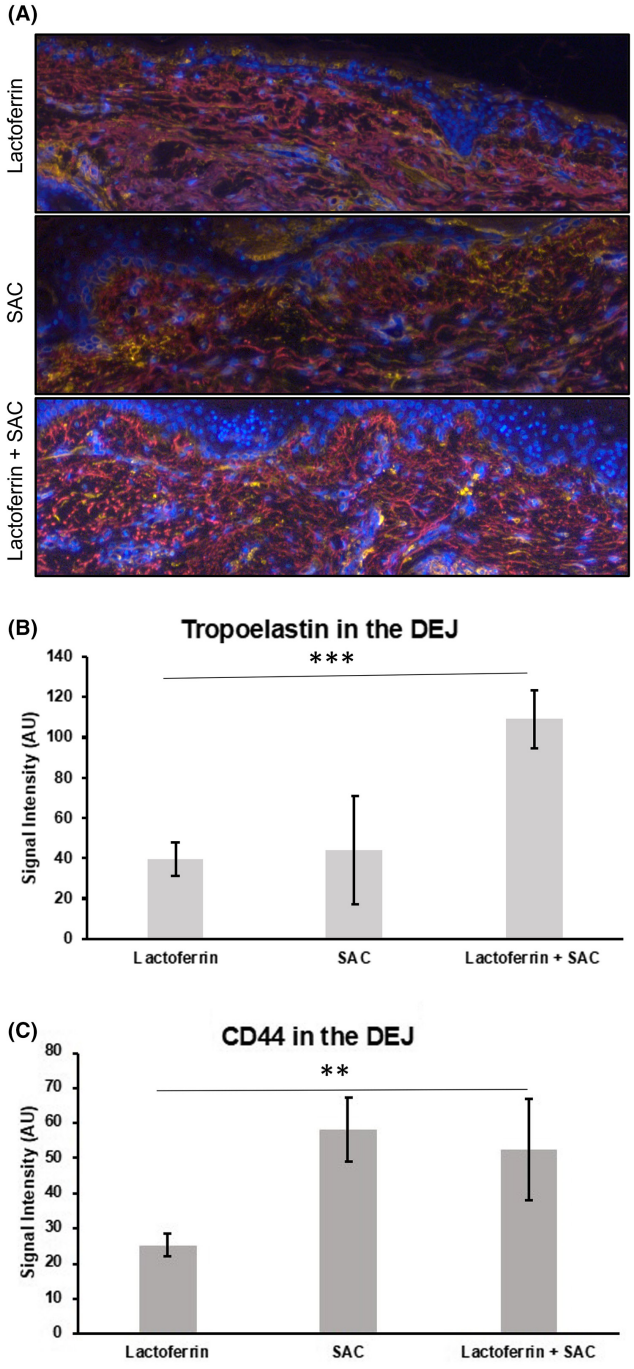


FIGURE 2 Lactoferrin and SAC Synergy Assessment of Elastin and CD44. (A) Discarded skin was cultured using the established ex vivo model and treated with lactoferrin or SAC alone or in combination for 7 days. The tissue was processed, and sections were stained to assess tropoelastin (red) and CD44 (yellow) by immunofluorescence. DAPI was used to detect the nuclei (blue). Representative images are shown. (B) The DEJ was marked, and the intensity of the red was quantified in ImageJ to assess tropoelastin. (C) The DEJ was marked in and the intensity of the yellow was quantified in ImageJ to assess CD44. The data are presented as the mean ± SD. **$p < 0.01$; ***$p < 0.001$.

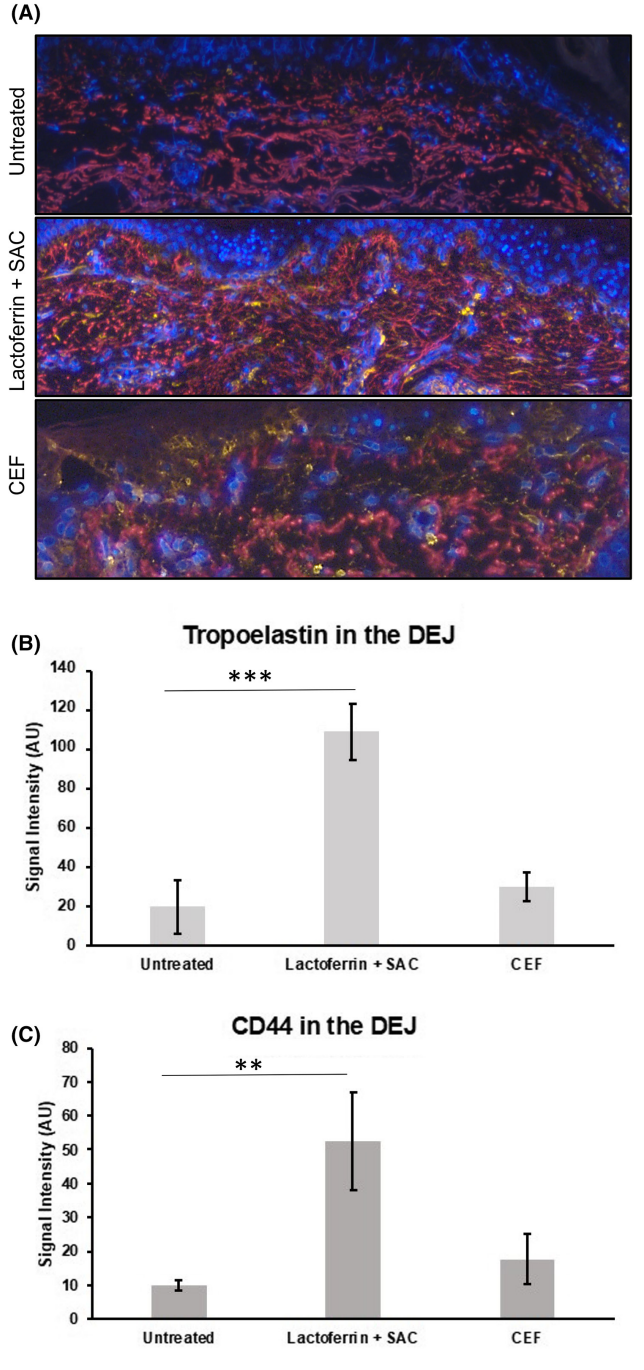


FIGURE 3 Lactoferrin and SAC compared to CEF. (A) Discarded skin was cultured using the established ex vivo model and left untreated or treated with lactoferrin and SAC in combination or CEF for 7 days. The tissue was processed, and sections were stained to assess tropoelastin (red) and CD44 (yellow) by immunofluorescence. DAPI was used to detect the nuclei (blue). Representative images are shown. (B) The DEJ was marked, and the intensity of the red was quantified in ImageJ to assess tropoelastin. (C) The DEJ was marked in and the intensity of the yellow was quantified in ImageJ to assess CD44. The data are presented as the mean ± SD. **$p < 0.01$; ***$p < 0.001$.

on elastin and confirming that the sodium salt indeed conserved elastin and prevented its destruction, the clinical trial conducted using the sodium ascorbate in a full formulationshowed not only elastin conservation but elastin stimulation in 5 of 5 biopsies undertaken.[5] The clinical studies were carried out using a full formulation[5] (C-Radical antioxidant, Alastin Skincare, Carlsbad, CA) and previous ex vivo

studies were undertaken using the SAC alone to measure the effect on elastin.[2] It was therefore necessary to look at individual components to explore the possibility that another constituent could synergistically increase the effect of elastin stimulation together with SAC.

Lactoferrin was chosen as the candidate as the compound has shown multiple benefits to the extracellular matrix, pigmentation effects, and other regenerative effects.[8–12] This was considered because of the diverse effects that had been demonstrated in the literature in contrast to the list of ingredients in the full formulation (Sodium Ascorbate; Lactoferrin; Ergothioneine; Green tea; Oleuropein; Ectoine; Centella asiatica; Carnosine; Ubiquinone (coenzyme Q10); Betaine). In particular the reports of lactoferrin initiating IGF-1 signaling[11] was a good starting point as IGF-1 signaling is also involved in elastin production.

In the previous ex vivo studies, we demonstrated the conservation of elastin by SAC in contrast to an L-Ascorbate formulation. The first segment of this study investigated the differences between isolated lactoferrin and SAC observing their effects on tropoelastin and hyaluronic acid HA (via CD44 staining) versus untreated samples. Figure 1 demonstrates that both lactoferrin and SAC conserve and promote elastin in the DEJ. Interestingly SAC significantly stimulated HA, another unreported and beneficial effect of this compound. Related to this finding the CD44 stain masks the tropoelastin stain to a certain extent, so the intensity and volume of stains in the entire papillary dermis were analyzed using Image J software, a semi-quantitative tool to assess intensity of staining. Figure 2 demonstrates that tropoelastin is conserved in individual test samples but when combined, SAC and lactoferrin significantly stimulated intact robust elastin fibers.

After establishing the individual components conserved elastin and the combined components stimulated elastin, an additional comparison was done with L-Ascorbate containing formulation (CEF) versus the combination of SAC and lactoferrin. Importantly here, as was observed in the first study,[2] the structure of the tropoelastin fibers was pertinent. The fibers in the CEF group were smaller and fragmented, whereas SAC and lactoferrin-treated group (and the full formulation C-Radical defense as demonstrated in initial study[2]) demonstrated intact, full, plumped elastin fibers.

An unexpected finding in this study was an added effect of SAC on HA. The study demonstrated significant stimulation of HA staining in all samples involving SAC alone. Although the combination of SAC and lactoferrin was not synergistic, it certainly showed stimulation of HA when combined (Figure 2). HA stimulation is very relevant to ECM regeneration,[13] DNA repair, and limiting reactive oxygen species generation,[14] which are all very important components of an antioxidant formulation.

Thus, the solution to the in vitro, ex vivo, and histological observations in clinical studies may have been uncovered, namely, SAC, together with lactoferrin synergistically stimulates elastin fiber formation. This added benefit to an antioxidant formulation that includes multiple antioxidants has the capacity to effectively quench free oxygen radicals, stimulate collagen fibers, and also uniquely stimulate elastin fibers in contrast to products that use L-ascorbate as their Vitamin C active or use other actives such as tetrahexyldecyl ascorbate, which is an L-ascorbic acid precursor that degrades to L-Ascorbate with the same issues.[15]

Limitations to the study include the fact that the full formulation was not tested against the comparator full formulation. However, the point here was to try to identify the active components responsible for the elastin stimulation seen when the full formulation was used. It would also be useful to compare gene expression of individual versus combined actives to see if elastin stimulating transcription genes also show synergistic upregulation.

## 5 | CONCLUSION

A series of studies were undertaken to validate the efficacy of constituents and that of a full antioxidant formulation. Aside from its efficacy in ROS management, the SAC constituent has been shown to be unique within Vitamin C variations for its ability to conserve elastin. Clinical studies demonstrated elastin stimulation by histology, not just conservation. From this current study, it would appear that the combination of SAC with lactoferrin is likely responsible for this additive stimulatory effect on elastin. This presents a significant advance in topical antioxidant applications.

### AUTHOR CONTRIBUTIONS

ADW—developed the science, designed studies, analysis, paper writing. MZ—data analysis, paper writing, study design.

### FUNDING INFORMATION

Funding applied for these studies was from Alastin, a Galderma company.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### DECLARATION

Dr Widgerow is Chief Scientific Officer Galderma, and Mary Ziegler is a consultant for Alastin, a Galderma company.

### ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the authors guideline page have been adhered to, and the appropriate ethical review committee approval has been received.

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