



Research Article

Pirfenidone inhibits epithelial–mesenchymal transition in keloid keratinocytes

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Abstract

Background: Keloids are benign fibroproliferative skin lesions that are difficult to treat and become a lifetime predicament for patients. Several treatment modalities have been put forth, but as yet no satisfactory approach to the prevention or treatment of keloids has been identified. The process of epithelial-to-mesenchymal transition (EMT) has been implicated in keloid scarring, as keloid keratinocytes display an EMT-like phenotype. This study investigated the potential of pirfenidone, an antifibrotic agent, to counteract EMT-like alterations in keloid keratinocytes, including gene expression, cell migratory and proliferative functions.

Methods: Normal and keloid keratinocytes were isolated from discarded normal skin tissues and from resected keloid tissues, respectively. Cells were quiesced for 24 h without epidermal growth factor DS-Qi1MCDigital and were exposed to transforming growth factor-beta1 (TGF- β 1; 10 ng/mL), with or without pirfenidone (400 μ g/mL), for an additional 24 h. The effects of pirfenidone on cytotoxicity, cell migration, cell proliferation, and on expression of genes and proteins involved in EMT were assayed. Statistical significance was determined by two-way ANOVA using Sigma Plot.

Results: We found that pirfenidone did not elicit any cytotoxic effect at concentrations up to 1000 μ g/mL. A statistically significant dose-dependent decrease in basal cell proliferation rate was noted in both normal and keloid keratinocytes when exposed to pirfenidone at concentrations ranging from 200 to 1000 μ g/mL. Pirfenidone significantly decreased basal cell migration in both normal and keloid keratinocytes, but a significant decrease in TGF- β 1-induced cell migration was seen only in keloid keratinocytes. Significant inhibition of the expression of TGF- β 1-induced core EMT genes, namely hyaluronan synthase 2, vimentin, cadherin-11, and wingless-type MMTV integration site family, member 5A along with fibronectin-1, was observed in both normal and keloid keratinocytes treated with pirfenidone. In addition, the protein levels of vimentin and fibronectin were significantly reduced by pirfenidone (400 μ g/mL) in both normal and keloid keratinocytes.

Conclusions: For the first time, this study shows the efficacy of pirfenidone in inhibiting the EMT-like phenotype in keratinocytes derived from keloids, suggesting that pirfenidone may counteract a critical contributor of keloid progression and recurrence.

Key words: Keloids, EMT genes, Cell migration, Proliferation, Pirfenidone, Fibronectin

Background

Keloids are abnormal scars that result from a dermal injury and have an ability to spread beyond the original wound margin to the neighboring skin or spontaneously develop in uninjured skin [1, 2]. Keloids are red and elevated, have a conspicuous appearance, and are associated with intermittent pain, persistent itching, and a sensation of contraction. Keloids are difficult to treat; surgical resection results in high recurrence rates and exacerbation after recurrence is common [3, 4]. Until now, the mainstays of prophylactic and therapeutic strategies for keloids include good soft tissue surgical technique, proper use of splinting and pressure dressings, administration of intralesional injections of corticosteroids and 5-fluorouracil (5-FU), laser therapy, radiation therapy, and cryosurgery. The application of established techniques of scar revision surgery aimed at reducing tension on the edges of the wound has also been performed when necessary, although this is more commonly used for hypertrophic scars or scars over joints [5]. However, keloids typically cannot be treated by surgery alone. For these cases, multimodal therapy, such as surgery followed by radiation, laser therapy, steroid injection, and/or 5-FU administration, may be most suitable. Although several treatment modalities have been put forth, as yet no satisfactory approach to the prevention or treatment of keloids has been identified. This presses for an immediate need for better therapy.

The pathogenesis of keloid development is still not fully understood, and most etiologies regard keloids as a chronic inflammatory and fibrotic disease [6, 7]. The main effector cells are fibroblasts, which are associated with excessive inflammation in the reticular dermis and the deposition of extracellular matrix (ECM) proteins [8]. Abnormal levels of inflammation and growth factors were recognized as fundamental factors in keloids [9]. Elevated levels of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and transforming growth factor- β 1 (TGF- β 1) have been observed as the principal components in the acceleration of keloid growth, progression, and recurrence [10]. Growing evidence suggests that cells other than fibroblasts (e.g., keratinocytes, mast cells, and macrophages) actively participate in scar pathogenesis [11]. In addition, considerable evidence suggests that epithelial-mesenchymal transition (EMT) is involved in the development of keloids [12–14]. EMT is a process characterized by the loss of epithelial characteristics in the epidermis (in particular, E-cadherin expression) and adoption of mesenchymal features (such as vimentin expression). The phenomenon whereby epithelial cells acquire mesenchymal traits has been observed in many physiological and pathological processes, including embryogenesis, inflammation, fibrosis, wound healing, and cancer progression [15]. Kuwahara et al. [16] showed increased expression of vimentin in the epidermis of keloid tissue compared with the epidermis of normal skin, suggesting that EMT may be involved in keloid generation. Several growth factors, including TGF- β and TNF- α , are implicated as major profibrotic cytokines involved in

EMT [17]. EMT-derived cells exhibit similar functions to mesenchymal stem cells (MSCs) with multilineage differentiation potential and the ability to migrate toward tumor cells and wound sites [18]. Previous studies provide evidence of the presence of EMT in keloid epidermis and skin appendages, suggesting that EMT might be involved in keloid formation; epithelial cells from the epidermis and skin appendages undergoing EMT could be the sources for a fraction of the fibroblasts/myofibroblasts with the invasive property [19].

Dysregulated TGF- β 1 signaling was shown to be a major contributor to pathological scarring after wound healing [20]. TGF- β 1 is expressed highly in hypertrophic scars, keloids, and fibroblasts derived from scar tissues [21, 22]. Our previous reports showed that in both normal and keloid keratinocytes, TGF- β 1 regulated expression of EMT-related genes, including hyaluronan synthase 2 (HAS-2), vimentin (VIM), cadherin-11 (CDH-11; OB-cadherin), wntless-type MMTV integration site family, member 5A (WNT-5A), frizzled 7 (FZD-7), ADAM metallopeptidase domain 19 (ADAM 19), and interleukin-6 (IL-6) [12]. Of all the growth factors, TGF- β 1 is well characterized for its multifunctional role in keloids. A key breakthrough will be to develop anti-EMT drugs to suppress both basal and TGF- β 1-mediated EMT signals, and thereby simultaneously reduce the synthesis of collagen and production of other key ECM proteins related to keloid formation. We have previously shown that TGF- β 1-induced EMT via both canonical and non-canonical signaling pathways in keloid keratinocytes and was inhibited using pharmacological inhibitors SB525334 (SMAD2 and SMAD3) and U0126 (ERK 1/2 and p38) [12]. It would be much more appealing to identify a small molecule that can be utilized to suppress both fibroblast and keratinocyte functions in keloids. In this context, we propose pirfenidone as a small molecule candidate to inhibit EMT phenomenon in keloid keratinocytes. Pirfenidone (5-methyl-phenylpyridin-2[H-1]-one), is an antifibrotic agent shown to effectively suppress liver and lung fibrosis [23] and it shows potential for the treatment of idiopathic pulmonary fibrosis (IPF), hepatic cirrhosis, and diabetic kidney disease. Pirfenidone is a currently FDA-approved oral drug for treating IPF. Pirfenidone has been proposed for therapeutic use in other types of fibrosis, such as epidural fibrosis, as pirfenidone inhibited fibroblast proliferation and suppressed collagen formation in a rat model of laminectomy [24]. Pirfenidone has also shown promise in treating burn wound-induced fibrosis [25], which warrants further investigation for its utility in preventing burn-induced skin scarring.

Recent studies by Jin et al. [26] showed the high sensitivity of lung fibrotic fibroblasts to pirfenidone compared to normal fibroblasts, which regulated TGF- β 1-mediated fibrotic responses. Our previous studies on Dupuytren's contracture, a palmar fascial fibrosis of the hand, demonstrated that pirfenidone has the potential to reduce TGF- β 1-induced SMAD and non-SMAD signaling pathways in Dupuytren's contracture-derived fibroblasts [27, 28]. *In-vitro* studies

on keloid fibroblasts show that pirfenidone could reduce contraction of collagen gels by inhibiting the downstream pathway of TGF- β 1 [29]. Also, pirfenidone was not only shown to inhibit proliferation of select cancer cells [30, 31] but was also shown to inhibit TGF- β 1-induced EMT in selected normal cells and cancer cell lines [32].

Until now, all the studies reported previously are on the efficacy of pirfenidone on fibroblasts derived from various diseases states. Importantly, no studies to date have investigated the role of pirfenidone on keratinocytes derived from keloids. Furthermore, epithelial cells from the epidermis and skin appendages undergoing EMT could be the sources for a fraction of the fibroblasts/myofibroblasts with the invasive property [12, 33]. EMT plays a crucial role in wound healing and may be involved in the dermal fibrosis associated with keloid formation. This study was undertaken to determine the effects of pirfenidone on EMT in keloid keratinocytes and its effect on functional properties, mainly cell migration and proliferation.

Methods

Human tissue samples

Keloid scar and normal skin samples were obtained with the approval of the University of Cincinnati Institutional Review Board (IRB; Study ID# 2013–2166), in accordance with the Declaration of Helsinki Principles, from patients at the Shriners Hospitals for Children—Cincinnati and the University of Cincinnati Medical Center. Keloid scar samples were obtained with informed consent from patients undergoing elective scar excision procedures. Written consent was obtained from parents or legal guardians of participants under the age of 18, with written assent obtained from pediatric patients age 14 or over, before sample collection. Patient information was anonymized, and samples were de-identified prior to analysis. Collection of de-identified normal skin samples from plastic surgery procedures was classified as “not human subjects research” by the University of Cincinnati IRB using discarded tissue. Strain numbers were used to enable de-identification and were assigned sequentially to all skin or scar samples collected by the laboratory, including those used for this study. For the current study, experiments were performed using four different donor strains for each cell type, normal and keloid, and demographics are provided in Table 1.

Cell culture

Primary cultures of keratinocytes were established from discarded normal skin and keloid scar as described previously [34]. In brief, tissue samples were cut into 2–3 mm strips and were incubated in Dispase II (Roche Applied Science, Indianapolis, IN) to separate dermis from the epidermis. Epidermal strips were treated with 0.025% trypsin (Sigma-Aldrich, St. Louis, MO), followed by neutralization with 10% fetal bovine serum (Invitrogen/Thermo Fisher Scientific, Inc., Waltham, MA) and filtration through Falcon® 70- μ m

Table 1. Demographics information on donor strains of normal and keloid patients

Strain (n)	Age (years)	Race	Sex	Body site
Normal skin				
783	45	White	Female	Abdomen
888	15	African American	Male	Abdomen
815	17	White	Female	Breast
879	12	White	Female	Thigh
Keloid scar				
917	23	African American	Female	Ear
823	17	African American	Male	Ear
795	20	African American	Female	Neck
821	12	White	Male	Abdomen

cell strainers (Corning, Inc., Corning, NY) to release keratinocytes, which were inoculated into tissue culture flasks coated with collagen (Coating Matrix; Invitrogen/Thermo Fisher Scientific). Keratinocyte growth medium consisted of MCDB 153 with 0.06 mM calcium chloride, supplemented with 0.2% bovine pituitary extract (Hammond Cell Tech, Windsor, CA), 1 ng/mL epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ), 5 μ g/mL insulin (Invitrogen/Thermo Fisher Scientific), 0.5 μ g/mL hydrocortisone (Sigma-Aldrich), and 1 $\times$ penicillin–streptomycin–fungizone (Invitrogen/Thermo Fisher Scientific). Cells were subcultured when they reached 80–90% confluence onto tissue culture flasks without collagen coating, using media as described above but with 0.2 mM calcium chloride. Cells were used between passages 2 and 5 for all the experiments. All assays were performed with technical triplicates in addition to biological replicates.

Cell proliferation assay

Primary normal and keloid keratinocytes (5×10^4 /well) seeded on a 24-well plate were grown overnight in complete MCDB 153. The following day, the cells were washed with 1 $\times$ phosphate-buffered saline (PBS) and quiesced with MCDB 153 lacking EGF for 24 h. Cells were treated with concentrations of 0–1000 μ g/mL pirfenidone (Toronto Research Chemicals, Toronto, CA) for an additional 24 h. After this treatment period, the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) was performed to determine the proliferative efficiency. Cells were placed in fresh media and dye solution according to the manufacturer's instructions and incubated at 37°C for 2 h, followed by the addition of solubilization/stop solution and another incubation for 1.5 h at 37°C. Results were obtained using a SpectraMax 384 Plus microplate reader (Molecular Devices) at 570 nm of 200- μ L aliquots placed into a Falcon Tissue Culture Treated 96-well plates. Optical densities were obtained using SoftMax Pro (v3.1.2). Data are represented as fold change in proliferation

± standard error of the mean (SEM) as compared to non-treated control (NTX) normal and keloid keratinocytes.

Cytotoxicity assay

Primary normal and keloid keratinocytes (5×10^4 /well) seeded on a 24-well plate were grown overnight in supplemented MCDB 153 medium, as described above. The following day, the cells were washed with $1 \times$ PBS and quiesced with MCDB 153 lacking EGF for 24 h. Cells were then treated with varying concentrations of pirfenidone (0–1000 µg/mL; Toronto Research Chemicals) and/or TGF- β 1 (10 ng/mL; Peprotech, Rocky Hill, NJ) for an additional 24 h. After this treatment period, a lactate dehydrogenase (LDH) cytotoxicity assay was performed on the cell culture supernatant according to the manufacturer's instructions (Sigma Aldrich). Optical densities were obtained using SoftMax Pro (v3.1.2). Data are presented as fold change in cytotoxicity as compared to NTX for both normal and keloid keratinocytes.

In-vitro wound-healing assay

Primary normal and keloid keratinocytes were cultured to confluence in Falcon Tissue Culture Treated six-well plates (3×10^5 /well) and allowed to grow for 24 h at 37°C. On each plate, a horizontal line was drawn under each well to facilitate consistent imaging of the same area at multiple time points. The cells were then washed with $1 \times$ PBS, and the media was changed to MCDB 153 without EGF and incubated for 24 h at 37°C. Following this incubation, media was removed and using a P1000 pipet tip, a vertical wound was made across the monolayer of cells. Each well was then washed twice with $1 \times$ PBS and imaged for time zero. Cells were then treated with pirfenidone (400 µg/mL) and/or TGF- β 1 (10 ng/mL) in quiescent media for 24 h at 37°C. A 24-h post-treatment image was taken. Using Image J software (NIH), the width of the scratched areas was measured for both time zero and 24 h post-treatment. These distances were used to calculate percent closure using the formula: (time zero distance – 24-h distance/time zero distance) $\times$ 100. Cell migration was expressed as a fold change compared to no treatment cells $\pm$ SEM.

RNA isolation and quantitative real-time RT-PCR

Normal and keloid keratinocytes grown in six-well plates (3×10^5 /well) were lysed using a cell scraper with RLT lysis buffer (Qiagen Inc., Valencia, CA) with β -mercaptoethanol. Total RNA was isolated (Qiagen RNeasy Mini Kit, Qiagen Inc.) from normal- and keloid-derived keratinocytes treated and untreated with pirfenidone (400 µg/mL) and TGF- β 1 (10 ng/mL) and was subjected to real-time RT-PCR to determine the gene expression levels of select EMT genes. QIAshredder columns were used to homogenize samples followed by running the samples through the gDNA eliminator column to remove any genomic DNA present in the sample. cDNA was prepared using the superscript Vilo cDNA kit (Invitrogen/Thermo Fisher Scientific). Sybr green

primer sets for the following human gene products were purchased from Qiagen Inc.: GAPDH (cat. no. PPH00150F-200), HAS2 (cat. no. PPH13147A-200), VIM (cat. no. PPH00417F-200), CDH11 (cat. no. PPH00667A-200), WNT-5A (cat. no. PPH02410A-200) and FN1 (cat. no. PPH00143B-200). Real-time PCR was performed using a StepOnePlus Real-Time PCR System using the following protocol: denaturation 95°C for 10 min, then 40 cycles of amplification at 95°C for 15 s followed by annealing and extension at 60°C for 1 min. The comparative $2^{-\Delta\Delta CT}$ method [35] was used to determine the expression levels of target genes after normalization to GAPDH expression. The data are presented as fold changes in relative expression levels compared to NTX for both normal and keloid keratinocytes $\pm$ SEM.

Immunocytochemistry

Normal and keloid keratinocytes grown in glass chamber slides at a density of 5×10^3 and fixed with 4% paraformaldehyde in PBS for 30 min and then blocked with 2% BSA in PBS at pH 8.0. After blocking, cells were permeabilized using 0.05% Triton X in PBS (pH 7.6). After PBS washes the cells were incubated overnight with primary antibodies which included peptide affinity-purified rabbit polyclonal antibody vimentin (1:200 dilution; cat. no. AP2739a, Abgent, Inc., San Diego, CA) and antifibronectin antibody (1:200 dilution; cat. no. ab2413, Abcam, Cambridge, MA). Following primary antibody incubation, cells were incubated for 1 h at room temperature with labeled secondary antibody chicken anti-rabbit, Alexa Fluor 488 (1:400 dilution; cat. no. A21441, Life Technologies, Thermo Fisher Scientific), followed by coverslipping using Vectashield mounting media[®] with DAPI (cat no. H-1200, Vector Laboratories, Maravai LifeSciences, San Diego, CA) to counterstain cell nuclei. Cells were viewed and photographed using Eclipse 90i microscope and photographed with a DS-Qi1MC Digital Microscope Camera (Nikon Instruments Inc., Melville, NY).

Statistical analyses

Statistical analyses were performed using SigmaPlot for Windows Version 11.0 (SyStat Software, Inc., San Jose, CA). Data were analyzed by a two-way analysis of variance (ANOVA) to identify statistically significant differences among groups; post-hoc pairwise multiple comparison procedures were performed using the Holm–Sidak method. A value of $p < 0.05$ was considered statistically significant.

Results

Pirfenidone inhibits cellular proliferation and migration in both normal and keloid-derived keratinocytes without cytotoxicity

Excessive proliferation [36] and migration of keratinocytes [34, 36] is a characteristic disorder of keloids. Pirfenidone's role on the basal proliferative capacity of keratinocytes was investigated with different concentrations of pirfenidone

(0, 200, 400, 600, 800, and 1000 $\mu\text{g/mL}$). The results showed that pirfenidone inhibited basal proliferation of both normal and keloid keratinocytes in a dose-dependent manner (Fig. 1a).

To determine whether the antiproliferative effects were mediated by cellular toxicity, an lactate dehydrogenase (LDH) release assay was performed. Human-derived normal and keloid keratinocytes were exposed to various concentrations (0–1000 $\mu\text{g/mL}$) of pirfenidone for 24 h. Results indicated that pirfenidone showed no significant cytotoxicity on both normal and keloid keratinocytes, suggesting that pirfenidone elicited an antiproliferative effect in a non-cytotoxic manner (Fig. 1b). We also determined that cells treated with various concentrations of pirfenidone for an extended period of time (48 and 72 h) did not elicit any significant cytotoxicity (data not shown).

It has been suggested that in the wound-healing process, epithelial cells acquire migratory and invasive behavior, similar to cells undergoing EMT. We have previously reported that keloid keratinocytes exhibit increased migration rates compared with normal keratinocytes [34] followed by a recent report by Chen et al. [36]. Analysis of migration in normal and keloid keratinocytes showed that TGF- β 1 induced migration of normal and keloid-derived keratinocytes (Fig. 1c). This was inhibited by the addition of both 200 and 400 $\mu\text{g/mL}$ of pirfenidone, and a statistically significant decrease was noted in both keloid keratinocytes, and normal keratinocytes (Fig. 1c). Morphological changes consistent with increased adhesion were observed in keloid keratinocytes cultured in 200 or 400 $\mu\text{g/mL}$ pirfenidone (Figure S). Because pirfenidone at a concentration of 400 $\mu\text{g/mL}$ showed potency in inhibiting both cell migratory and proliferative properties of keloid keratinocytes, this concentration was considered optimal for determining the effects on EMT genes.

Basal and TGF- β 1-induced expression of core EMT genes was significantly reduced by pirfenidone

We analyzed five EMT genes, namely HAS2, CDH-11, VIM, WNTA along with FN-1, which we have previously shown to be elevated in keloid keratinocytes compared to normal keratinocytes [34]. Importantly, HAS2, VIM, CDH-11, and WNT5A are considered core EMT genes [37]. We previously reported increased expression of these genes in both normal and keloid keratinocytes when exposed to TGF- β 1 [12]. Interestingly, we found differences in expression of these core EMT genes in normal and keloid-derived keratinocytes in response to pirfenidone. Treatment with pirfenidone had no significant effect on basal expression of HAS2, VIM, CDH11, WNT5A, or FN1, in the absence of TGF- β 1 stimulation. Pirfenidone significantly inhibited TGF- β 1-induced HAS2, VIM, CDH11, and FN1 expression in both normal and keloid keratinocytes (Fig. 2a–c, e). Pirfenidone reduced TGF- β 1-induced expression of WNT5A in both normal and keloid keratinocytes, but the decrease was only statistically significant in keloid keratinocytes (Fig. 2d).

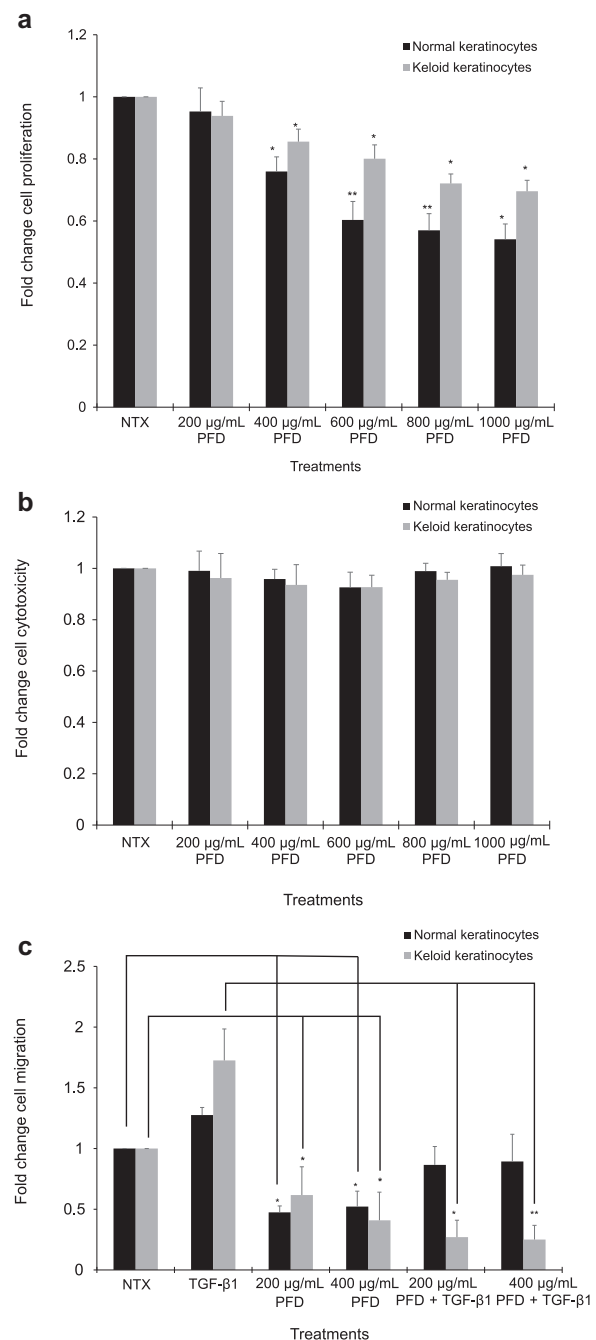


Figure 1. Effect of pirfenidone on cytotoxicity, proliferation, and migration on normal and keloid keratinocytes. Proliferative, cytotoxicity, and cell migration were determined using (a) non-radioactive cell proliferation assay, (b) LDH cytotoxicity assay, (c) *in-vitro* scratch assay. Keratinocytes cultured in MCDB-153 medium were quiesced for 24 h without EGF and then exposed to TGF- β 1 (10 ng/mL), pirfenidone (0, 200, 400, 600, 800, and 1000 $\mu\text{g/mL}$). Representative images of the scratch assay performed on normal and keloid keratinocytes are shown. Four different donor strains were used for each of normal, and keloid keratinocytes, and experiments were done in triplicates on each strain. Data are shown as mean $\pm$ SEM of the averages of the mean collected from the triplicate reads and expressed as fold changes differences comparing NTX of normal and keloid keratinocytes. Two-way analysis of variance (ANOVA) was used to determine statistical significance. $p < 0.05$ was considered a statistically significant difference. * $p < 0.05$; ** $p < 0.001$. The pirfenidone-treated group was compared to non-treatment groups of normal and keloid-derived keratinocytes. NTX non-treated control, EGF epidermal growth factor, TGF- β 1 transforming growth factor- β 1, LDH lactate dehydrogenase

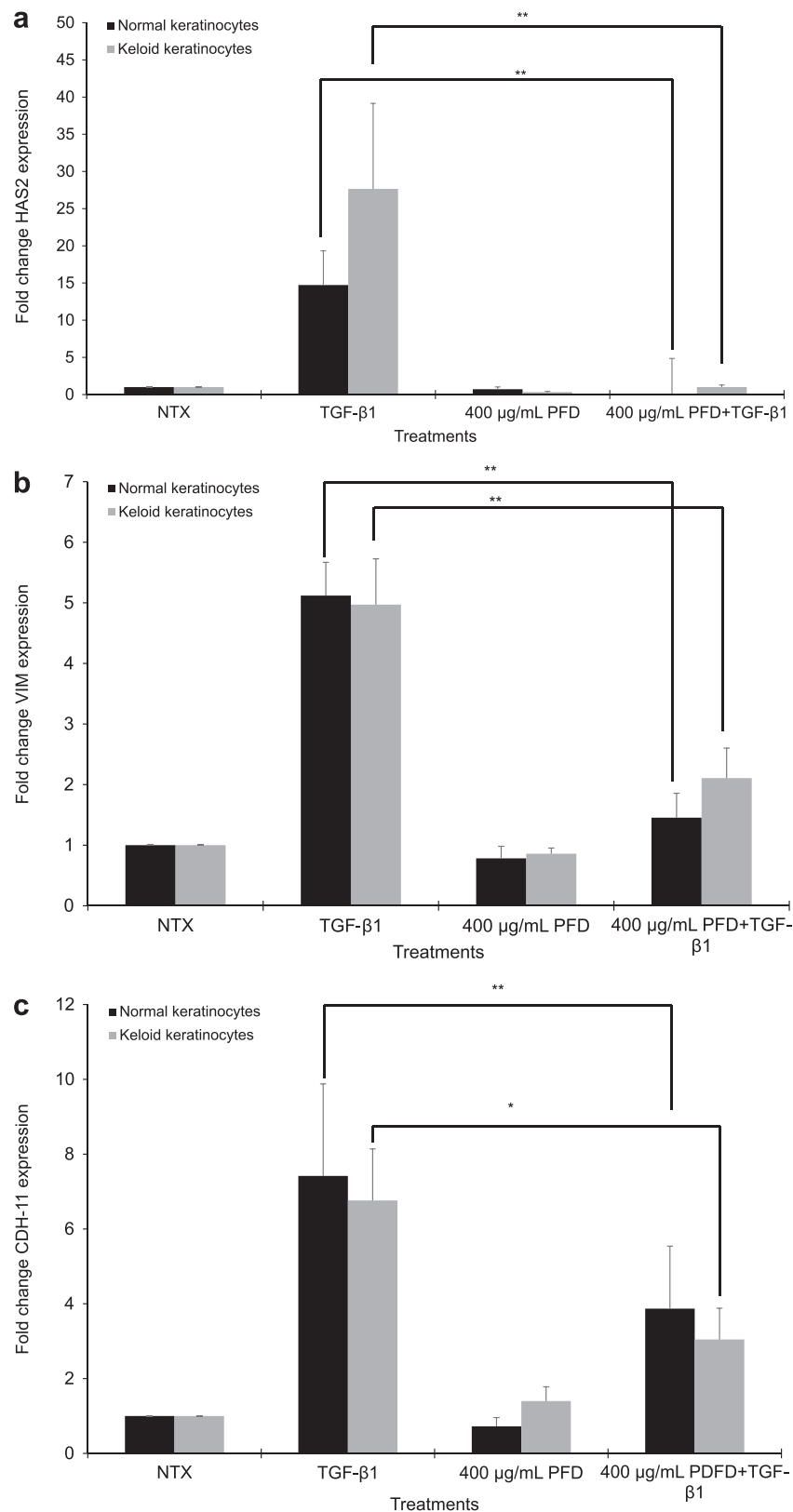


Figure 2. Effect of pirfenidone on EMT genes in normal and keloid keratinocytes. Four different donor strains each of normal and keloid keratinocytes grown in MCDB-153 with specific growth factors and culture conditions were quiesced in medium without EGF for 24 h. After 24 h cells were exposed to TGF-β1 (10 ng/mL) and pirfenidone (400 µg/mL) for an additional 24 h. After 24 h, RNA was extracted, and real-time RT-PCR was performed on (a) HAS2, (b) VIM, (c) CDH-11, (d) WNT5a, and (e) FN-1. Experiments were performed in triplicate on each of four donor strains, and data are presented as fold change expression compared to NTX normal and keloid keratinocytes. Values are mean ± SEM of experimental triplicates for all four donor cell strains per cell type. Two-way analysis of variance (ANOVA) was used to determine statistical significance. $p < 0.05$ was considered a statistically significant difference. * $p < 0.05$; ** $p < 0.001$. EGF epidermal growth factor, EMT epithelial mesenchymal transition, FN-1 fibronectin-1, HAS2 hyaluronan synthase-2, TGF-β1 transforming growth factor-β1, NTX non-treated control, VIM vimentin

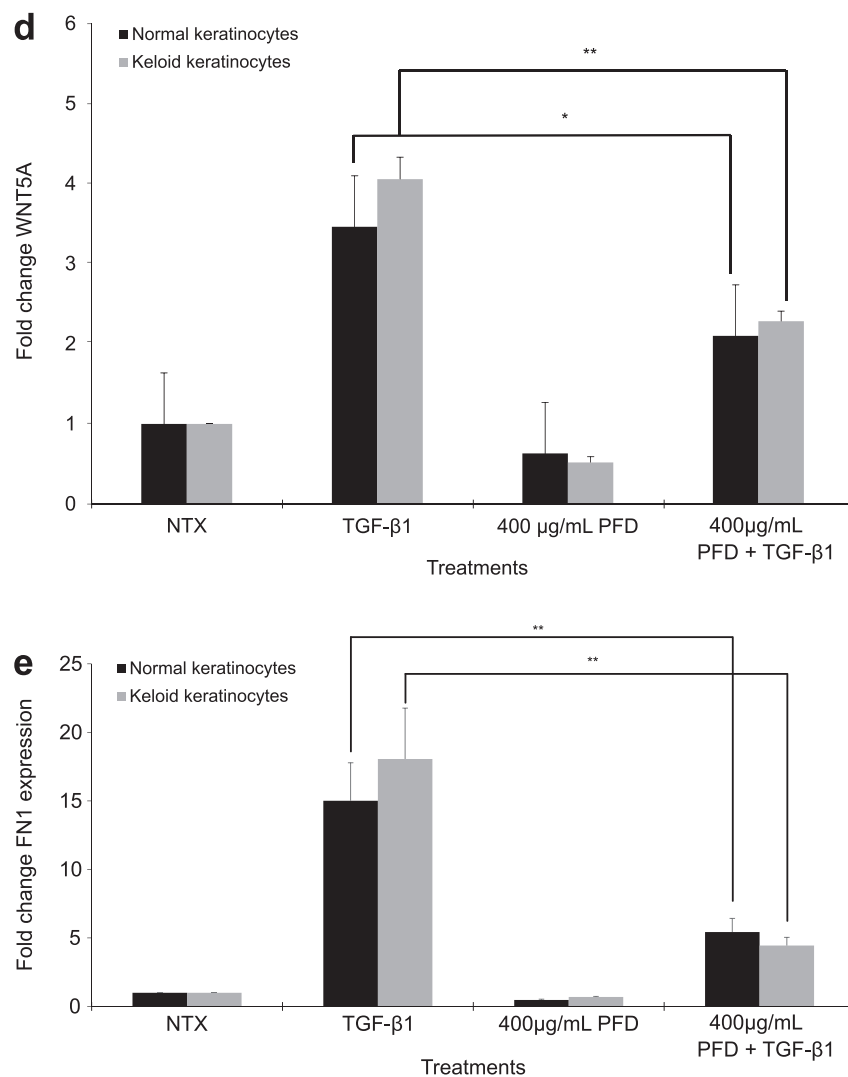


Figure 2. continued

Pirfenidone reduced both basal and TGF- β 1-induced protein levels of VIM and FN-1 in both normal and keloid keratinocytes

To ascertain whether the changes occurring at the mRNA level are recapitulated in protein expression, immunofluorescence was performed on the EMT genes VIM and FN-1. Interestingly, pirfenidone inhibited both basal and TGF- β 1-induced expression of VIM and FN-1 protein in both normal and keloid keratinocytes (Fig. 3a, b).

Discussion

For the first time, we report the effect of pirfenidone, a known antifibrotic agent, on keratinocytes derived from normal skin and keloid scars. We show that pirfenidone can inhibit TGF- β 1-mediated increases of EMT genes and both proliferative and cell migratory efficiency of normal and keloid keratinocytes. Notably, some of these effects were more pronounced in keloid keratinocytes. Studies have reported the involvement of keloid keratinocytes in

the progression of keloids by influencing the biological properties of fibroblasts [38, 39]. EMT process occurring in keratinocytes could significantly enhance the number of fibroblasts with an ultimate increase in the production of ECM components. A previous study reported the potency of pirfenidone in suppressing the contraction of keloid fibroblasts in collagen gels with or without the addition of TGF- β 1 by downregulating connective tissue growth factor (CTGF) and alpha-smooth muscle actin (α -SMA) [29]. EMT plays a significant role in keloid pathology. Keloid is a complex disease with multiple events occurring at the same time and if a small molecule like pirfenidone can target the overt activities of fibroblasts and keratinocytes simultaneously, this will be of benefit for the treatment of keloids. In a limited number of cancer cell lines, pirfenidone has proven to be effective in inhibiting the EMT process. Pirfenidone was shown to be effective in inhibiting TGF- β 1 and fibroblast growth factor-2 (FGF-2)-induced EMT in human adenocarcinoma cell lines [32]. Similarly, in non-small cell lung cancer, pirfenidone inhibited TGF- β 1-induced

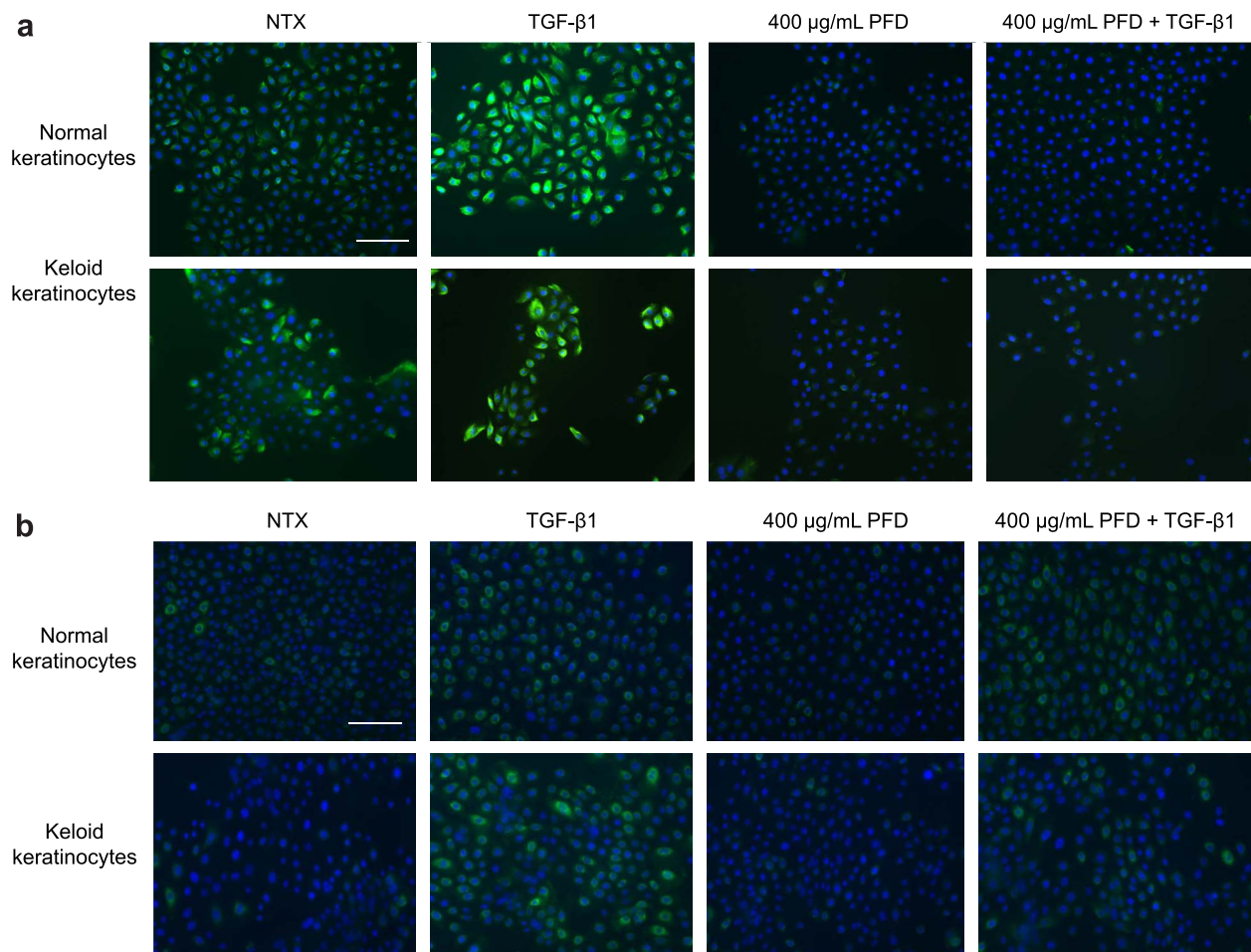


Figure 3. Effect of pirfenidone on EMT proteins in both normal and keloid keratinocytes. Two different donor strains each of normal and keloid keratinocytes were subjected to immunofluorescence after the cells were exposed to TGF- β 1 (10 ng/mL) and pirfenidone (400 μ g/mL). Cells were fixed using 4% paraformaldehyde and were incubated with primary antibody against vimentin (a) and fibronectin (b) and incubated with fluorescently labeled secondary antibody chicken anti-rabbit, Alexa Fluor 488, following which cells were mounted using DAPI and visualized and photographed using an Eclipse 90i microscope and photographed with a DS-Qi1MC Digital Microscope Camera. Shown here is the representation of image captured from two different cultures each of normal and keloid keratinocytes. All images shown were captured in the same magnification. Scale bar: 100 μ m. EMT epithelial mesenchymal transition, TGF- β 1 transforming growth factor- β 1, NTX non-treated control

EMT by inhibiting SMAD2 phosphorylation, reducing TGF- β 1 receptor 1 expression and inhibiting the translocation of SMAD2 from the cytoplasm into the nucleus [40]. One of the common aspects shared by keloid and malignant tumors is the capacity of prolonged cellular proliferation. Cancer progression is associated with migration, proliferation, invasion, and metastasis [41]. Except for metastasis, the other events occur in keloids, which characterizes keloid as a benign tumor and not malignant, which makes a distinction from cancers [42]. In this context, the drugs that interfere with EMT in cancer cells might also be effective in keloids.

We previously reported involvement of EMT in keloid pathology based on an EMT-like gene expression signature [34]; later, Yan et al. [33] reported observations of EMT-like phenomena in keloid tissues along with the increased expression of TGF- β 1 and SMAD phosphorylation. Our group has also observed reduced expression of E-cadherin, and increased active β -catenin in vivo, in keloid epidermis, consistent with EMT. We further showed that TGF- β 1 regulated the

expression of EMT genes, and reverted the expression of these EMT genes by inhibiting the canonical and non-canonical TGF- β 1 signaling pathways [12]. Interestingly, another study implicated microRNA (miRNA) in keloid EMT. This study showed that miR-21-5p was upregulated in keloid epidermis, which might be responsible for invasion and recurrence of keloids due to the enhanced EMT phenotype and the stem cell-like characteristics of keloid keratinocytes [43].

Few limited approaches have been put forth to inhibit the EMT phenomenon in keloids. A recent study by Zhang et al. [44] proposed hyperbaric oxygen therapy. The authors proposed this therapy from the previously published studies by Ma et al. [14] wherein they demonstrated that the EMT phenomenon exists in keloid tissue and that under hypoxic conditions, hypoxia-inducible factor-1 alpha (HIF-1 α) upregulates the expression of vimentin and fibronectin and downregulates the expression of E-cadherin and zonula occludens-1 in keloid keratinocytes. In light of that study, Zhang et al. [44] reported that keloid tissue excised from

patients undergoing hyperbaric oxygen treatment once a day for 7 days showed decreased expression levels of HIF-1 α , fibronectin, and vimentin, suggesting that hyperbaric oxygen treatment has protective effects against the EMT phenomenon during keloid development. Another recent study reported that metformin, an anti-diabetic drug, can reverse EMT by downregulating the HIF-1 α /pyruvate kinase M2 signaling pathway, thereby inhibiting keloid development [45]. For the present study, our focus was to determine the effect of pirfenidone on the five core EMT genes, namely, HAS2, VIM, CDH-11, WNT5A, and *FN-1*. All of these genes have been implicated in keloid EMT, and our previous findings have determined that TGF- β 1-regulated expression of HAS2, VIM, WNT5A, and CDH-11 can be downregulated by inhibiting both canonical and non-canonical TGF- β 1-induced signaling pathways [12].

HAS2, an enzyme that induces hyaluronic acid (HA) biosynthesis, is known to be induced by TGF- β 1 and mediates EMT. We have previously shown that HAS2 is overexpressed in keloid keratinocytes compared to normal keratinocytes and HAS2 contributes to increased cell migration and altered gene expression. We also reported that decreasing HAS2 expression using 4-methylumbelliferone (4MU) reduced migration of keloid keratinocytes [46]. Our present findings showed significant inhibition of mRNA expression of TGF- β 1-induced HAS2 expression in keloid keratinocytes than normal keratinocytes, and observed significant decrease in the basal and TGF- β 1-induced migratory capacity of keloid keratinocytes when induced with pirfenidone, indicating that pirfenidone inhibition of HAS2 may have decreased migration in keloid keratinocytes. The role of pirfenidone on HAS2 expression in keloid fibroblasts is not yet known and warrants investigation. Interestingly, in vocal scar fold fibroblasts HAS2 mRNA expression was increased by the addition of 1 mg/mL of pirfenidone and authors contribute to this as a positive effect as HAS2 induces hyaluronic acid production and is the critical component for the restoration of vocal fold pliability [47]. *FN-1* and *VIM* are well characterized as mesenchymal EMT genes in keloid keratinocytes [14]. In human lung adenocarcinoma cell lines, pirfenidone effectively inhibited the expression of *FN-1* and *VIM* and reverted the process of EMT [32]. There have been no prior studies that investigated the effect of pirfenidone on the expression of EMT genes WNT-5a and CDH-11. Studies by Igota et al. [48] showed the involvement of the WNT5a/ β -catenin signaling pathway in keloid fibroblasts, thereby promoting keloid progression. WNT5A expression is also shown to be upregulated in pancreatic cancer, skin cancer, and gastric cancer along with non-small cell lung cancer [49]. We have previously shown an increased expression of WNT-5A in keloid versus normal keratinocytes, as well as its induction by TGF- β 1; this increase was abrogated by inhibiting the TGF- β 1 signaling pathways [34]. Similarly, CDH-11 is a hallmark of the EMT process, and high expression is seen in keloid keratinocytes [34]. Given the established role of CDH-11 in cancer, it is perceived as a therapeutic target, and similarly, CDH-11's prominent role in keloids makes it an

attractive target. Direct demonstration of inhibition of CDH-11 by pirfenidone is yet to be seen in cancer cells, but our present study strongly suggests the ability of pirfenidone in inhibiting CDH-11 expression in keloid keratinocytes.

Our previous studies on Dupuytren's disease (DD)-derived fibroblasts, a fibrotic condition of the palm, demonstrated that pirfenidone significantly inhibited TGF- β 1-induced ECM proteins, mainly type-I and type-III collagen and *FN-1*, and also reduced the expression of α -SMA along with migratory and proliferative effects [27]. Because all the fibrotic characteristics of DD fibroblasts are also observed in keloid fibroblasts, it warrants in-depth investigation of pirfenidone in keloid fibroblasts. The present study explored the utility of pirfenidone in keloid keratinocytes and establishes its efficacy in reducing EMT. These findings make pirfenidone a strong candidate to be evaluated on patients, and a quicker clinical translation can be achieved as extensive evaluation on the safety profile has already been conducted on patients orally administered with pirfenidone suffering from IPF. Notable side effects due to oral administration of pirfenidone are skin rash, nausea, and dyspepsia, which can be circumvented by local target administration of pirfenidone. We envision utilizing pirfenidone as an injectable formula to directly target keloids. In the future, our hope is that pirfenidone can be used on clinically diagnosed keloids to prevent growth and injected onto the site of keloids that have been resected to prevent recurrence.

Conclusions

Our findings from this study strengthen the idea of using pirfenidone in clinical settings for treating keloid lesions. Previous studies have shown the effectiveness of pirfenidone in suppressing the fibrotic properties of fibroblasts in various fibrotic disease. Especially in keloid fibroblasts, contraction of collagen gel was reduced by the addition of pirfenidone by inhibiting the downstream pathway of TGF- β 1. Our study shows for the first time that pirfenidone is potent in inhibiting EMT occurring in keloid keratinocytes and also inhibited the cells' ability to migrate and proliferate. TGF- β 1 is a prominent growth factor in the growth and progression of keloids. Interestingly, the pronounced effect of pirfenidone was seen on TGF- β 1-induced EMT genes in both normal and keloid keratinocytes, suggesting that the growth factor effects can be negated in both keloid fibroblasts and keratinocytes for a positive outcome. Additional studies will be needed to define the signaling mechanism(s) through which pirfenidone limits the actions of TGF- β 1 in keloid keratinocytes.

Abbreviations

ADAM19: ADAM metalloproteinase domain 19; CDH-11: cadherin-11; ECM: extracellular matrix proteins; EGF: epidermal growth factor; EMT: epithelial-mesenchymal transition; *FN-1*: fibronectin-1; FZD-7: frizzled-7; HAS2: hyaluronan synthase-2; IL-6: interleukin-6; MSCs: mesenchymal

stem cells; TGF- β 1: transforming growth factor- β 1; TNF- α : tumor necrosis factor- α ; VIM: vimentin; WNT5A: wingless-type MMTV integration site family.

Supplementary material

Supplementary material is available at *Burns & Trauma Journal* online.

Declarations

Not applicable.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LS developed the study idea, designed and planned the experiments, critically analyzed, and interpreted the data, and drafted the manuscript. AE and JMH were involved in the study discussion and planning of the experiments. AE collected data, analyzed, performed statistical analyses, and provided comments on the manuscript. JMH isolated and cultured primary keratinocytes from normal skin and keloid scars, performed immunofluorescence and provided her expertise in real-time RT-PCR and read the manuscript and provided comments. EG performed real-time RT-PCR and participated in the study discussion. DMS assisted with primary cell culture, actively participated in the study discussion, provided reagents and critically analyzed and interpreted the data along with LS. DMS reviewed the manuscript, provided comments, revised and approved the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Keloid tissues were obtained after informed consent from patients undergoing keloid resection and were through the approval from the University of Cincinnati Institutional Review Board (Study ID #2013–2166). Normal skin collected from de-identified surgical discard tissue designated as “not human subjects research” by the University of Cincinnati Institutional Review Board.

Consent for publication

All authors provide their consent to publish.

Conflicts of interests

The authors declare that they have no competing interests.

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