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Cholesterolgenic Inhibition Causes Permanent Hair Follicle Damage by Activating Fibrosis Via the Angiotensin Receptor

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Abstract

Primary Cicatricial Alopecia (PCA) is a type of inflammatory hair loss disorder resulting in the permanent damage of the pilosebaceous structure due to fibrosis. Various internal and environmental stimuli caused the breakdown of hair follicle cells. Cholesterol is a crucial component in the formation and differentiation of hair follicles and the skin's overall health. The loss of hair follicles and aberrant cycles were caused by inhibiting or obstructing the cholesterol biosynthetic pathways. This study suggests that cholesterologenic changes like precursor formation and inhibition in the hair follicle, trigger inflammation, fibrogenic signaling and lead to fibrosis. TGFβ-SMAD pathways related to the fibrogenic process were significantly expressed during the experimental condition. Angiotensin II receptor, AGTR1, showed a profound effect on the hair follicle cells. Real-time PCR analysis and immunohistochemistry of the patient's scalp biopsies, HHFORS cells, and mice tissue sample revealed that the fibrotic genes were significantly activated after the treatment of BM15766, a cholesterol biosynthesis inhibitor, and 7-DHC, a sterol precursor. Our study confirmed that fibrosis is developed in the late stage of PCA by the dysregulation of cholesterol biosynthesis pathways in the hair follicle cells.

Keywords: Aryl hydrocarbon Receptor, Angiotensin II, Primary Cicatricial Alopecia, Autoimmune Disorder, Peroxisome Proliferator-Activated Receptors γ, Transforming Growth Factor β

1. Introduction

Cicatricial Alopecia (CA) is an uncommon inflammatory hair loss disorder that permanently destroys stem cells in the hair follicles or pilosebaceous structures replaced by fibrotic tissues (scar tissue). It is classified into primary and secondary. In Primary Cicatricial Alopecia (PCA), the hair follicle is the main target of inflammation. PCA is divided into lymphocytic, neutrophilic and mixed based on the type of immune cell infiltrate. Inflammation can arise due to faulty immune response, severe pathological or fungal infection, chemical irritants, or potential allergens and inhibition of cholesterol biosynthesis. The distinguishing feature of the disorder is the destruction of the hair follicle due to many external and/or internal stimuli, which eventually leads to fibrosis. Diverse biological molecules and their pathways are associated with the normal functioning of the hair follicles and pilosebaceous unit. Cholesterol is a sterol that promotes the epidermal permeability barrier and is indispensable for hair follicle growth and differentiation. It is a precursor for the production of local steroid hormones[1], influences

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keratinocyte differentiation[2], corneocyte desquamation, barrier repair, and melanogenesis[3] among other things. Current evidence suggests that altered lipid homeostasis may have a remarkable role in Hair Follicle (HF) biology[4]. The hair follicle is one of the principal skin appendage-bearing hair shafts where lipids are important. Inhibition of Cholesterol biosynthesis in the experimental mice models resulted in loss of HF growth, abnormal cycling, and activation fibrosis[5]. Panicker *et al.*,[5] pointed out significant downregulation of genes associated with cholesterol biosynthesis in affected and unaffected scalp biopsy from PCA patients. Scarring type hair loss was seen in the mutated Emopamil-Binding Protein (EBP) of Conradi Hunermann syndrome, whose cholesterol biosynthetic pathways were also altered[6] OMIM 302960. Excessive or sustained production of TGFβ is a critical molecular mediator of tissue fibrosis[7].

Various genes and signaling pathways are included in the process of scar formation. TGFβ-SMAD signaling genes mainly contribute to the pathogenesis, and PPARγ genes down regulate the fibrosis-related processes, including cellular inflammation and differentiation[8]. Increased expression of Col1A1 and Col1A2, two distinct genes, is a common hallmark of fibrotic diseases leading to the synthesis of Type I Collagen[9]. Transforming Growth Factor β (TGFβ) is a crucial cytokine that initiates and terminates tissue repair and whose sustained production contribute to the development of tissue fibrosis[10]. A recent work explained the role of inflammatory proteins and cytokines in PCA's pathogenesis[11]. Inhibition of Cholesterol biosynthesis also upregulated TGFβ, an established inducer of catagen and fibrosis[12–14]. The phosphorylation of SMAD2 and SMAD3 primarily carried out TGFβ actions on cells by activating the TGFβ type I receptor and TGFβ signaling[15]. TGFβ signaling results in a potent stimulant, and it initiates the expression of many pro-fibrotic genes and a marked increase in their related proteins[16]. A shred of increasing evidence implicates that the TGFβ family exerts a vital function in the pathogenesis of dermal scarring[17,18]. Two separate pathways control the synthesis of different Col1A1 polypeptides: the TGFβ activation protein pathway and the SMAD signaling pathway[19]. The Renin-Angiotensin system (RAS), an endocrine axis, plays a significant role in converting Angiotensin I to Angiotensin II. Angiotensin II is the classic endocrine hormone that participates in the inflammation and fibrogenesis of many organs including heart, lung, kidney, liver, and arteries[20]. Angiotensin Type 1 Receptor (AGTR1) controls the biologically active component of angiotensin II. RAAS may involve in the pathogenesis of alopecia areata[21]. TGFβ and Angiotensin II induce fibrosis via the Angiotensin Type 1 Receptor (AGTR1) in different cardiovascular and renal systems tissues, including pulmonary fibrosis and systemic sclerosis[22]. Angiotensin Receptor (AGTR1) has a stimulatory effect on the TGFβ-SMAD signaling pathway[23]. Lung Fibrosis is evident in a patient with SARS- CoV infection. They activate TGFβ/SMAD signaling pathways, and the receptor Angiotensin-Converting Enzyme 2 (ACE2) is a negative regulator of particular fibrosis[24].

The regulatory potential of PPARγ in lipid metabolism, cholesterol homeostasis, and controlling the inflammatory response suggests a crucial for this nuclear receptor in the pilosebaceous unit maintenance[4,25]. PPARγ plays a significant role in decreasing the fibrotic process by antagonizing TGFβ1 and cutaneous lesions[26,27]. In scalp tissue from patients with lichen planopilaris (LPP, a form of PCA characterized by follicular inflammation and fibrosis), a significant reduction in PPARγ expression is found in both affected and unaffected HFs[2] Lichen planopilaris (LPP5). One of the most important receptors for xenobiotics is the Aryl Hydrocarbon Receptor (AhR)[28]. Studies show that overactivation of the AhR in the liver leads to an increased expression of several fibrogenic genes[29]. Cholesterol is synthesized from the simpler molecule by a complex 37-step process. 7-Dehydrocholesterol Reductase (DHCR7) is an enzyme that functions in the last step of cholesterol biosynthesis (conversion of 7DHC to Cholesterol). BM15766 is a pharmacological inhibitor of the enzyme DHCR7[30]. So in the present study, we investigated the fibrosis in altered cholesterol biosynthesis on the different types of PCA, Human Hair Follicle-Outer Root Sheath Cells, HHFORS (*in vitro*), and Mouse model (*in vivo*). There are lacunae in the connection between cholesterol inhibition and intermediate sterol accumulation in the hair follicle dysfunction and scar formation and, we also try to establish the significance of the hormonal mechanism of angiotensin II in the pilosebaceous unit.

2. Materials and Methods

2.1 Human Tissue (Scalp Biopsy)

All research experiments involving human subjects were carried out based on the written approval by the

Human Research Ethics Committee of the University of Kerala (No.ULECRIHS/UOK/2016/3). Written informed consent was obtained from the humans involved in this study. The histopathologic observation of the lymphocytic, neutrophilic, and mixed types of PCA were already described in clinical conditions[5]. Four mm scalp biopsies were collected from the affected and non-affected areas of the patients with a different type of PCA. Scalp biopsy of three patients of each type of PCA was analysed, and scalp biopsy collected from three healthy people was considered the normal sample. The healthy volunteers confirmed that there was no hair disorder or inflammatory disorders. However, all patients had active symptoms of Cicatricial Alopecia including itching, burning, pain, and progressive hair loss. Tissue samples were collected and stored at -80°C for RNA extraction and subjected to qRT-PCR analysis.

2.2 *In vivo* and *in vitro* experiments

According to the Institutional Animal Ethics Committee's written approval, animal experiments were conducted at the University of Kerala (IAEC-KU-18/2016-17-ZOO-SRP (1)). Seven-week-old female C57BL/6 mice were divided into four groups. Depilation of the head region of the mice helped to synchronize the hair cycle was performed in previously published work[3]. The scalp hair was shaved and a depilatory agent was applied. 25mM 7DHC (Sigma, India) and 4mM BM15766 (Sigma, India) were treated as the test for 15 days. The chemicals or the vehicles (DMSO/Ethanol) were applied on the mouse head topically every day. Cutaneous tissue from the treatment area was collected under euthanasia and stored at -80°C. Tissues were used for Real-Time PCR analysis and embedded in paraffin wax subjected to histological analysis.

Human Hair Follicle Outer Root Sheath (HHFORS) cells were grown in mesenchymal cell media with growth supplements as per the manufactures specifications. Primary cell lines were purchased from Science Cell Research, USA. Cells were subcultured, and the third-fourth passage was seeded at a density of 6 x 10^7 cells in a 96 well plate. 7DHC (Sigma, India) was dissolved in ethanol, and BM15766 (Sigma, India) was dissolved in DMSO. 7DHC and BM15766 were treated in the serum-free medium of HHFORS cells. Using the MTT cell proliferation assay, the optimal concentration for the treatment of 7DHC (25µM) and BM15766 (4µM)[5] were determined. HHFORS cells were also treated with vehicles (Ethanol and DMSO). Control and treated samples were used for RNA isolation, continued by microarray and qRT- PCR analysis.

2.3 RNA Isolation and Microarray Analysis

RNA isolation and microarray analysis were conducted as explained previously[5]. Using TRIzol reagent (BR Biochem Life Sciences, India), the total RNA from each scalp biopsy, HHFORS cells, and mice tissue were extracted according to the manufacturer's instructions. Then purification of the sample was done using RNeasy Mini columns (Qiagen India Pvt Ltd.). The spectroscopic method was used for RNA quantification and subjected to microarray and real-time PCR experiments. Microarray analysis of the BM15766-7DHC treated HHFORS was done HG U133 plus 2.0 Array.

2.4 Quantitative Real-time PCR

SYBR Green-Labelled PCR primers for all selected genes purchased from G-Biosciences (Geno Technologies). RT-PCR was accomplished on an ABI StepOneTM Sequence Detection System (Applied Biosystems, India). Samples of the experiments include PCA Samples, Compound treated (7DHC & BM15766) mice and HHFORS samples, and Control/Vehicle samples. The

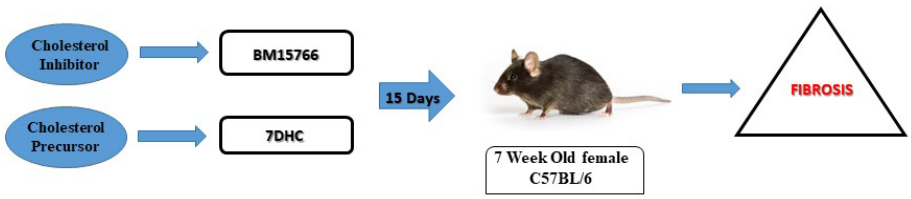


Figure 1. Schematic representation of *in vivo* Experiments.

Primer Name (Human)	Sequence		Expected product size
TGFβ	Forward	5′-AACCCACAACGAAATCTATG-3′	146 bp
	Reverse	5′-CTTTTAACTTGAGCCTCAGC-3′	
SMAD2	Forward	5′-GAGAGTTGAGACACCAGTTTTGC-3′	86bp
	Reverse	5′-ATAGTCATCCAGAGGCGGAAGTT-3′	
SMAD3	Forward	5′-GGAGACACATCGGATGCAA-3′	105bp
	Reverse	5′-GGGTCAACTGGTAGACAGCC-3′	
Col1A1	Forward	5′-TCCCAGGGTGGCTTCTGATA-3′	156bp
	Reverse	5′-GAGTCGGGGACACTTACAGC-3′	
AGTR1	Forward	5′-GTCCAAAGGCTCCACAGCTC -3′	263bp
	Reverse	5′-GGCCACAGTCTTCAGCTTCA -3′	
PPARγ	Forward	5′-TTATCATCTGCGTGAGCCAA -3′	144bp
	Reverse	5′-TGGCATCTCTGTGTCAACCA-3′	
CYP1A1	Forward	5′-ACCATCCCCCACAGGAAGCTAT-3′	317bp
	Reverse	5′-TCAGGGCTCTCAAGCACCTA-3′	
CYP1B1	Forward	5′-TACGGCGACGTTTTCCAGAT-3′	177bp
	Reverse	5′-CTCCGAGTAGTGGCCGAAAG-3′	
DHCR7	Forward	5′-CCCCTGGCTAGAGGGTAGG-3′	106bp
	Reverse	5′-TCAACCGGCTAAAGTCCTGC-3′	
EBP	Forward	5′-GGTTTGCAGTGTGTGGGTTC-3′	131 bp
	Reverse	5′-GTATCGGCTGTCTCCCTTGG-3′	
GAPDH	Forward	5′-TGGTATCGTGGAAGGACTCATGAC-3′	189 bp
	Reverse	5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′	
Primer Name (Mice)	**Sequence**		**Expected product size**
TGFβ	Forward	5′-ACTGGAGTTGTACGGCAGTG-3′	123bp
	Reverse	5′-GGGGCTGATCCCGTTGATTT-3′	
SMAD2	Forward	5′-GGGCTGTGACTTAAGGACCC-3′	202bp
	Reverse	5′-TGGCAAAGTCATAGGCCCTG-3′	
SMAD3	Forward	5′-GTACCCGTGGGAACCCAAAT-3′	159bp
	Reverse	5′-AACTCTGGAGAACTTGCCCG-3′	
Col1A1	Forward	5′-GGGGCAAGACAGTCATCGAA-3′	171bp
	Reverse	5′-GAGGGAACCAGATTGGGGTG-3′	
AGTR1	Forward	5′-GGTTGGAACCTGCGGAGTAG-3′	98bp
	Reverse	5′-CAGCATCATCCAGTCCCTCC-3′	
PPARγ	Forward	5′-CTGCTGGGGATCTGAAGGC-3′	196bp
	Reverse	5′-ATCACGGAGAGGTCCACAGA-3′	
CYP1A1	Forward	5′-AGGTGGTAGTTCTTGGAGCTT-3′	169bp
	Reverse	5′-GCAGAATACGGTGACAGCCA-3′	
CYP1B1	Forward	5′-CCAAGTGGCCTAACCCAGAG-3′	127bp
	Reverse	5′-TGCCATCCGATGCACCTC-3′	
GAPDH	Forward	5′-GCATCTTCTTGTGCAGTGCC3′	74bp
	Reverse	5′-TACGGCCAAATCCGTTCACA-3′	

expression of target genes in all samples was quantified using the ΔΔCT method, as explained in the ABI StepOneTM SDS manual (Applied Biosystems, India).

2.5 Immuno Fluorescence

Adding coverslip on six-well chamber slides, HHFORS cells were seeded at 10 X 10^3 per well. After treating BM15766 and 7DHC, cells were washed with PBS and fixed in ice-cold methanol. Slides were blocked in 5% FBS and then incubated with TGFβ-1 Polyclonal Antibody (1:500), SMAD2/SMAD3 Polyclonal antibody (1:100) and Col1A1 Polyclonal antibody (1:100) (Real Gene, India). Goat anti- Rabbit IgG (HRP conjugated) Secondary antibodies purchased from G-Biosciences (Geno Technologies). Nuclei were visualized by DAPI staining (Scientific Solution), and cells were analyzed using a fluorescence microscope (Leica).

2.6 Immuno Histochemistry

Mice treated with 7DHC and BM15766 were subjected to euthanization, then the skin of the treatment area (head region) was collected and placed in formalin overnight. Samples were embedded in paraffin wax based on the procedure used for the normal histology and stained with Hematoxylin and Eosin. For immunostaining of mouse skin tissue TGFβ-1 Polyclonal Antibody (1:500), SMAD2/SMAD3 Polyclonal antibody (1:100) were purchased from Real Gene, India. Goat anti- Rabbit IgG (HRP conjugated) Secondary antibody was purchased from G-Biosciences (Geno Technologies). Images were captured with LABOMED Lx 500 Binocular Microscope, and images were analyzed using Image J software.

3. Results

Based on qRT-PCR data and histological features, the cholesterol inhibitor-precursor treated samples and PCA patient samples showed fibrogenesis or scar development in hair follicle cells. The expression of *TGFβ-SMAD* signaling genes and *Col1A1* genes was drastically increased. *AGTR1* was shown to be highly expressed in hair follicle cells. *PPARγ,* which regulates lipid metabolism, and *DHCR7,* which regulates cholesterol production, were considerably downregulated. The interaction of the AhR gene *CYP1A1* and *CYP1B1* with *PPARγ* validated their cross-talk relationship in PCA inflammation and fibrosis.

3.1 PCA Samples Triggered Fibrotic Genes

PCA samples were collected from the patients with lymphocytic, neutrophilic, and mixed variants. Lichen Planopilaris (LPP), Frontal Fibrosing Alopecia (FFA), Central Centrifugal Cicatricial Alopecia (CCCA) are the major types of Lymphocytic PCA. Cicatricial Alopecia due to Neutrophilic inflammation consists of Folliculitis Decalvans (FD), and Tufted Folliculitis (TF). The third type of PCA with a mixed infiltrate (Lymphocytic and Neutrophilic) comprises Dissecting Cellulitis (DC). The real-time PCR was carried out with primers specific for the TGF signaling gene *TGF*β, SMAD2, SMAD3, and Col1A1. All these fibrotic genes were crucially expressed in different PCA subtypes of the affected sample compared with the unaffected sample. *TGF*β expression was elevated in the lymphocytic type of PCA as well (FFA, ~2.1 fold, LPP, ~1.2 fold, and CCCA, ~1.3 fold), and in the mixed type of PCA, DC was highly upregulated with a fold change of 1.8 compared to other types (Figure 1A). *SMAD2* and *SMAD3* genes of both Lymphocytic and Neutrophilic subtype PCA were significantly upregulated. *SMAD2* (TF, ~1.5 fold and DF, ~2 fold: FFA, ~1.1 fold, LPP, ~2.5 fold and CCCA, ~2.7 fold and DC, ~1.9 fold). *SMAD3* (TF, ~2.1 fold and DF, ~2.5 fold: FFA, ~1.8 fold, LPP, ~3.3 fold and CCCA, ~1.7 fold and DC, ~1.6 fold) (Figure 1 B, C). *Col1A1* expression was activated in the mixed type and lymphocytic PCA sample(TF, ~2 fold, and DF, ~9.5 fold: FFA, ~6.2 fold, LPP, ~2 fold, and CCCA, ~4.2 fold and DC, ~3.5 fold) (Figure 1D).

3.2 Inhibiting cholesterol biosynthesis promotes fibrosis signals in HHFORS

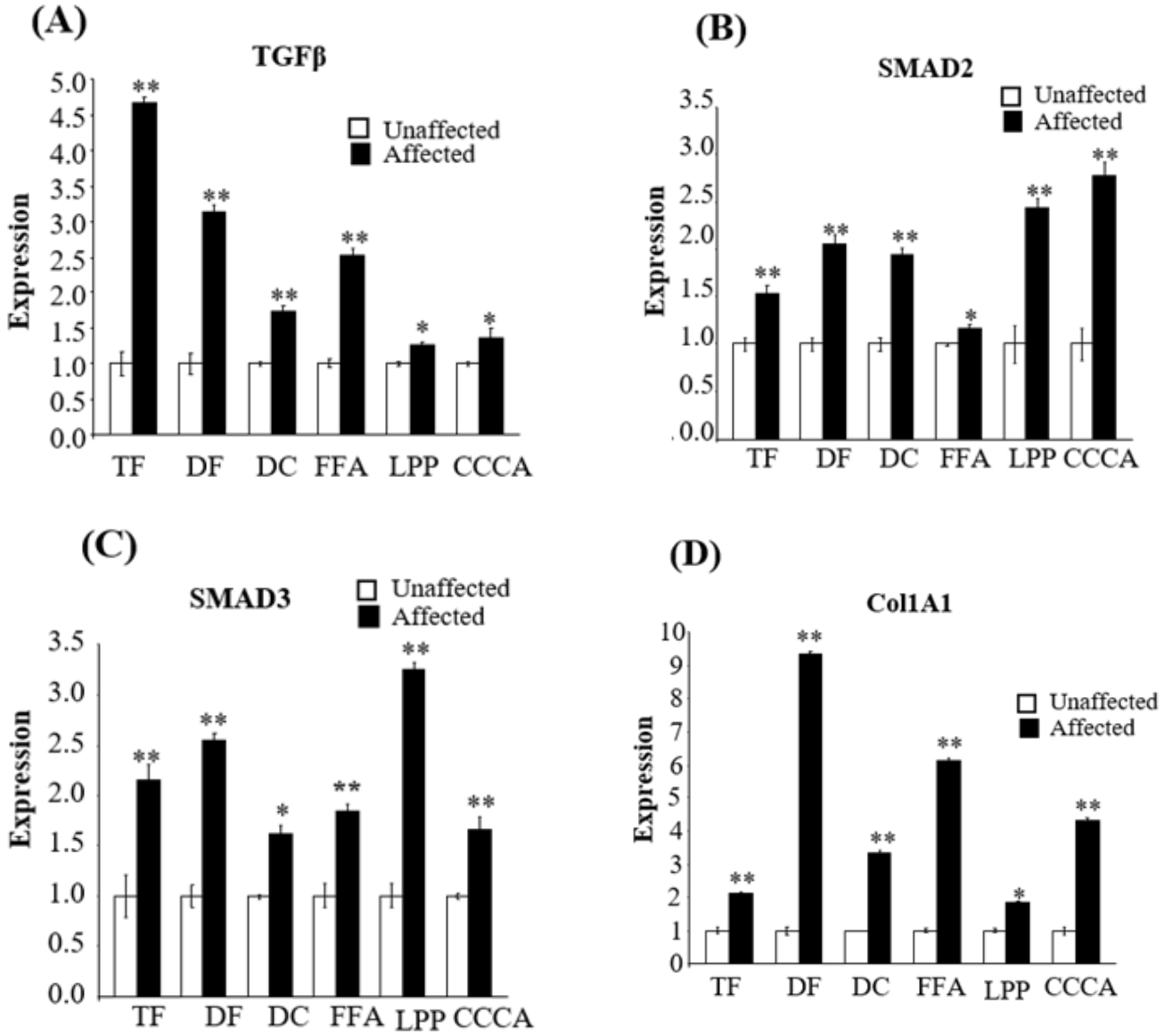
IPA analysis of the microarray data for 7DHC and BM15766 treated HHFORS cells is shown in Figure 1(E-F) (IPA Toxlist). IPA Toxlist was used to analyze the microarray data to evaluate toxicity-associated pathways. It is a data analysis tool in the IPA pathway analysis and determining the toxicity associated pathways in the experimental condition, with the observed gene expression changes. Figure 1(E-F) (Toxlist IPA) shows significant pathways with gene expression changes in HHFORS cells treated with 7DHC and BM15766. From the analyzed data, critical toxic pathways affected in cells treated with 7DHC were TGFβ signaling, Aryl hydrocarbon signaling, and hepatic fibrosis (Figure 1E). The toxic pathways involved in BM-15766 treated cells were cytochrome p450, xenobiotic metabolism, and hepatic fibrosis (Figure

1F). TGFβ signaling, hepatic fibrosis, and cytochrome P450 pathways appear to be the most significant pathways associated with fibrosis in HHFORS cells. This data confirms that inhibited cholesterol biosynthesis pathways led to the TGFβ signaling fibrosis in HHFORS.

3.3 *In vitro* study Revealed Activated TGFβ Signaling Genes and Protein

From IPA Pathway analysis, we identified that fibrosis-related pathways were significantly activated in the BM15766 & 7DHC treated HHFORS. So, we had to confirm that cholesterolgenic changes impact the expression of fibrosis-related genes. We treated HHFORS cells with 7DHC or BM15766 to check scarring genes are activating with this background. Expression of fibrotic genes was evaluated by Real-time PCR, with untreated groups, and the expression of TGFβ signaling genes was markedly increased in the treatment group. *TGFβ* was elevated in HHFORS cells treated with BM15766 (Figure 2A-E). *SMAD2* was explicitly upregulated in BM15766, but there was no change in cells treated with 7DHC. Following the treatment with 7DHC and BM15766, *SMAD3, Col1A1*, and *AGTR1* were significantly increased in HHFORS cells.

Immunocytochemistry of HHFORS cells treated with 7DHC and BM15766 showed that TGFβ signaling proteins were highly expressed (Figure 2F-J). We



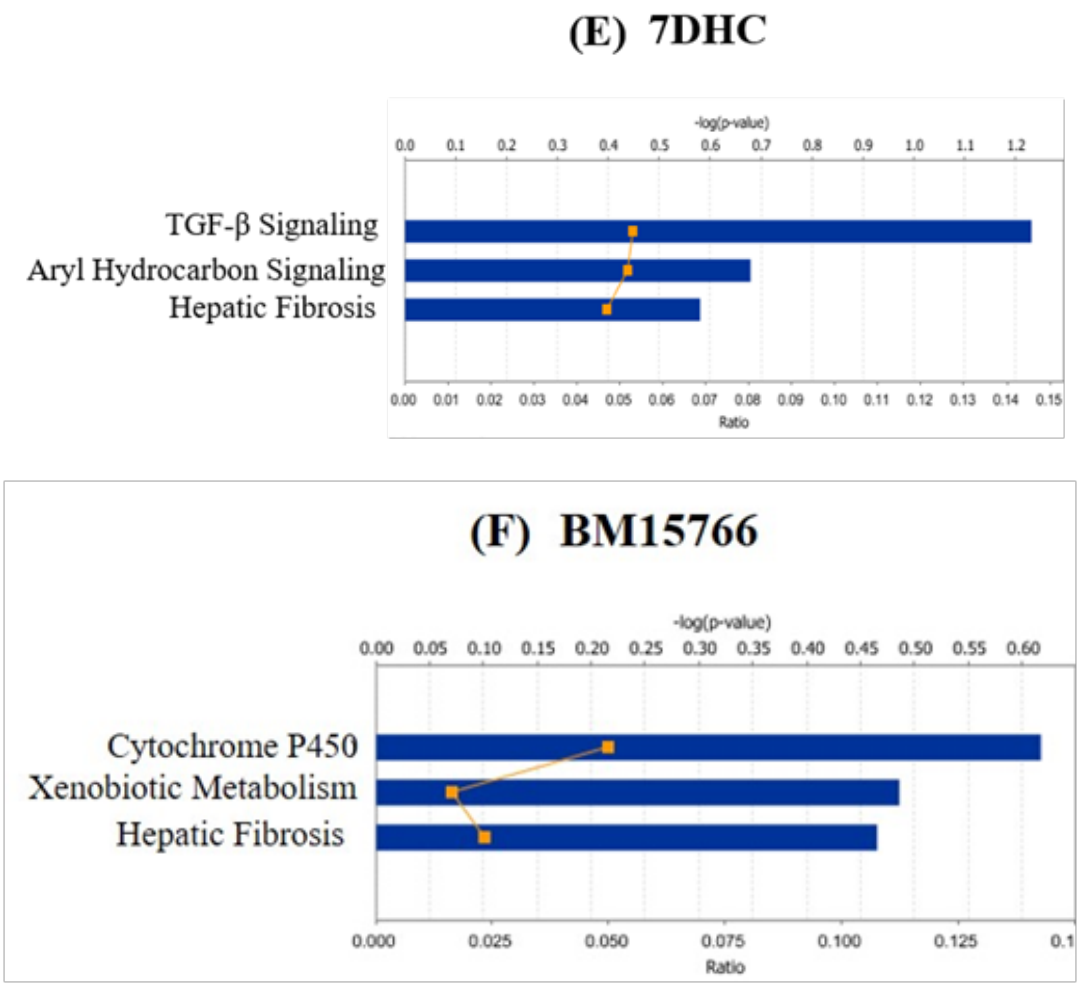


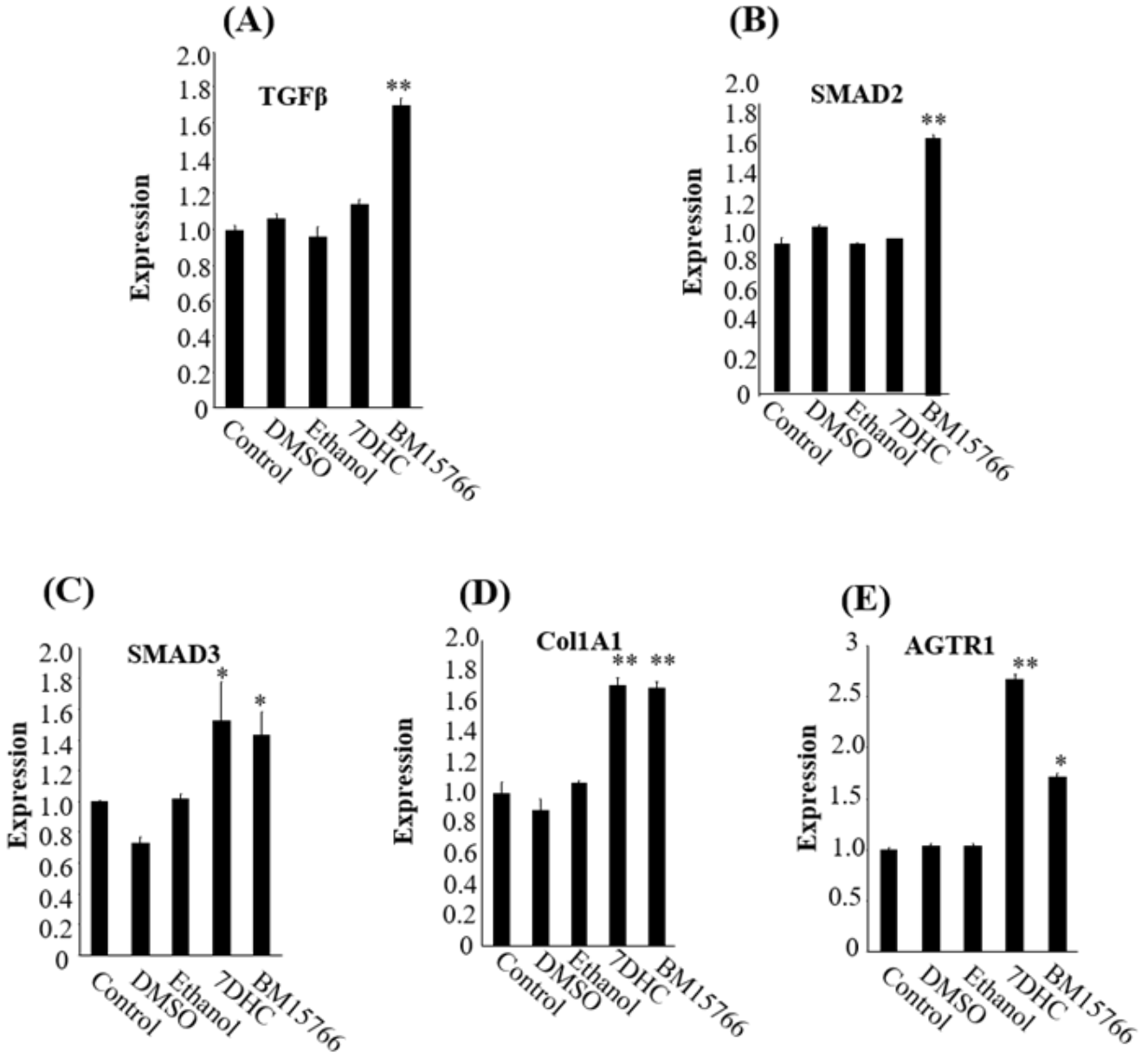
Figure 1(A-D): PCA samples triggered fibrotic genes. The Real -time PCR validation of *TGFβ, SMAD2, SMAD3, Col1A1* gene expression in the Primary Cicatricial Alopecia sub-groups TF, DF, DC, FFA, LPP and CCCA (*p<0.05, **p<0.01). Compared with unaffected samples, *TGFβ, SMAD2, SMAD3, Col1A1* gene expression significantly increased in all affected samples of PCA sub- groups. The unpaired t-test was used for statistical analysis. TF-Tufted Folliculitis, DF-Folliculitis Decalvans, DC-Dissecting Cellulitis, FFA-Frontal Fibrosing Alopecia, LPP-Lichen Planopilaris, CCCA-Central Centrifugal Cicatricial Alopecia.

Figure 1(E) and (F) Inhibition of cholesterol biosynthesis promotes fibrosis signals in HHFORS. TGFβ Signalling, AhR Signalling, Hepatic Fibrosis were the main biological pathways distressed by the treatment of HHFORS cells with (E) 7DHC and (F) BM15766. The number of differentially expressed genes (molecules), significant biological functions affected and their p values have been analysed using IPA analysis. The fold change of greater than or equal to 1.5, shows the significance of variation in the vehicle and 7DHC or BM15766 treatment ($p<0.05$ using a two-tailed unpaired *t*-test was observed).

examined the TGFβ signaling proteins expression in HHFORS cells using immunocytochemistry with specific antibodies. Confocal microscopic images representative of four different treatments of TGFβ, SMAD2, SMAD3, Col1A1, and AGTR1 are shown in the figure. Increased fibrotic signaling proteins accumulated in the single staining of antibodies and increased nuclear accumulation of TGFβ, SMAD2, SMAD3, Col1A1, and AGTR1 proteins identified in the merged treatment.

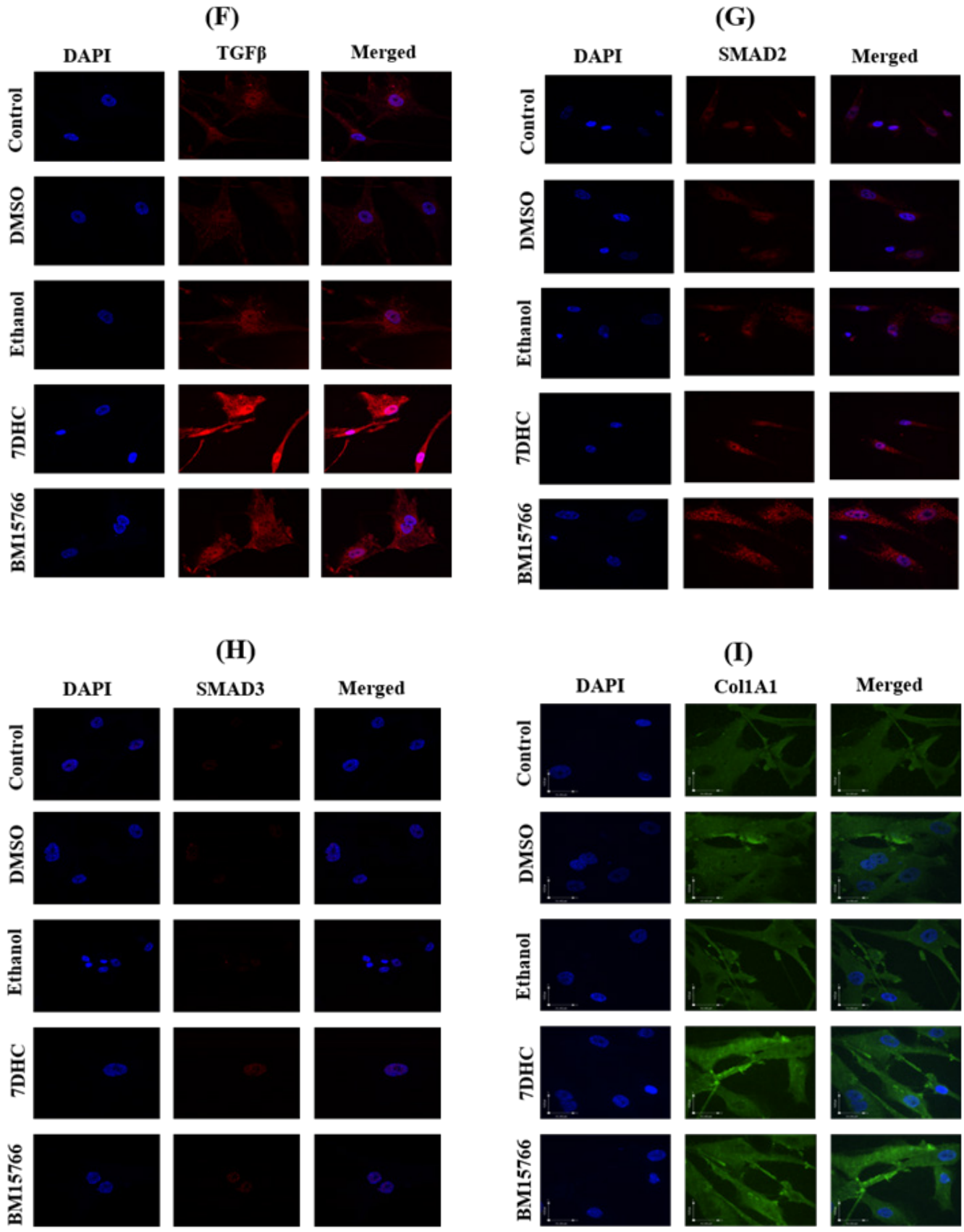
3.4 7DHC and BM15766 Affect Fibrotic Genes and Proteins *in vivo*

TGFβ signaling genes (such as *SMAD2*, *SMAD3*, and *Col1A1)* were dramatically elevated in 7DHC- treated



animals (Figure 3A-B). The expression of TGFβ signaling genes *TGF*β, *SMAD2*, *SMAD3*, and *Col1A1* were increased in BM-15766-treated mice, but the *AGTR1* signaling gene was down-regulated with no change.

Immunohistochemical staining was performed on a deparaffinized, formalin-fixed tissue section using appropriate antibodies (Figure 3C-F). The position and expression of TGFβ signaling proteins were analyzed using immunohistochemical (IHC) techniques. Normal or control skin of mice has modest protein expression of fibrotic gene TGFβ, SMAD2 and SMAD3 all over the skin. At the same time, intense staining was evident in both inner and outer root sheath cells of hair follicles in 7DHC and BM15766 treated groups. Immunolocalization for phosphorylated TGFβ, SMAD2, and SMAD3 indicated that downstream TGFβ signaling had differential expression in dermal fibroblast of 7DHC and BM15766 treated mice. The dystrophic hair follicle and perifollicular


(F)
DAPI
TGFβ
Merged
Control
DMSO
Ethanol
7DHC
BM15766
(G)
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SMAD2
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DMSO
Ethanol
7DHC
BM15766
(H)
DAPI
SMAD3
Merged
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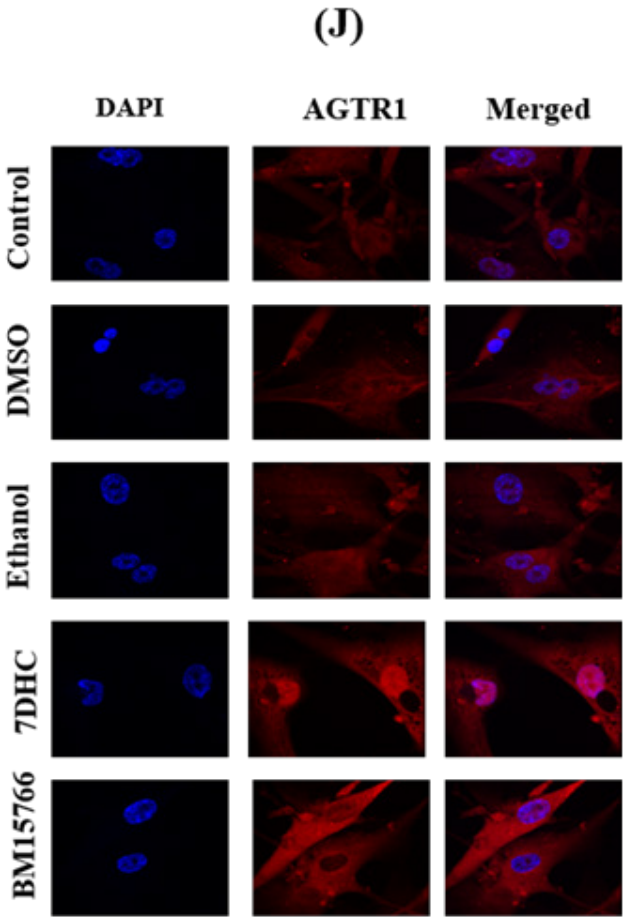

Figure 2. ***In vitro*** **study revealed activated TGFβ signaling genes and protein. (A-E)** The Real -time PCR validation of *TGFβ, SMAD2, SMAD3, Col1A1* and *AGTR1* gene expression in 7DHC and BM15766 treated HHFORS (Human Hair follicle Outer Root Sheath Cells) cells (*p<0.05, **p<0.01). Compared with Control and Vehicle (DMSO) group *TGFβ, SMAD2, SMAD3, Col1A1* and *AGTR1* gene expression significantly increased. The unpaired t-test was used for statistical analysis. **(F-J)** Confocal immunoflourescent images represent the four separate treatments of TGF*β*, SMAD2, SMAD3, Col1A1 and AGTR1 antibodies. TGF*β*, SMAD2, SMAD3, Col1A1 and AGTR1 proteins were significantly expressed in the 7DHC and BM15766 treated groups.

fibrosis were observed in the treatment. 7DHC and BM15766 treated mice section reveals a significant change in dermal thickness and density in the skin's outer layer compared to vehicle-treated animals. The 7DHC and BM15766 treated sections showed an increased level of fibroblasts. For TGFβ, staining in ethanol-treated skin showed reticular fiber in the papillary dermis.

3.5 BM15766 and 7DHC Affect Mouse PPARγ and AhR Signaling

PPARγ is downregulated in cholesterol precursor-7DHC administrated mice, according to gene expression analysis (Figure 3G). The Aryl Hydrocarbon Receptor (AhR) is a xenobiotic chemical sensor that regulated xenobiotic-metabolizing enzymes like Cytochrome P450. The cytochrome P450 enzymes were coded by the genes *CYP1A1* and *CYP1B1*. Expression of both the xenobiotic genes (*CYP1A1* and *CYP1B1*) was dramatically elevated in BM15766 treated mice (Figure 3H).

3.6 AhR and PPARγ Cross-Talk were Observed in 7DHC and BM15766 Treated HHFORS

PPARγ is a transcription factor that modulates the lipid metabolism in organisms. The *CYP1A1* and *CYP1B1* genes directly targeting AhR and xenobiotic genes were

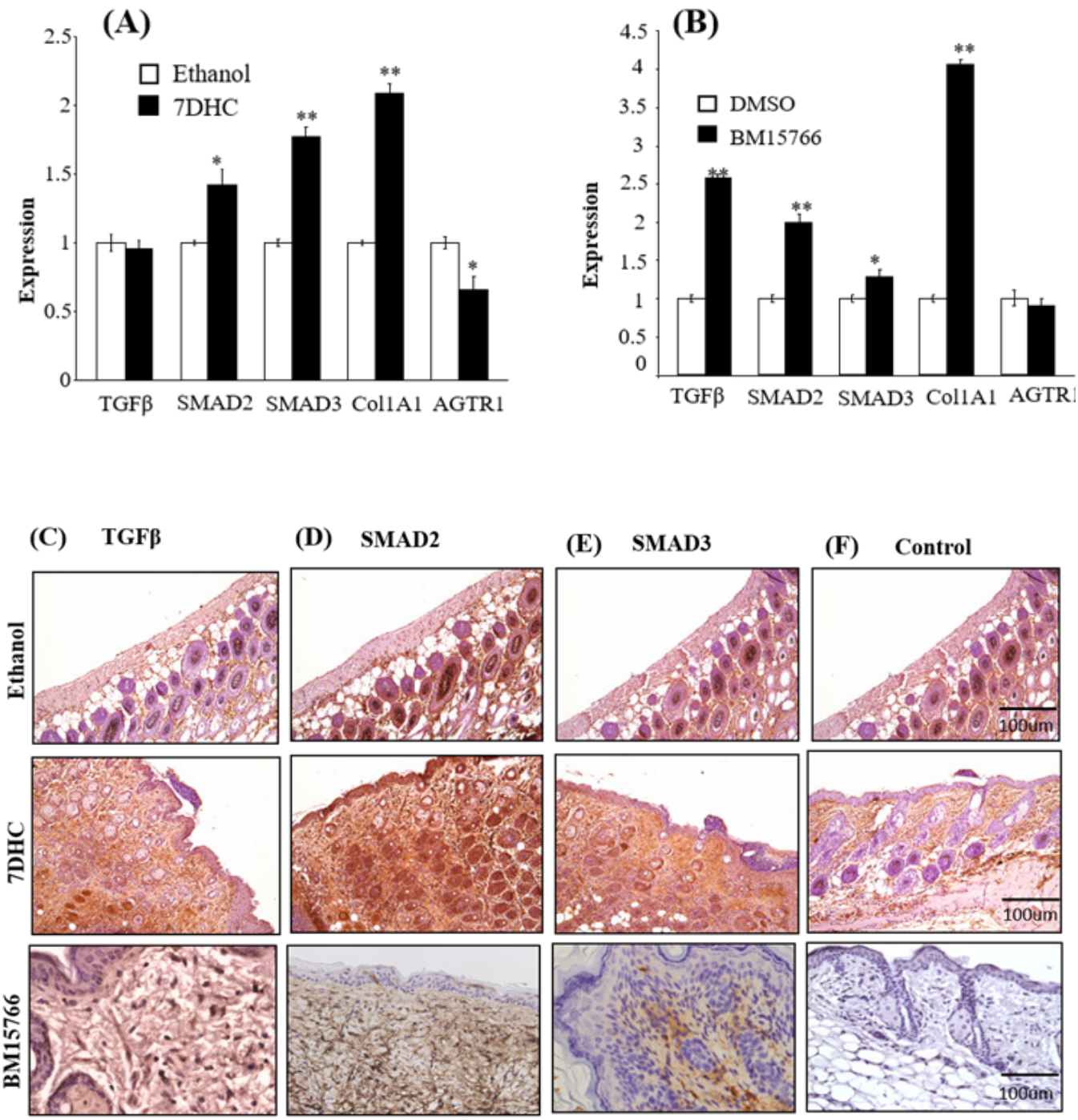

Figure 3(A-E) 7DHC and BM15766 affect fibrotic genes and proteins ***in vivo*.** The Real-time PCR validation of *TGFβ*, *SMAD2, SMAD3, Col1A1* and *AGTR1* gene expression in 7DHC and BM15766 treated mouse skin (*p<0.05, **p<0.01). Compared with Vehicle (Ethanol and DMSO) group *TGFβ, SMAD2, SMAD3, Col1A1* and *AGTR1* genes expression were significantly upregulated. **(C-F)**. Immunohistochemical staining was performed on deparafffinized, formalin fixed mouse tissue section using with TGFβ, SMAD2, SMAD3 antibodies. Control skin expressed that modest immunostaining all over the epidermis. Acute staining were present in the 7DHC and BM15766 treated groups. Dystrophic hair follicle, perifollicular fibrosis and increased number of fibroblast were present in the treated groups.

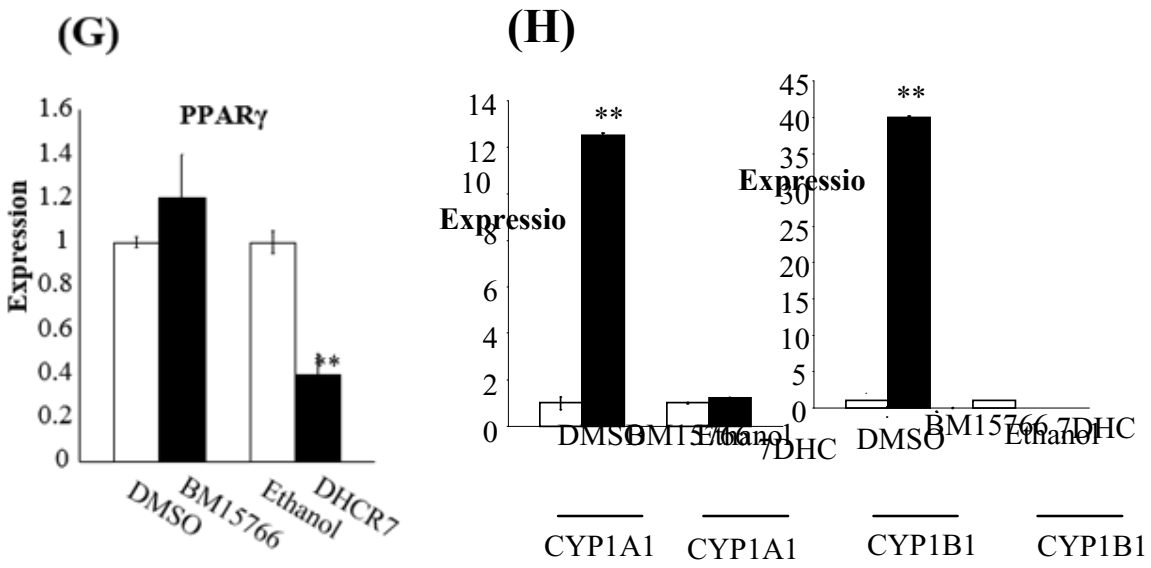

Figure 3(G-H) BM15766 and 7DHC affect mouse PPARγ and AhR Signaling. *PPARγ* gene expression in the 7DHC treated group is downregulated, and AhR gene like *CYP1A1* and *CYP1B1* were significantly upregulated in BM15766 treated group.

upregulated in 7DHC and BM15766 treated tissues (Figure 4A-B). While, *PPARγ* gene expression was downregulated in 7DHC and BM15766 treated tissues (Figure 4C). The cross-talk analysis revealed an inverse relationship between AhR and *PPARγ*. Evidence indicated that the AhR suppresses *PPARγ* gene expression in response to dioxin-like compounds[33,34]. The *PPARγ* gene expression was significantly decreased in the experimental condition, and it seems lipid metabolism was altered. This cross-talk analysis of AhR genes and *PPARγ* confirmed that the action of BM15766 and 7DHC as a xenobiotic or external chemical leads to the destruction of aspect of lipid metabolism such as cholesterol biosynthesis; therefore, the *PPARγ* gene is down-regulated. *PPARγ* inhibition or AhR activation causes scarring by induction of TGFβ signaling.

3.7 Cholesterol-Related Genes were Inhibited under *in vitro* Condition

Gene expression analysis confirmed that cholesterol-related genes like DHCR7 and EBP were significantly decreased in 7DHC and BM15766 treated cells. Thus it indicates that cholesterol biosynthesis is inhibited by BM15766 and 7DHC (Figure 4D-E).

4. Discussion

The most important discovery of our research was how inhibition of cholesterol biosynthesis induce fibrosis and lead to permanent hair loss, ultimately leading to permanent loss of the hair follicle. Furthermore, several fibrotic genes were considerably expressed in the diverse experimental setting (*in vitro, in vivo,* and patients sample). Hair follicles with overactive fibrogenic responses cause tissue deterioration and disrupt hair follicle homeostasis. TGFβ signaling and SMAD pathways have been identified as significant positive regulators of tissue fibrosis in the hair follicle in numerous *in vitro*, *in vivo*, and scalp studies. This study also discovered that the endocrine Angiotensin II receptor significantly impacts human hair follicles.

Various PCA studies proved that inflammatory reactions represent disease pathogenesis, and changes within the hair follicles trigger the inflammation[4]. The gene expression analysis showed that TGFβ signaling genes were activated in the different PCA samples, compared with affected versus unaffected areas of scalp tissues. The data confirm that fibrosis occurs in the late stage of alopecia. DHCR7 and EBP were significantly downregulated in the experimental conditions, substantiating that cholesterol synthesis altered the hair

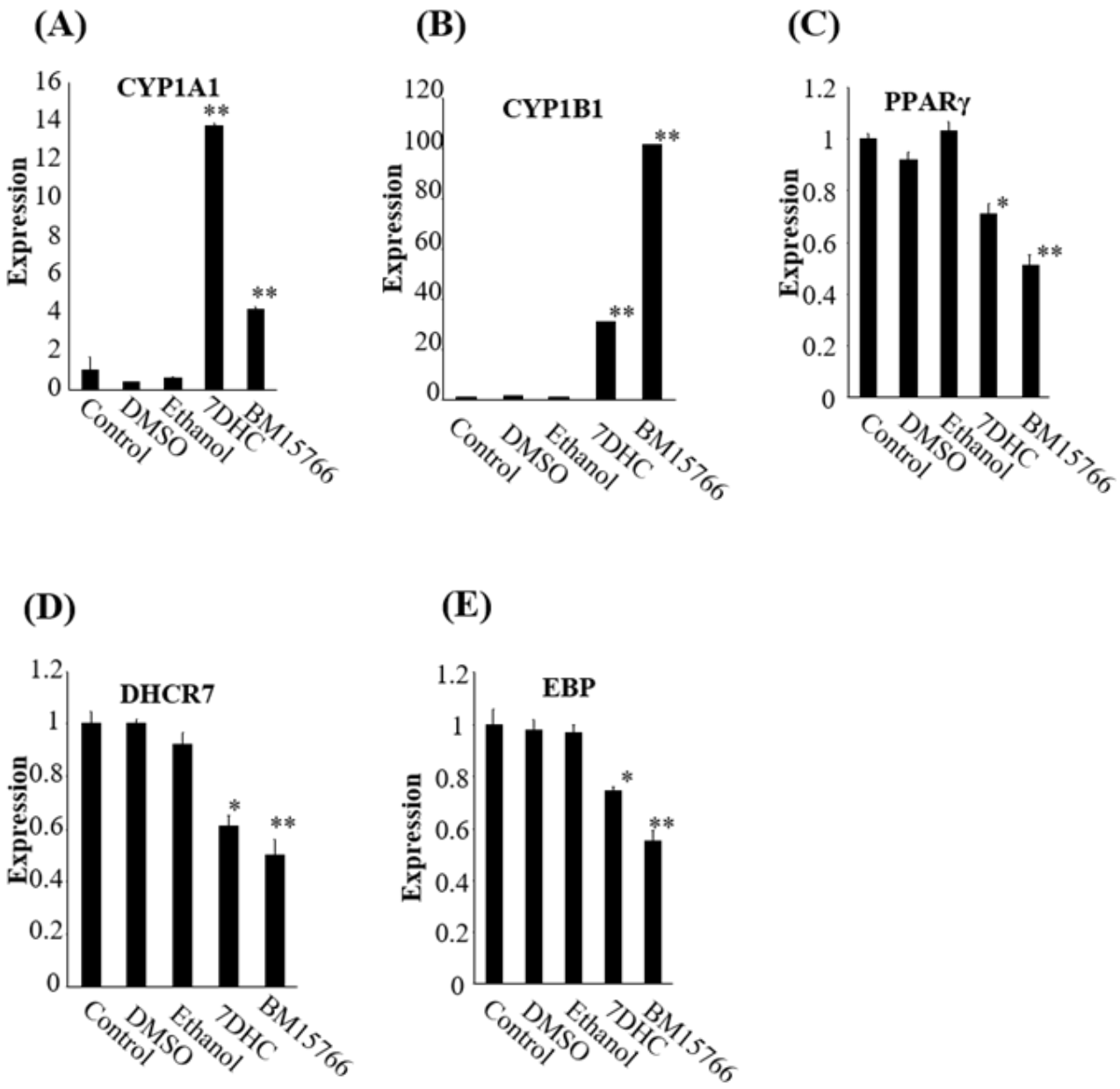

Figure 4(A-C). AhR and PPARγ cross-talk were observed in 7DHC and BM15766 treated HHFORS. The real-time PCR confirmation of *CYP1A1, CYP1B1* and *PPARγ* gene expression in BM15766 and 7DHC treated HHFORS cells (*p<0.05, **p<0.01). Compared with Control and Vehicle (DMSO) group *CYP1A1, CYP1B1* genes expression were significantly increased and *PPARγ* gene was significantly downregulated. There is an inverse relation between expression of AhR target genes and *PPARγ*.

Figure 4(D-E). Cholesterol related genes were inhibited under *in vitro* condition. *DHCR7* and *EBP* genes were downregulated in the 7DHC and BM15766 treated HHFORS cells (*p<0.05, **p<0.01).

follicle. Toxlist generated from IPA analysis of BM15766 and 7DHC treated HHFORS confirmed that the different signaling pathways were significantly expressed in the experimental conditions. TGFβ signaling, aryl hydrocarbon signaling, hepatic fibrosis, and xenobiotic metabolism were the critical pathways affected in 7DHC and BM15766 treated hair follicle cells.

In vitro analysis of the study disclosed those different fibrotic genes like TGFβ, SMAD2, SMAD3, Col1A1, and AGTR1 were significantly expressed after treating cholesterol biosynthesis inhibitors. TGFβ is the prominent cytokine that stimulates extracellular matrix synthesis and involves tissue repair/wound healing and tissue fibrosis. TGFβ signaling promotes the expression of many pro-fibrotic genes and increases the corresponding protein production. TGFβ plays a significant role in developing fibrosis in numerous organs, including systemic sclerosis[3]. TGFβ activates SMAD2 and SMAD3 in most cell types. In epidermal keratinocytes, TGFβ activates both SMAD2 and SMAD3[36]. Angiotensin II promotes early SMAD signaling through the AGTR1 –mediated ERK-1/2 MAPK pathway, resulting in type I collagen accumulation[37].

Immunocytochemistry of TGFβ signaling proteins in HHFORS cells treated with 7DHC and BM-15766 showed that TGFβ, SMAD2, SMAD3, Col1A1, and AGTR1 proteins were highly expressed. Increased nuclear accumulation of TGFβ, SMAD2, SMAD3, Col1A1, and AGTR1 proteins was identified in the merged images. The regulated cytoplasmic and nuclear retention may play a role in determining SMAD distribution between the cytoplasm and the nucleus in the uninduced cells. Upon TGFβ induction, signals from the receptor for TGFβ superfamily members are transduced to the nucleus by the SMADs[38]. After TGFβ stimulation, the SMADs predominantly enter the nucleus[39]. SMAD2 and SMAD3 are continuously shuttling between the cytoplasm and nucleus during active TGFβ signaling.

We next confirmed the fibrogenic process in the cholesterolgenic inhibition of PCA through *in vivo* mice model. Fibrotic signaling genes TGFβ, SMAD2, SMAD3, and Col1A1 were significantly expressed in the treatment of 7DHC and BM15766 in mice. The topical application of TGFβ accelerates wound healing[40]. Increasing evidence indicates that the TGFβ family exerts an essential function in the dermal scarrings pathogenesis[17,18]. Research in experimental animal model have demonstrate the *in vivo* role of TGFβ in fibrosis[41]. Two weeks of intravenous injections of TGFβ1 produced serious systemic effects in rats, including marked fibrosis in the kidneys and liver and at the injection site[42]. TGFβ1 and collagen are increased in tissue sections from patients with systemic sclerosis[43], keloids, and hypertrophic scars[44] from burns.

The histological analysis also demonstrated that fibrotic proteins cause morphological alterations in epithelial tissues and the pilosebaceous unit. Immunohistochemical analysis revealed phosphorylated TGFβ, SMAD2, and SMAD3 in the dermal fibroblast of 7DHC-treated animals, demonstrating that TGFβ signaling was differentially expressed. Dermal TGFβ signaling regulates the extracellular matrix deposition and organization[45]. TGFβ1 protein was detected by immunostaining in fibrosis areas but not seen in regions of the normal liver[46]. In transgenic mice, fibroblastic postnatal TGFβ signaling induction recapitulates scleroderma's biochemical, clinical, and histological features[47]. Fibrosis markers such as TGFβ, Col1A1, αSMA and murine hepatic fibrosis histological features became apparent in the *in vitro* and *in vivo* treatment with TCDD[48].

Angiotensin II reactivates Col1A1 and TGFβ mRNA and protein expression[20]. Our results from *in vitro* and *in vivo* studies show that AGTR1 expression is greatly elevated. Furthermore, TGFβ and AGTR1 have been associated with local fibrosis in many tissues. They communicate with one another by using the same set of SMAD proteins. This may cause hair follicle cells to express type I collagen and create extracellular matrix. Angiotensin II-induced cutaneous fibrosis in mice supports the association between AGTR1 and fibrosis[49]. Human skin contains a whole renin-angiotensin hormonal system, which plays an important role in maintaining the skin's homeostasis[50]. The renin-angiotensin hormonal system affects how complicated the hair follicle is, and any changes to that system cause hair loss. This shows that the hormone action of angiotensin AGTR1 may play a role in the homeostasis mechanism of HF.

In the present work, the AhR gene CYP1A1 and CYP1B1 were increased considerably in the cholesterol inhibitor BM15766 and precursor 7DHC treated murine skin. One study showed that overactivation of the AhR in the liver leads to an increased expression of several fibrogenic genes[29]. Therefore, we hypothesized that activation of the AhR could trigger biological pathways associated with fibrosis. The extensive possibility of PPARγ in lipid metabolism and inflammatory responses suggests that this nuclear receptor has a significant role in maintaining hair follicle health[25]. Our observation

revealed that gene expression of PPARγ was significantly downregulated in the cholesterol inhibitor, BM15766, and precursor 7DHC treated mice. Cross-talk analysis of AhR genes and PPARγ confirms that xenobiotic metabolism and PPARγ signaling are very different from each other. Cross-talk analysis of AhR-PPARγ participates in the regulation of connective tissue, activation of mesenchymal cells, differentiation, and cell survival by linking with the metabolism and fibrogenesis[51]. In human fibrosis diseases, PPARγ expression is decreased, as in lung, scarring, alopecia, liver, and kidney diseases[52–54]. PPARγ agonists decrease the deposition of TGFβ-induced collagen and myofibroblast differentiation[55]. As a result of our cross-talk analysis findings, we believe that AhR down regulation in conjunction with PPARγ over expression confirms that xenobiotic metabolism activates cholesterol biosynthesis inhibition, resulting in PCApathogenesis.Thus PPARγ is a transcription factor that potentially inhibits the TGFβ and SMAD protein system[56].

Expression of cholesterol biosynthetic pathway-related genes was significantly decreased DHCR7, EBP, which confirms the cholesterol synthesis programs were halted. There is no change in lanosterol level when accumulation of 7-dehydrodesmosterol in DHCR-7 deficient mice, but desmosterol level was reduced significantly at the time of decreased cholesterol level in the hair[57]. Mutations to EBP in Conradi Hunermann Syndrome are associated with Follicular atrophoderma and patchy scarring alopecia[6]. Understanding the fibrosis-related signaling pathways and cholestereologenic influence in the pathogenesis of PCA may allow the identification of a therapeutic target for this chronic inflammatory disease. The inclusion of PPARγ agonist was already used as a therapeutic strategy in PCA pathogenesis. Cross talk connection with AhR receptor and PPARγ can be incorporated into further therapeutic research. Tissue samples from people with various forms of alopecia have been found to exhibit elevated levels of AGTR1 expression or activity, suggesting that this gene serves as a diagnostic biomarker for the diseases. To better understand the pathophysiology of alopecia, future cutaneous biology research should taken into account the expression or activity of the RAS effector hormone angiotensin II, and furthermore, hormonal mechanisms in the pilosebaceous unit may be of assistance. This study pointed out that fibrosis is followed by the involvement of coactivated pathogenic pathways that affect the inflammation in the hair follicle. Antifibrotic treatment methods can be incorporated into the pathogenesis of PCA. To reach a conclusion about hair loss treatment modalities, more studies and hypothesis research are required.

5. Conclusions

Our findings imply that deregulation of cholesterologenic pathways in hair follicle cells leads to fibrosis in the late stage of Primary Cicatricial Alopecia. The inflammatory response, HF destruction, and fibrotic signaling are all mediated by cholesterol precursors in PCA patients, resulting in persistent scarring or fibrosis. In this study, it was found that there is a strong link between HF sterol status and fibrosis in PCA patients.

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