The Effect of Intense Pulsed Light Treatment on the Expression of Transforming Growth Factor-β in Acne Vulgaris

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Dedication

This thesis is dedicated to my family

(Dad, Mom, Mahnaz & Munim),

without whom I am incomplete.

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Declaration

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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Table of Contents

List of Figures

List of Tables

Abbreviations

ABC	Avidin-biotin peroxidise complex
ACTB	β-actin
ALA	Aminolevulinic Acid
ANOVA	One-way analysis of variance
AP-1	Activator protein-1
APN	Aminopeptidase N
ATRA	All-trans retinoic acid
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CamKII	Ca^{2+}/calmodulin-dependent protein kinase II
CBS	Central biotechnology services
cdk	Cyclin dependent kinase
COL1A2	Type I procollagen gene
Co-Smad	Co-mediator Smad
CRH	Corticotropin-releasing hormone
Ct	Threshold cycle
CTGF	Connective tissue growth factor
CYP17	Cytochrome P450 17A1
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotide triphosphate
DP IV	Dipeptidyl peptidase IV
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
FGF	Fibroblast growth factor
FGFR2	Fibroblast growth factor receptor 2
FoxO1	Forkhead box O transcription factor 1
Foxp3	Forkhead box p3

FRET	Förster resonance energy transfer
GADD34	Regulatory subunit of the protein phosphatase 1 holoenzyme
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycosyl phosphatidyl inositol
GUSB	β-glucuronidase
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HPR	Hydroxyphenyl retinamide
HSP	Heat shock protein
IOD	Integrated optical density
I-Smad	Inhibitory Smad
LAP	Latency-associated peptide
LHE	Light and heat energy
LLC	Large latent complex
LTBP	Latent TGF-β -binding protein
LTGF-β	Latent TGF-β
ICAM-1	Intercellular adhesion molecule-1
IPL	Intense pulsed light
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor-1
IL	Interleukin
LED	Light-emitting diode
MAD	Mothers against decapentaplegic
MAL	Methyl aminolevulinate
MAPK	Mitogen-activated protein kinase
MH domain	MAD homology domain
MMP	Matrix metalloproteinase
MT-MMP	Membrane type matrix metalloproteinase
NA-PDL	Non-ablative pulsed-dye laser
NF-κB	Nuclear factor-κB
NLS	Nuclear localisation signal
OCT	Optimal cutting temperature
PAGE	Polyacrylamide gel electrophoresis

PAI-1	Plasminogen activator inhibitor type 1
PAR-2	Protease- activated receptor-2
PASI	Psoriasis area and severity index
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Triton
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDL	Pulsed dye laser
PDT	Photodynamic Therapy
PGK1	Human phosphoglycerate kinase 1
PI3K	Phosphoinositol-3-kinase
PKC	Protein kinase C
PP1c	Catalytic subunit of protein phosphatase 1
PPAR	Peroxisome proliferator-activated receptor
PPM1A	A metal ion-dependent protein phosphatase
pRB	Protein product of the retinoblastoma gene
PY motif	Proline-tyrosine motif
RCF	Relative centrifugal force
RGD	Arginine-Glycine-Aspartate
RQ	Relative quantity or Relative quantification
R-Smads	Receptor-associated Smads
RT-PCR	Reverse transcription polymerase chain reaction
SARA	Smad anchor for receptor activation
SBE	Smad-binding element
SCC	Squamous cell carcinomas
SDS	Sodium dodecyl sulphate
SGSGSG	Glycine-serine repeat
SiRNA	Small interfering RNA
SLC	Small latent complex
Smurf	Smad ubiquitination regulatory factor
TEMED	N,N,N′,N′-Tetramethylethylenediamine

t-Flavanone	Trans-3,4'-Dimethyl-3-hydroxyflavanone
TFRC	Human transferrin receptor (p90, CD71)
TIMP	Tissue inhibitor of metalloproteinase
TLDA	TaqMan® low density array
TLR	Toll-like receptor
TGF-β	Transforming growth factor-beta
TGF-βR	TGF-β receptor
Th1	Type 1 helper T-cell
Th2	Type 2 helper T-cell
TNF-α	Tumour necrosis factor-alpha
Treg	Regulatory T-cell
TRT	Thermal relaxation time
TSP-1	Thrombospondin-1
u-PA	Urokinase plasminogen activator
UVA	Ultraviolet A
UVB	Ultraviolet B
VCAM-1	Vascular cell adhesion molecule-1

Presentations and Published abstracts

1. Ali MM, Gonzalez ML, Ruge FS, Porter RM. *In vivo* effects of IPL in the resolution of inflammatory acne vulgaris. Presented orally at the 26th Annual Postgraduate Research Day, School of Medicine, Cardiff University; 11 November 2011.

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3. Ali MM, Gonzalez M, Porter R. The role of transforming growth factor beta in the resolution of inflammatory acne vulgaris following treatment with intense pulsed light. Br J Dermatol. 2011 Jul;165 Suppl 1:133(PD10).
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4. Ali MM, Gonzalez ML, Ruge FS, Porter RM. The immunomodulatory effect of intense pulsed light on acne vulgaris. J Invest Dermatol. 2011 Apr;131 Suppl 1:S86(516).
   Presented as a poster at the 71st Society of Investigative Dermatology (SID) annual meeting, Phoenix, Arizona, USA; May 4 – 7, 2011.

5. Ali MM, Gonzalez ML, Ruge FS, Porter RM. The role of transforming growth factor beta in light induced resolution of acne vulgaris. Br J Dermatol. 2011 July; 164(4):923(P17).
   Presented as a poster at the British Society of Investigative Dermatology (BSID) annual meeting, Manchester, UK; April 11–13, 2011.

6. Ali MM, Gonzalez ML, Ruge FS, Porter RM. Intense pulsed light treatment in acne vulgaris. Presented as a poster at the 25th Annual Postgraduate Research Day, School of Medicine, Cardiff University; 19 November 2010. Secured the third place for the poster prize.

7. Ali MM, Gonzalez ML, Ruge FS, Porter RM. Effect of intense pulsed light treatment on transforming growth factor beta expression in acne vulgaris. J Invest Dermatol. 2010 Sep;130 Suppl 2:S63(377). Presented as a poster at the 40th ESDR annual meeting, Helsinki, Finland; September 8-11, 2010.

Abstract

The mechanism of action of IPL in acne treatment is not clearly understood, but an immunomodulatory role has been suggested. Furthermore, inflammatory cytokines and matrix degrading enzymes play a key role in acne pathogenesis. Therefore, curbing the production of these mediators may assist acne resolution. In photorejuvenation studies, IPL has been shown to induce the expression of a key immunomodulatory cytokine, TGF-β. Interestingly, TGF-β has been demonstrated to mediate immunosuppression, inhibition of keratinocyte proliferation and MMP-1 repression through a Smad3-mediated signalling pathway. Therefore, we sought to investigate the in vivo effects of IPL used for acne treatment. Biopsies obtained from 20 patients with inflammatory acne vulgaris at baseline and post-IPL treatment (48 hrs after the first treatment and 1 week after the final treatment) were immunohistochemically analysed to investigate the expression of TGF-β1, TGF-β2, TGF-β3, Smad3, MMP-1 and IL-8. Digital images were semi-qualitatively assessed using image analysis software. In addition, quantitative PCR analysis of TGF-β1, Smad3 and IL-8 was performed on biopsies from seven cases. Immunohistochemical analysis demonstrated that IPL elicited a statistically significant increase in epidermal TGF-β1 expression. However, no statistically significant difference was observed in the expression of TGF-β2/β3. Increased nuclear immunolocalisation of Smad3 was demonstrated in the post-IPL biopsies, which was statistically significant. Although not statistically significant, both IL-8 and MMP-1 expression showed a downward trend in the majority of cases. No statistically significant change was detected in the gene expression of TGF-β1, Smad3 and IL-8, which may be attributed to the small sample in which PCR was carried out. The data from this study suggests that Smad3-mediated TGF-β1 signalling may play a role in IPL-induced resolution of acne vulgaris. The therapeutic effect of TGF-β1 in inflammatory acne vulgaris could be attributed to its immunosuppressive effect and its ability to inhibit matrix degradation and keratinocyte proliferation.

Chapter 1: The Pathogenesis of Acne Vulgaris: Current Concepts

1.1 Introduction

This thesis is divided into seven chapters. Chapter 1 reviews our current understanding of acne vulgaris and its pathogenesis. Chapter 2 describes the mechanisms of intense pulsed light (IPL) in the skin and its use in acne vulgaris. Chapter 3 gives us an insight on transforming growth factor beta (TGF-β). Chapter 4 explores the potential role of TGF-β in acne. Chapter 5 (*Materials and Methods*) outlines the research methodology employed to complete this study. Chapter 6 (*Results*) summarises the data gathered from the experiments carried out. Finally, Chapter 7 (*Discussion*) provides insights based on these results and how these results relate to the available literature. It also speculates on the potential impact of this research on acne management and what future studies may be undertaken.

1.2 Acne vulgaris

Acne vulgaris is a chronic inflammatory cutaneous condition involving the pilosebaceous unit (a structure that comprises the hair follicle and sebaceous gland). It commonly occurs in adolescence and young adulthood[1,2]. However, acne per se can affect all ages ranging from neonatal and infantile acne to adult-onset acne[2]. It is clinically characterized by seborrhoea and the formation of a variety of lesions ranging from non-inflammatory comedones [closed (whiteheads) or open (blackheads) comedones] to inflammatory papules, pustules and cysts that predominantly occur on the skin of the face, the upper back and the upper chest[3]. In addition to these features, acne has been reported to impact the psychological and social well being of the affected individuals[1,3].The severity of acne may range from the mild comedonal form to severe nodulocystic acne.[4] The severity is generally assessed by the number, type, and distribution of lesions[4]. There are currently many grading systems available for assessing the severity of acne[4]. However, none of them have been accepted as the globally standardized grading system[4].

1.2.1 Pathogenesis

Despite its common occurrence, the pathogenesis of acne is not completely understood[5]. Nevertheless, it is generally considered to be a multifactorial disease that involves[6]:

- Abnormal follicular hyperkeratinisation and differentiation
- Enlargement of the sebaceous gland and increased sebum production as a result of increased androgen sensitivity
- Colonisation of the pilosebaceous unit with *Propionibacterium acnes* (*P. acnes*), and
- Inflammation and immunological host reaction

Currently, there is increasing evidence on the involvement of hereditary factors, hormones, skin lipids, inflammatory signalling, and neuropeptides in this multifactorial process[7]. Recent studies and advances in molecular genetics have shed additional light on the pathophysiology of this complex disease. This chapter summarises the current understanding of the pathogenesis of acne vulgaris.

1.2.1.1 Genetic factors

Family studies have demonstrated a strong familial clustering of acne vulgaris and have suggested that hereditary factors may be important in determining acne susceptibility[8-10]. Furthermore, a prospective epidemiologic study on 151 acne patients with or without a family history of acne reported that presence of a positive family history of acne was associated with an earlier onset of acne, increased number of comedones and was therapeutically more challenging[11]. Interestingly, two recent studies showed that an increase in the risk of developing moderate to severe acne was associated with a positive maternal history of acne suggesting that a potential X-chromosome-linked genetic risk factor may have an influence on acne susceptibility and severity[10,11].

Nevertheless, it is a well understood fact that clustering of diseases in families may be attributed to either shared genetic or environmental influences[12]. Therefore, it is

difficult to draw definitive conclusions from these studies. In contrast, twin studies comparing monozygotic and dizygotic twins permit approximate estimation of the environmental and genetic contribution to the disease[12].

Kirk KM et al[13] reported high heritability for facial acne in adolescent twins recruited from an Australian twin registry, with estimated heritability (genetic variance/phenotypic variance) for acne risk and severity between 0.5 and 0.9 for girls, and between 0.7 and 0.8 for boys.

Furthermore, Bataille et al[14] conducted a large adult twin study on 458 pairs of monozygotic and 1099 pairs of dizygotic twins (all women; mean age: 46 years) to investigate the relative contribution of genetic and environmental factors on acne. Genetic modelling using acne scores showed that 81% of the variance of the disease was attributable to additive genetic effects and the remaining 19% was attributed to environmental factors[14]. This study suggested a strong genetic basis for acne vulgaris. However, the data for this study was collected retrospectively[14].

Evans et al[3] conducted a prospective twin study of acne development in a large sample of twins (n = 778 pairs) during adolescence and reported that the severity of acne at all of the involved sites in this age group (12 to 16 years) was strongly influenced by genetic factors. This research group are also currently performing a genome scan to identify individual susceptibility loci[3].

In an earlier twin study, which investigated sebum excretion in 40 pairs of adolescent acne twins, it was suggested that sebum excretion may be under genetic control but the development of clinical lesions is influenced by environmental factors[15]. However, it is difficult to draw conclusions from this study as it was limited by its small sample size.

Although family and twin studies have reported a genetic influence, its precise effect has not been elucidated. A few candidate genes have been proposed to be associated with the pathogenesis of acne vulgaris (**Table 1.1**). Most of these are gene polymorphisms of cytokines involved in the initiation and maintenance of the immune response in acne lesions such as interleukin-1α (IL-1α), toll-like receptor (TLR) and

tumour necrosis factor-α (TNF-α) gene polymorphisms[16-19]. The other polymorphisms identified are related to steroid hormone metabolism such as the human androgen receptor gene CAG repeat length polymorphisms and CYP17 -34C/C homozygote (also known as cytochrome P450 17A1 is a member of the cytochrome P450 superfamily and is a key enzyme involved in steroid hormone biosynthesis)[20-23].

Recently, Tasli et al[24] conducted a study on 115 acne patients and 117 healthy subjects at the Pamukkale University Hospital, Turkey and reported that the frequency of an insulin-like growth factor-1 (IGF-1) polymorphism was significantly different in acne patients when compared to healthy controls ($p = 0.0002$). They also found a significant association between the IGF-1 (CA) 19 polymorphism and severity of acne ($p = 0.015$)[24]. These results suggest that this IGF-1 polymorphism may be involved in determining the susceptibility to and severity of acne vulgaris in Turkish patients. However, further studies on a larger sample and in different populations are warranted to corroborate these findings.

Table1.1 Genetic polymorphisms associated with acne vulgaris

Gene	Polymorphism	Comment	Reference
Interleukin-1α (IL-1α)	Minor T allele of the IL1A +4845(G>T)	Positive association with susceptibility for acne vulgaris and severity in Caucasian population from Romania	Szabó et al[16], 2010
Toll-like receptor-2 (TLR-2)	753Gln allele of TLR2 Arg753Gln	Risk factor for acne vulgaris in Chinese Han patients	Tian et al[17], 2010
Tumour necrosis factor-α (TNF-α)	196R allele of TNFR2 M196R TNFα -857 minor T allele TNFA-308 G/A polymorphism	Risk factor for acne vulgaris in Chinese Han patients Protective factor for acne in Caucasian population from Romania Predisposition to acne in Turkish population and in a female Caucasian population from Romania	Tian et al[17], 2010 Szabo et al[18], 2011 Baz et al[19], 2008 Szabó et al[18], 2011
Androgen receptor	CAG repeat length polymorphism Short CAG and GGN polymorphism	Acne susceptibility in the male Chinese Han population. May affect androgen mediated gene expression in hair follicles and sebaceous glands in androgenic skin disorders Associated with acne risk in a North-east Chinese population.	Yang et al, 2009[20] Sawaya et al[22], 1998 Pang Y et al[21], 2008
Cytochrome P450 17A1	CYP17 -34C/C homozygote	Associated with significantly increased risk of developing severe acne in a Chinese male population	He L et al[23], 2006
Insulin-like growth factor-1 (IGF-1)	IGF-I (CA) 19 polymorphism	May be involved in determining the susceptibility to and severity of acne vulgaris in Turkish patients	Tasli et al[24], 2011

The presence of acne lesions in a few complex genetic syndromes may broaden our understanding of the influence of genetics in acne pathogenesis[25]. Apert syndrome is an inherited autosomal dominant condition characterised by craniofacial and limb deformities due to synostoses of the bones of the distal extremities, vertebra and cranium. In addition to these features, patients with this syndrome were also found to have moderate to severe acne involving their forearms, face, back, and chest[26].

The mutations of Apert syndrome are gain of function mutations, which increases fibroblast growth factor receptor 2 (FGFR2) interactions and its affinity for fibroblast growth factors[25]. This FGFR2-gain of function mutation (Ser252Trp mutation) was also noted in unilateral acneiform naevus, a variant of naevus comedonicus, which is characterised by acneiform lesions, hypopigmentation and hypotrichosis[27]. The increased FGFR2b-signalling consequent to this mutation is considered to be responsible for the dermatological manifestations in these two conditions[26]. FGFR2b is predominantly expressed in the spinous layer of the epidermis and is also expressed in sebaceous glands and hair follicles[26]. It has been reported to play a significant role in regulating epithelial proliferation and differentiation[27].

Interestingly, an elevated expression of interleukin-1 alpha (IL-1α), one of the cytokines implicated in the early stages of acne development, was observed in Ser252Trp *FGFR2* mutated human osteoblasts[28]. Moreover, androgens are reported to stimulate the expression of FGF7 and FGF10[27]. These two ligands interact with FGFR2b and regulate epithelial proliferation[27]. Based on these findings, it has been proposed that in acne vulgaris, androgen-induced overstimulation of cutaneous FGFR2b signalling may induce IL-1α and this could in turn result in the hyperproliferation and activation of infundibular keratinocytes and sebocytes[26]. Furthermore, in a recent detailed review by Melnik et al[29] it has been suggested that some of the currently used treatments for acne (such as anti-androgens, benzoyl peroxide, azelaic acid , tetracyclines, erythromycin and retinoids) may exert their therapeutic effects by attenuating FGFR2 signal transduction. The data available so far appears promising but is far from being conclusive. Future studies that are conducted in acne patients are necessary to evaluate the potential role of FGFR2-signalling pathways in acne pathogenesis.

The available literature on the genetic influence of acne is limited. A better understanding of the genetic determinants involved may assist in clarifying the molecular events leading to the development of acne and in identifying novel therapeutic avenues for this complex disease. In addition, it may help explain the variability of acne in terms of presentation and the response to treatment. Although, genetic susceptibility cannot be ruled out in the pathogenesis of acne, genetic factors alone do not fully account for the acne risk. Environmental influences may also play a role and may potentially act as modifiers of gene expression.

1.2.1.2 Dietary and lifestyle factors

As discussed above, the results of genetic studies support the role of environmental influences in addition to genetic predisposition in the aetiology of acne. Diet may figure as one of these environmental influences. Until recently, diet was considered to have no association with acne[30]. However, a recent spate of studies conducted in the latter half of the last decade reinvigorated the interest in the association between diet and acne[31-35].

Population-based studies suggest that the prevalence of acne is lower among rural, non-westernised populations when compared to urban, westernised populations, and that acculturation affects acne prevalence as a result of change in environmental factors such as dietary habits[36,37]. Western diets that are rich in refined carbohydrates and low in omega-3 fatty acids have been implicated in acne[33]. Moreover, milk, dairy products and high-glycaemic index foods have been linked with acne risk[34-36]. Diets including these products are said to stimulate insulin and IGF-1, which in turn potentiates androgen signalling and results in increased sebaceous gland activity and probably acne[33]. On the contrary, low-glycaemic-index foods have been shown to reduce androgen levels[31,32].

Smith et al[31,32] conducted a prospective, controlled, 12-week, parallel, dietary intervention study to compare the effect of a low glycaemic-load diet with a high glycaemic-load diet on the clinical and endocrine aspects of acne vulgaris in 43 male patients with mild-to-moderate acne. At 12 weeks, there was a reduction in total lesion

counts, free androgen index and weight and an increase in insulin sensitivity and insulin-like growth factor binding protein-1 in the low glycaemic-load diet group when compared with the high glycaemic-load diet group. However, the authors could not rule out the contribution of weight loss to the overall effect[31,32]. These findings suggested that dietary factors could play a role in acne pathogenesis, but further studies are needed to validate these findings and to determine the independent effects of weight loss and dietary intervention.

The available evidence does not prove that diet has a causal role in acne but demonstrates that it may influence it to a certain degree. Further well-designed prospective, randomised trials are essential to fully clarify the role of dietary factors in acne.

1.2.1.3 Hormones

Various hormones such as androgens [testosterone, dehydroepiandrosterone sulphate (DHEAS) and dihydrotestosterone (DHT)], progesterone, growth hormone, insulin, insulin-like growth factor-1 (IGF-1) and corticotropin-releasing hormone (CRH) have been implicated in acne pathogenesis[6,38]. Acne onset coincides with adrenarche when there is a surge in the production of DHEAS, a testosterone precursor[39]. With the onset of puberty, androgen-mediated stimulation of the sebaceous gland results in increased sebum production in both sexes[39,40]. Androgens are thought to play a vital role in the pathogenesis of acne by influencing the proliferation and differentiation of sebocytes and infrainfundibular keratinocytes and by inducing lipogenesis[7]. Androgens are considered to regulate the genes responsible for sebaceous gland growth and sebum production[6]. DHT is produced from testosterone within the skin, by the action of the type 1 isoenzyme of 5α-reductase[41]. These androgens then form a complex with the nuclear androgen receptors and interact with DNA to regulate genes involved in cell growth and lipid production[38].

Infrainfundibular keratinocytes and sebocytes in the pilosebaceous unit possess androgen receptors and the androgen metabolising enzyme system that have the ability to synthesize androgens *de novo* from cholesterol or by converting weaker androgens

to more potent ones[41]. The activity of these enzymes have been reported to be increased in the sebaceous glands of patients with acne[41].

Although conditions of androgen excess and hyperandrogenism have been associated with acne, most of the patients with acne have normal circulating androgen levels[40,42]. It is hypothesised that the local excess androgen production in the skin and/or the elevated expression and sensitivity of androgen receptors in the pilosebaceous unit may contribute to the formation of acne lesions[7]. Interestingly, androgen-insensitive subjects who lack functional androgen receptors do not produce sebum nor do they develop acne[43]. Furthermore, acne-prone skin has been demonstrated to possess a higher androgen receptor density and 5α-reductase activity than uninvolved skin[44-46]. Therefore, alterations in androgen metabolizing enzymes and androgen receptor levels may be implicated in acne pathogenesis.

To determine whether DHT has a potential role in the production of inflammatory cytokines, Lee et al[47] compared the expression of IL-1, IL-6, and TNF-α before and after addition of DHT to cultured sebocytes using immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR). Up-regulation of IL-6 and TNF-α after addition of DHT compared with the control were observed with both the techniques[47]. This study suggests that DHT may not only be involved in sebum production but may also contribute to the production of pro-inflammatory cytokines in acne[47].

Apart from androgens, other hormone systems such as Insulin/IGF-1 (as discussed in the previous section) and CRH have been demonstrated to potentiate androgen signalling and promote lipogenesis and sebocyte differentiation[38]. More details on the role of CRH will be discussed in the following section.

1.2.1.4 Stress

Emotional or psychosocial stress has been reported to initiate or exacerbate acne[48,49]. However, the exact mechanism by which it does so is not clear[48]. There are two potential explanations: hormonal changes and/or neuropeptide production. Hormones

that exacerbate acne, such as glucocorticoids and adrenal androgens are believed to be released during periods of emotional stress[49]. Current evidence suggests that neuropeptides with hormonal and non-hormonal activity may influence the development of inflammation in acne, which in turn might partially explain the pathologic significance of neurogenic and psychogenic aspects in the disease process[50]

Neuropeptides (NP) are a heterogenous group of biologically active peptides, present in neurons that may contribute to the cross-talk between the nervous and immune systems of the skin[48]. Cutaneous NPs are either directly derived from sensory neurons, from keratinocytes or from mast cells[48]. Stress has been demonstrated to elicit the release of a NP called substance P that can induce inflammation by the release of pro-inflammatory cytokines and chemokines[48]. Immunohistochemical studies revealed that substance P-immunoreactive nerve fibers are found in close apposition to the sebaceous glands[50]. Facial skin specimens from acne patients showed more numerous substance P-containing nerve fibers around the sebaceous glands and an increase in expression of neutral endopeptidase (the principal proteolytic substance P-degrading enzyme) compared to healthy controls[50]. Moreover, *in vitro* experiments using sebaceous gland organ cultures, demonstrated that substance P stimulated the proliferation and differentiation of sebaceous glands and upregulated lipogenesis in sebaceous cells[48]. The findings of these studies suggest that substance P and its degrading enzymes may be involved in acne pathogenesis. Taking into account that stress can elicit substance P release from peripheral nerves, it is tempting to speculate that this neuropeptide could be partially involved in stress-induced exacerbation of acne[48].

Corticotropin-releasing hormone (CRH), a neuropeptide and central coordinator for stress responses, has been demonstrated to increase sebaceous lipogenesis, stimulate androgen signalling and affect immune and inflammatory processes[51]. The existence of a complete CRH system in human sebocytes has been established[51,52]. Furthermore, the expression of the complete CRH system was found to be more abundant in the sebaceous glands of acne-involved skin, when compared to sebaceous glands of uninvolved and normal skin[52].

These findings suggest a role for neuropeptides in the pathopysiology of acne. These neuropeptides possibly activate immune and inflammatory processes in response to stress leading to the development and/or exacerbation of acne. Future studies should explore this association and determine whether stress management strategies could prevent or assist in the resolution of acne.

1.2.1.5 Sebum and sebaceous lipids

The association between sebum and acne development is still ambiguous. It is generally accepted that sebum provides an anaerobic, lipid-rich environment for the proliferation of P. acnes[53]. In addition to this, increasing amount of data seem to confirm the presence of alterations in sebum constituents in acne patients[54]. The components of human sebum include cholesterol, cholesterol esters, squalene, fatty acids, diglycerides, triglycerides, and wax esters[53]. Apart from decreased amounts of linoleic acid in sebum from acne patients, modifications of the sebum composition due to altered ratio between saturated and unsaturated fatty acids and lipid peroxidation by-products, particularly squalene peroxide have been implicated in acne pathogenesis[54,55]. These qualitative alterations in sebum are considered to play a role in comedo formation[54]. Furthermore, lipid peroxidation products are also capable of triggering an inflammatory response by inducing the production of pro-inflammatory cytokines (IL-6 and IL-8) and are capable of activating peroxisome proliferator-activated receptors (PPARs)[56]. PPARs are members of the nuclear hormone receptor family[55]. Three receptors have been identified in sebocytes: PPAR-α, PPAR-γ and PPAR-δ[55]. PPAR activation is reported to regulate differentiation and proliferation, lipid metabolism, inflammation, and apoptosis[57,58]. With respect to its role in sebaceous lipogenesis, there have been contradictory results, with some studies showing that it stimulates sebaceous lipogenesis and others reporting an inhibition of sebaceous lipogenesis with PPAR activation[57,59]. This may be attributed to the different *in vitro* models used in these studies. Further studies are warranted to elucidate the specific effects of PPAR isoforms on lipogenesis.

Recently, Schuster et al[58] reported that PPAR activators, particularly activators of PPAR-δ, have an anti-apoptotic effect in SZ95 sebocytes *in vitro*. The authors

suggested that activators of PPAR-δ may induce a sebostatic effect through the inhibition of sebocyte apoptosis involved in holocrine secretion and this may be beneficial in acne vulgaris[58].

Further *in vivo* studies are needed to confirm the distinctive effect of each subtype of these receptors and to determine whether agonising or antagonising the activation of these receptors would mediate a therapeutic effect in acne vulgaris.

1.2.1.6 Propionibacterium acnes

Propionibacterium acnes (*P*. acnes) is a gram-positive anaerobic bacteria that is considered as a skin commensal and normally found in the sebaceous follicles[60,61]. Increased numbers of *P. acnes* have been commonly found in the follicles of acne patients[61]. *P. acnes* has been implicated in the pathogenesis of acne for several years. The improvement of acne with the use of antibiotics and failure to improve with antibiotic resistance had reaffirmed this belief[62]. However, antibiotics used in the treatment of acne are known to have anti-inflammatory properties as well[62]. Furthermore, the presence of *P. acnes* as a member of the resident microbial flora of healthy human skin and the lack of an association between cutaneous *P. acnes* density and the severity of acne has made its role in acne pathogenesis debatable[61].

Advances in molecular genetics and the decoding of the *P. acnes* genome have revived the interest in its potential role in acne pathogenesis[63]. The decoding of the *P. acnes* strain KPA171202 genome has corroborated some of the previously held views on the role of *P. acnes* in acne pathogenesis (such as involvement of bacterial lipases in breaking down sebum and biofilm formation to protect the organism) and has also suggested additional mechanisms (tissue-degrading enzyme systems such as endoglycoceramidases and sialidase/neuraminidases that may contribute to follicular wall damage) for its action in acne[63]. Lomholt and Killian[64] recently performed phylogenetic reconstruction and genetic analysis on 210 isolates of *P. acnes* from patients with acne, those with opportunistic infections, and from healthy carriers. They demonstrated that particular clones of *P. acnes* are strongly associated with moderate to severe acne while others are associated with health. More recently, Brzuszkiewicz et

al[60] carried out genomic and transcriptomic analysis of distinct *P. acnes* strains that highlighted the genomic basis for strain diversity and suggested that the pathogenic potential of different *P. acnes* strains in acne is determined by the phylotype of the causative strain, favourable growth conditions and genetic predisposition of the host to respond immunologically to *P. acnes.*

Current research focuses on whether the immunopotentiating role of *P. acnes* is relevant to acne pathogenesis. *P. acnes* strains belonging to different phylotypes are reported to differ in their immunostimulatory activity, suggesting that the severity of acne may depend upon the phylotype of the causative strain[60]. This assumption has been reinforced by immunological observations *in* several *in vitro* and *in vivo* studies, demonstrating that distinct strains of *P. acnes* induce different inflammatory responses in host keratinocytes, sebocytes, and monocytes that selectively triggers the secretion of various pro-inflammatory cytokines, chemokines and antimicrobial peptides and also influences the growth and differentiation of these cells[65-69]. These distinct immune responses may probably influence the clinical course of acne[65].

In addition to triggering inflammation by the release of bacterial enzymes (such as lipases, proteases and hyaluronidases), *P. acnes* is also considered to activate these responses through a group of pathogen-associated pattern recognition receptors known as Toll-like receptors (TLRs)[70]. They are involved in recognizing microbial components and thereby initiating and regulating cutaneous immune responses[7]. Eleven TLRs have been identified so far in humans[7,70]. Of these, TLR-2 and TLR-4 have been implicated in acne pathogenesis[71]. TLRs are expressed on keratinocytes, sebocytes, monocytes/macrophages, Langerhans cells, T- and B-lymphocytes, mast cells and endothelial cells[7,67,70,71]. Activation of TLRs by their ligands results in the initiation of several signalling cascades and also activates transcription factors such as nuclear factor-κB (NF-κB) and AP-1 (activator protein-1), which, in turn, promotes the expression of genes responsible for production of chemokines (e.g. IL-8), cytokines (e.g. TNF-α, IL-1β, IL-6 and IL-12), anti-microbial peptides (e.g. human beta-defensins), and adhesion molecules[65,67,69-71].

New targeted therapies and vaccine-based strategies against *P. acnes* are currently being investigated. Nakatsuji et al[72,73] explored the possibility of using vaccination

against *P. acnes* as a potential management option. This group developed a heat-inactivated *P. acnes* vaccine[72] and another vaccine targeting *P. acnes* surface sialidase[73], which were administered intranasally into mice. Immunisation of the mice with either of these vaccines provided in vivo protective immunity against intradermal *P. acnes* challenge and also decreased *P. acnes*-induced IL-8 production[72,73]. Although the concept of acne vaccines appears promising, the question of whether it would confer protective immunity in humans and more so prevent the development of acne lesions is still not answered.

Apart from its role in the inflammatory phase of acne, *P. acnes* also influences the differentiation and proliferation of keratinocytes, and *P. acnes* extracts have been implicated in the augmentation of sebaceous lipogenesis[68,74,75]. Isard et al[74] recently reported that *P. acnes* can induce comedone formation by stimulating the IGF-1 system, which in turn induces keratinocyte proliferation. It has also been proposed that *P. acnes* may contribute to microcomedone formation via its biofilm, which may act as an adhesive that holds the shed keratinocytes together forming a plug in the infundibulum of the hair follicle[76].

Although much information about *P. acnes* has been gathered with the advent of genomics, its exact role and extent of involvement in acne pathogenesis is yet to be completely determined.

1.2.1.7 Immunological factors

The real challenge is to identify the initiating event of acne vulgaris. Follicular hyperproliferation and hyperkeratinization leads to the formation of the microcomedone, the earliest subclinical acne lesion[7,77]. The exact mechanism responsible for hyperproliferation (of basal keratinocytes) and abnormal hyperkeratinization of the follicular epithelium has still not been clearly defined. Microcomedo formation has been attributed to several factors including interleukin-1 alpha (IL-1α), androgens, *P. acnes*, abnormal integrin expression and qualitative sebum lipid alterations[55,78-80].

Inflammation was previously considered as a secondary event, but has been demonstrated to occur as an early event in acne development[77]. Jeremy et al[77] provided evidence for involvement of inflammatory events in the initial stages of acne development. They revealed an increase in CD4+ T-cells, macrophages and IL-1α activity in the skin around the uninvolved pilosebaceous follicles obtained from acne patients compared to normal control skin and reported that subclinical inflammatory events occur in the clinically uninvolved skin of acne patients prior to hyperproliferative or abnormal differentiation events[77]. These findings substantiate the necessity to apply topical treatments not just to the clinically observable lesions but to the apparently non-involved skin in order to curb acne progression.

IL-1α secreted by infundibular keratinocytes is considered to stimulate comedo formation and induce an innate immune response[55,78]. However, the trigger for increased IL-1α secretion is not known. It has variably been attributed to local irritation, release of substance P, release of bacterial heat shock proteins and increased FGFR2-signalling[25,55].

The receptor for IL-1α belongs to the same family as TLRs and TLR activation mimics the action of IL-1α to a certain extent and also induces its synthesis[25]. As discussed in the previous section, *P. acnes* have been demonstrated to trigger the release of pro-inflammatory cytokines via TLRs by activating transcription factors such as NF-κB and AP-1[65,67].

Kang et al[5] provided *in vivo* evidence for the activation of NF-κB and AP-1 in acne lesions. Consistent with NF-κB and AP-1 activation, they found a marked increase in inflammatory cytokines (TNF-α, IL-1β, and IL-8) and matrix metalloproteinases (MMPs), in acne lesions[5].

TNF-α and IL-1β upregulate the expression of adhesion molecules such as ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule-1) on endothelial cells that are required to slow the flow of circulating inflammatory cells for their eventual diapedsis into the inflamed tissue[5].

Trivedi et al[81] performed gene array expression profiling on skin biopsies obtained from acne patients, which revealed that genes involved in inflammatory pathways and extracellular matrix remodelling were upregulated in acne lesions. Of these, MMP-1 (92-fold), MMP-3 (64-fold) and IL-8 (52-fold) were the genes with the greatest fold increase in expression in acne lesions[81].

Therefore, among the several mediators involved in the acne inflammatory process this thesis will focus on IL-8 and MMP-1.

Interleukin-8

Interleukin-8 (IL-8) is a member of the CXC chemokine family and is also referred to as CXCL8 (Chemokine, CXC motif, ligand 8)[82]. Chemokines are chemotactic peptides that play a crucial role in the regulation of immunological responses[82]. IL-8 is produced in the skin by a variety of cell types in response to inflammatory stimuli, including macrophages, monocytes, keratinocytes, sebocytes and endothelial cells[82-84]. It is one of the major mediators of the innate immune response, and apart from being a potent chemoattractant for inflammatory cells such as neutrophils it also functions as an angiogenic factor, activates inflammatory cells and promotes keratinocyte growth[83,84]. IL-8 has been implicated in mounting an inflammatory response in acne lesions[5,81]. Upregulation of IL-8 expression in acne is predominantly attributed to NF-κB activation and it is further induced by pro-inflammatory cytokines such as IL-1α, TNF-α and IL-1β[5,66,83].

Furthermore, contact of keratinocytes with microbial agents was demonstrated to significantly induce IL-8 production[83]. The effect of *P. acnes* on IL-8 production has been the focus of several studies. *P. acnes* has been demonstrated to induce IL-8 production and IL-8 mRNA expression in human monocytic cells, keratinocytes and sebocytes through the activation of TLR-2 and transcription factor NF-κB[65-67,85]. Additionally, *P. acnes* vaccines attenuated IL-8 production in human sebocytes[72,73]. Recently, reactive oxygen species produced by *P. acnes* stimulated keratinocytes were also shown to induce IL-8 production[86]. A detailed understanding of the pathways regulating IL-8 production is necessary to delineate its role in acne pathogenesis.

In acne, IL-8 is considered to stimulate the recruitment and accumulation of neutrophils in to the follicles, which in turn results in the formation of pustules and destruction of the follicular walls by proteases released from the neutrophils[87]. Abd El All and team[88] found significantly increased expression of IL-8 in lesional skin compared to non-lesional skin of patients with acne vulgaris. Significant associations existed between IL-8 immunoreactivity and the degree of epidermal hyperplasia and follicular hyperkeratosis. Further, dermal IL-8 expression correlated with dermal angiogenesis and the extent of dermal inflammatory infiltrate[88].

These studies reveal that inflammatory processes play a vital role in the development of acne, and targeting inflammatory mediators such as IL-8 may be a viable therapeutic option in treating this condition.

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, which can degrade a wide variety of extracellular matrix (ECM) components[89]. MMPs play an important role in the human skin both during physiological and pathological states[89]. They are implicated in normal tissue remodelling as well as during inflammatory matrix remodelling, neovascularization, wound healing, and malignant transformation[90]. They can be produced by diverse cell types in the skin (such as fibroblasts, keratinocytes, macrophages, endothelial cells, mast cells, neutrophils and eosinophils) and their activity can be specifically inhibited by TIMPs (tissue inhibitors of metalloproteinases)[89]. In addition, MMP activity can be regulated by a variety of cytokines and growth factors such as IL-6, TNF-α, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor-beta (TGF-β) depending upon the cell type[89]. According to their substrate specificity and primary structure they are classified as collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and -26) and membrane type MMPs (MT-MMPs)[90].

Significant upregulation of MMPs (MMP-1, MMP-3 and MMP-9) have been demonstrated in skin obtained from acne patients[81,90,91]. AP-1 is a critical regulator of induced expression of MMP-1, -3, and -9[5]. MMP-1, also referred to as interstitial

collagenase, plays a vital role in mature collagen degradation as it has the distinctive capacity to initiate site-specific cleavage of the triple helix of type I and other fibrillar collagens[5]. Kang et al[5] demonstrated a significant 2.6-fold increase ($p < 0.04$) in degraded/fragmented collagen in acne lesions compared to normal skin. Thereby, providing evidence of matrix degradation in inflammatory acne.

The precise role of MMP in acne pathophysiology is still unclear. It probably participates in abnormal hyperproliferation, rupture of the pilosebaceous follicle wall, dissemination of inflammation and in the genesis of scars[55,90].

Papakonstantinou et al[90] investigated the expression of MMPs and TIMP in facial sebum specimens obtained from acne patients, and reported that the sebum contains proMMP-9, MMP-1, MMP-13, TIMP-1, and TIMP-2, possibly originating from keratinocytes and sebocytes. Thus, suggesting that MMP and TIMP of epithelial origin may be involved in acne pathogenesis.

In a subsequent *in vitro* study, *P. acnes* was demonstrated to induce MMP-9, MMP-1, and TIMP-1 transcript in primary human monocytes[92]. This was further supported by a more recent study, which showed that the protease activity of *P. acnes* could induce the expression of MMP-1, -2, -3, -9, and -13 via the activation of protease-activated receptor-2 (PAR-2) that are present on keratinocytes[91]. Based on these findings, the authors proposed that on exposure to *P. acnes* with protease activity, PAR-2-induced AP-1 activation may stimulate keratinocytes to produce MMPs[91]. This study also demonstrated that keratinocytes are an important source of MMPs in acne.

Therefore, curbing the activity of these matrix degrading enzymes or the molecular pathways leading to their production would be desirable in controlling inflammation and preventing scarring in patients with acne vulgaris. The immunopathogenesis of acne vulgaris is illustrated in **Figure 1.1**.

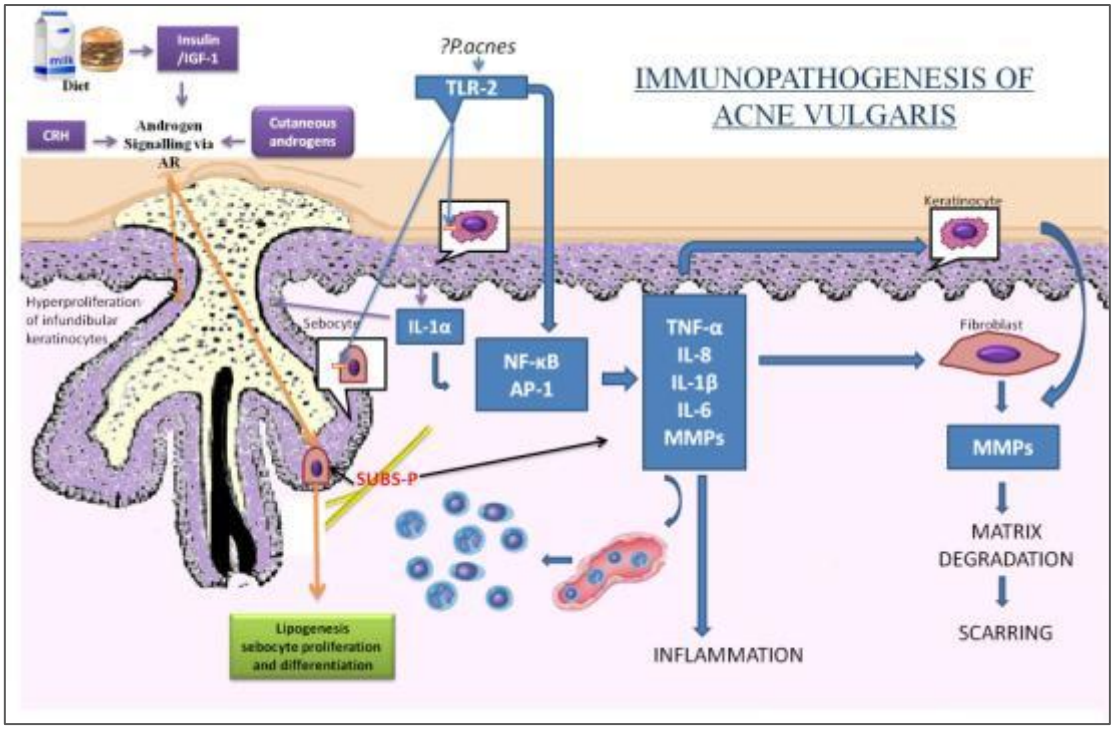

Figure 1.1 Schematic representation of the current understanding of the immunopathogenesis of acne vulgaris. Triggering of TLR-2 by *P. acnes* may lead to the activation of NF-κB and AP-1, which leads to the release of pro-inflammatory cytokines, chemokines and matrix degrading enzymes that may ultimately result in inflammation and scarring. Also, illustrated is the effect of diet and androgen signalling on the sebocytes and infundibular keratinocytes.

1.2.1.8 Forkhead box O transcription factor 1

Based on the premises that Forkhead box O transcription factor 1 (FoxO1) plays a pivotal role in the negative regulation of the several factors that are implicated in acne pathogenesis (androgen receptor activation, cell proliferation, apoptosis, lipid and glucose metabolism, oxidative stress and innate immunity), Melnik[93] recently proposed a hypothesis that nuclear deficiency of FoxO1 may be responsible for acne development. The author proposed that activation of the phosphoinositol-3-kinase (PI3K)/Akt kinase pathway by growth hormone signalling (puberty) or increased insulin/IGF-1 signalling (dietary) resulted in export of FoxO1 from the nucleus to the cytoplasm. As a result of the decreased nuclear levels of FoxO1, its effect on the various target genes is lost, leading to androgen receptor mediated signal transduction,

follicular keratinocyte hyperproliferation, augmented sebaceous lipogenesis, activation of immune responses (due to upregulation of TLR2) and increased MMP activity. Subsequently, the same author proposed that the upregulation of nuclear FoxO1 is the mechanism by which systemic isotretinoin exerts its therapeutic effect[94]. This hypothesis is certainly appealing, but it is based on indirect evidence and at this point of time there is no direct data available to support this hypothesis.

1.2.2 Targeting the inflammatory component of acne vulgaris

Currently, a wide range of treatments are available for acne, both topical (such as retinoids, benzoyl peroxide and antibiotics) and systemic (such as isotretinoin, antibiotics and hormonal therapy). The findings from the aforementioned studies suggest that targeting the inflammatory component of acne, such as pro-inflammatory mediators and ECM degrading enzymes, is crucial for the management of acne vulgaris.

Inadequate control of inflammation in acne may lead to the undesirable sequelae of scarring[5]. Therefore, in addition to alleviating the clinical symptoms and the associated psychological stress, prevention of scarring is also a vital goal of acne treatment.

With the growing body of evidence on the involvement of inflammatory events from the very early stages of acne development up to the stage of scarring, it would be intriguing to assess the currently available acne treatments and the newer promising options on their potential to modulate these events.

Apart from modulation of cell proliferation and differentiation (anticomedogenic effect), topical retinoids also possess anti-inflammatory properties[95,96]. In several *in vitro* and *in vivo* studies, topical retinoids have been demonstrated to have an inhibitory effect on the activity of leukocytes and on the production and release of inflammatory mediators[95-97]. However, the exact mechanism by which the anti-inflammatory response occurs has not been completely clarified.

The systemic retinoid, isotretinoin, is considered to be the most effective treatment available for the management of severe inflammatory acne[5]. It is considered to exert its effect by suppressing sebaceous gland activity, by normalising abnormal follicular keratinisation, and by inhibiting inflammation[7]. Despite its effectiveness, the use of isotretinoin in acne management is limited because of its teratogenic potential and its alleged risk of depression/suicidal ideation[5]. In recent years, interest has been generated on the effect of retinoids on the expression of matrix metalloproteinases[90,92]. These studies showed that retinoids can modulate MMPs (MMP-9 and MMP-1) and TIMP expression and this may be responsible for shifting from a matrix-degradative phenotype to a matrix-preserving phenotype[90,92]. Therefore, the anti-inflammatory effect of retinoids may in part be attributed to inhibition of MMPs.

Both topical (clindamycin, erythromycin, and tetracycline) and oral (mainly macrolides and tetracycline) antibiotics are used to treat inflammatory acne. In addition to their antibacterial effect, these agents also have demonstrated anti-inflammatory activity[98,99]. A few studies have suggested that their anti-inflammatory effect may be attributed to the inhibition of bacterial lipases, chemotactic factors, reactive oxygen species generation by neutrophils, pro-inflammatory cytokines and matrix-degrading collagenases (MMP-1)[7,98-101]. However, development of bacterial resistance has raised considerable concerns on the use of antibiotics in the management of acne[102].

Furthermore, benzoyl peroxide was found to modulate the immune response by impeding the release of reactive oxygen species from human polymorphonuclear leucocytes in a dose-dependent manner[103].

In depth understanding of the various treatment modalities at the molecular level is necessary to explain their mechanisms of action and also to develop newer treatments for acne vulgaris.

1.3 Summary

The pathogenesis of acne is complex and probably involves the interaction of genetic, hormonal, immunological and environmental aspects (**Figure 1.2**). These interactions may trigger an innate immune response and result in inflammation. The recent findings may have enhanced our understanding of acne pathogenesis, but many elements of this complex disease still remain unsolved. The exact mechanism triggering the development of acne, and the precise sequence of events involved in acne development and in its remission continues to be shrouded in mystery. In addition, the discordance in the therapeutic response from one patient to another and the variability in clinical presentation among patients need to be explained. Ongoing research is attempting to clarify these issues. Nevertheless, the improved understanding of the key players in acne pathogenesis has given a boost to the research on the available and novel acne treatment modalities.

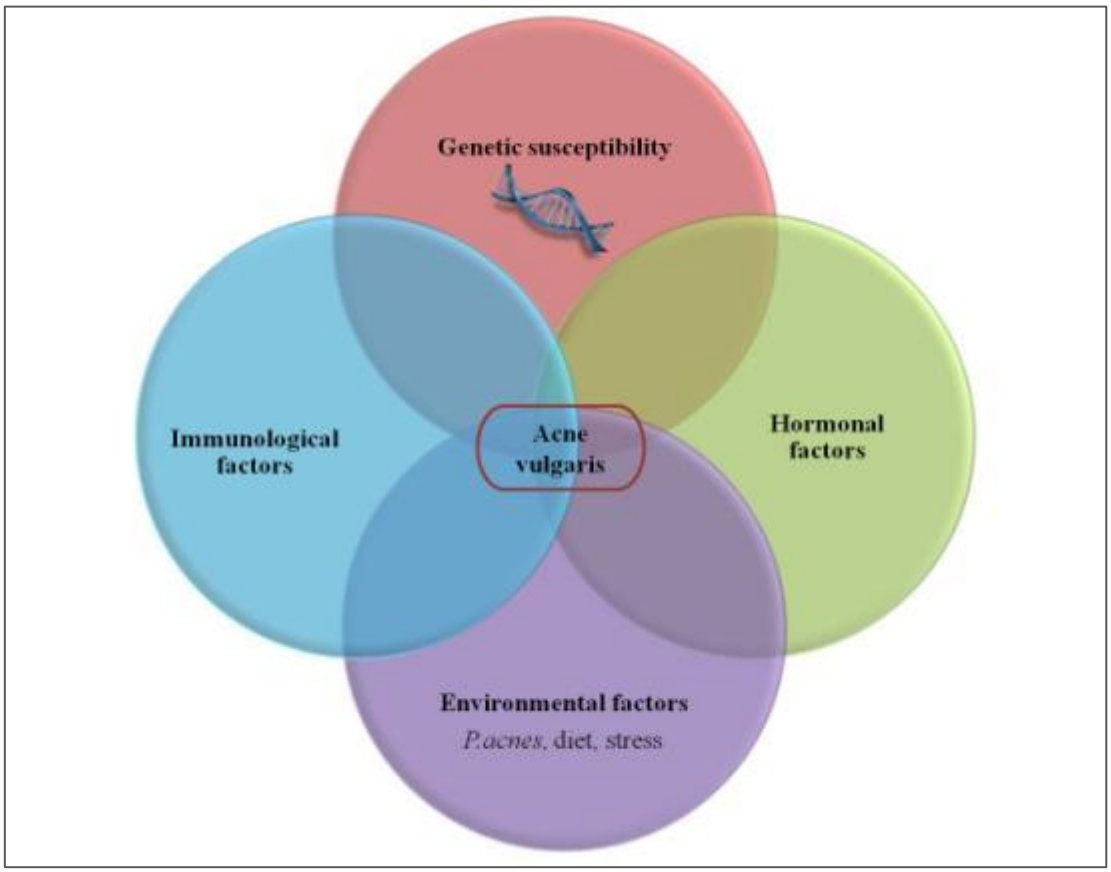

Figure 1.2 Interaction of the various factors contributing to acne pathogenesis.

Treatment of acne is tricky as the multifactorial character of acne has to be considered. Although conventional acne treatments such as retinoids and anti-microbials form the cornerstone of therapy, their use has been constrained due to the rise of antibiotic resistance, increased patient compliance issues and severe adverse effects such as teratogenicity. Light and laser therapy are increasingly being recognised as an alternative treatment option with a better safety profile. However, there are very few well-designed trials evaluating these treatments. Scientific evidence demonstrating the mechanism of action of these treatments would facilitate our judgement on their use in treating acne. In this thesis we attempt to determine the effect of intense pulsed light on acne-affected skin, with particular emphasis on the expression of cutaneous TGF-β, the role of which will be discussed in the later chapters. The next chapter throws light on the mechanisms of intense pulsed light in the skin and its use in acne vulgaris.

Chapter 2: Intense Pulsed Light

2.1 Introduction

It has been known for some time now that exposure to sunlight has a favourable effect on most cases of acne, which is probably attributed to its anti-inflammatory effect on follicular Langerhans cells or its potential for *P. acnes* destruction[104,105]. However, the wavelengths contributing to this beneficial effect have not been completely deciphered[105]. UVA and UVB treatment were found to have only a minimal beneficial effect in acne, which is overridden by its potential for carcinogenicity[106]. On the other hand, visible light therapy has been demonstrated to have a favourable effect on acne, but without the potential risks of UV irradiation[105,107]. Moreover, visible light has better penetration than UV irradiation. Sigurdsson et al[107] demonstrated a significant reduction in acne severity with visible light therapy. There was an overall reduction of 14% for full spectrum ($p > 0.10$), 22% for green ($p < 0.05$) and 30% for violet light ($p < 0.02$)[107]. Improvement was predominantly observed in the inflammatory lesions[107].

Various light-based therapies are currently being evaluated for the treatment of acne. These treatments are aimed at providing acne patients with non-invasive, effective, and more convenient therapy that produce rapid results with minimal downtime and relatively few adverse effects[108-110]. Furthermore, in comparison with lasers, light treatments are considered to be more convenient, cost-effective and minimally invasive[110]. Among these light therapies, much interest has been generated on the use of intense pulsed light (IPL) sources in the recent years. This chapter addresses the role of IPL therapy in the treatment of acne vulgaris.

2.2 Intense pulsed light (IPL)

IPL devices are high-intensity light sources that emit polychromatic, non-coherent visible light in the broad wavelength range of 515 to 1200 nm[111]. These devices use xenon flash lamps (gas-discharge lamps of high intensity filled with xenon gas) and computer-controlled capacitor banks, and band pass filters to generate pulsed polychromatic light of desired duration, intensity, and wavelength[112,113]. When an

electrical current is passed through the xenon gas in the flash lamp, bright light is produced[113]. Thus, these lamps convert electrical energy stored in capacitor banks into optical energy in a pulsed mode[113]. The wavelengths can be optimized depending on the target structure, its depth and the patient's skin type by using different convertible cut-off filters or adjusting the lamp type, or current density[111-113]. Owing to its flexibility in configuring the various parameters, IPL on its own or along with other therapeutic agents can be used for a wide range of dermatological indications, such as vascular lesions, pigmentary lesions, unwanted hair growth (photoepilation), photoaging or photodamaged skin, rosacea, hypertrophic scars or keloids, skin rejuvenation, and actinic keratoses[114-119]. Recently, IPL has been demonstrated to be beneficial in acne vulgaris as well[108-110,120-128].

The clinical effect of IPL is dependent upon both the properties of the light that irradiates the skin and the skin, itself. Therefore, a thorough understanding of the interactions between light and skin is important for the safe and effective use of IPL devices.

2.2.1 Light-skin interactions

Intense pulsed light emits light within the visible and near-infrared components of the electromagnetic spectrum[119]. Visible light (400–760 nm) is that portion of the electromagnetic spectrum that is perceptible to the human eye[129]. Light incident on the skin is absorbed, reflected, scattered, or transmitted (**Figure 2.1**), and the degree to which it is reflected, absorbed, and scattered determines the depth of penetration[130]. Incident light must pass through the stratum corneum before it reaches the underlying viable skin. Characteristics of the stratum corneum such as its thickness, composition and morphology affect the amount of light that passes through it[130]. In general, the reflectance of an incident beam from normal skin ranges from 4 to 7 %, and around 93 to 96 % may be either absorbed or scattered by the skin[130]. Scattering of light by the skin varies inversely with wavelength[131]. Hence, scattering decreases with the use of light of longer wavelengths. The absorption of light is mainly responsible for the desired biological effects on the tissue[129].

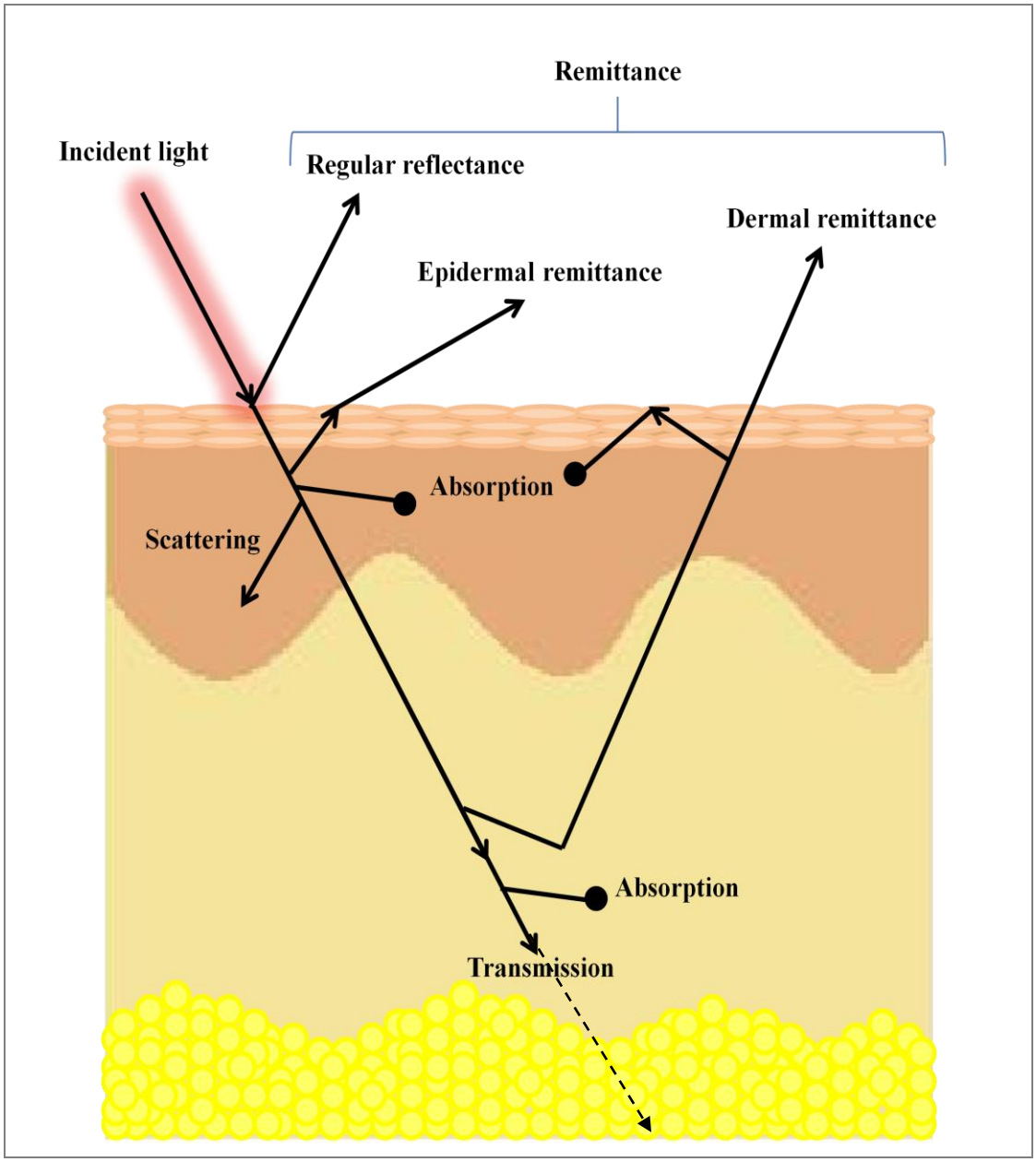

Figure 2.1 Schematic representation of the optical pathway in skin (Adapted from Anderson and Parish[130], 1989)

Chromophores are tissue structures that absorb photons, and the level of light absorption by the skin is determined by the type, concentration, amount and distribution of these chromophores[130]. The most common chromophores encountered in the skin are: haemoglobin and its derivates, melanin, and water[129]. The others include lipids, bilirubin, foreign tattoo ink etc.,[129,132]. These chromophores are wavelength-dependent and have unique absorption coefficients (degree of absorption by the chromophores at a particular wavelength)[129]. The absorption spectrum of the three major skin chromophores is depicted in **Figure 2.2**[133]. Melanin and haemoglobin

are the predominant chromophores in the visible spectral range[132]. Whereas, water and lipids are the main chromophores in the infrared spectral range[132]. The depth of penetration is also wavelength-dependent, the longer the wavelength of light the deeper the skin penetration[129]. The main target structures for Intense Pulsed Light treatment are melanin and blood vessels[129].

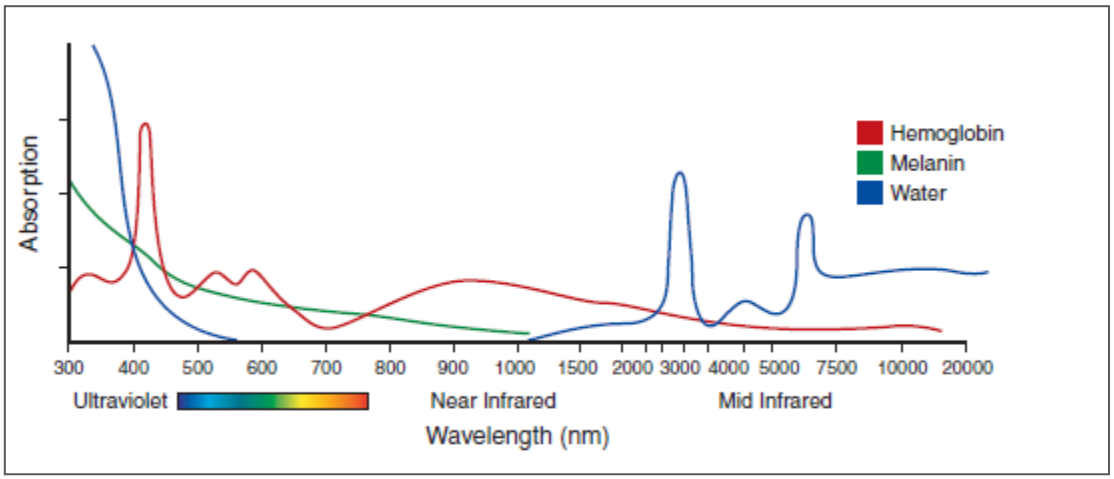

Figure 2.2 The absorption spectrum for the three main skin chromophores (Haemoglobin, Melanin & Water). Adapted from Kaufman[133], 2009

Most IPL devices have a number of parameters, which are configured by the operator according to the patient's skin type and skin condition. To enhance our understanding on the effects of IPL on skin, it is necessary to define some of these parameters:

Fluence: is defined as the total energy discharged per unit area of the target tissue in a single pulse[129,134]. It is measured in Joules (J) per unit area (cm^2) and is expressed as J/cm^2.

$$\text{Fluence} = \frac{\text{Power (W) x Time (s)}}{\text{Cross-sectional area of the laser beam } (cm^2)}$$

Power: measured in watts (W=J/s) represents the amount of energy delivered over a certain period of time[129,135].

Irradiance (sometimes referred to as intensity): rate of energy delivery per unit area to an object (watts/cm^2)[134].

Thermal relaxation time (TRT): is the time required for 50% of the heat generated in the target structure by absorption of a laser/light pulse to diffuse into the surrounding tissue[129]. The TRT is approximated by the formula, TRT = d^2 /gκ, where d = size of the target, κ = thermal diffusivity (about 2×10^{-3} cm^2/s for dermis), and g = geometrical factor[136]. The thermal relaxation time is influenced by the size of the target. The TRT is approximately equivalent to the square of the target size i.e., TRT (ms) ≈ d^2 (cm^2)[129].

Pulse duration: represents the time duration of exposure to the light beam in milliseconds (ms)[137]. The selection of the pulse duration is influenced by the thermal relaxation time of the target[129,134]. The pulse duration should be lower than or equal to the TRT of the target structure to prevent non-specific thermal damage to the surrounding tissue[112]. When the pulse is equal to or shorter than the TRT, the thermal damage is confined to the target structures[129]. In contrast, when the pulse is longer than the TRT, the heat diffuses into the surrounding structures resulting in non-specific thermal damage[129].

Wavelength (λ)*:* is the distance between two successive crests or troughs of the light wave[135]. It is measured in nanometres (nm). IPL devices have different cut-off filters that allow the desired range of wavelengths to enter the skin[111]. To exert maximum effect, the wavelength range should be near the peak absorption of the target chromophore and of adequate length to penetrate to the depth of the target[129].

Footprint (spot size): the spot size is equivalent to the cross-section of the beam of light[134,135]. It plays a role in the depth of penetration of light into the tissue. The larger the spot size, the greater the depth of penetration (**Figure 2.3**)[129,134].

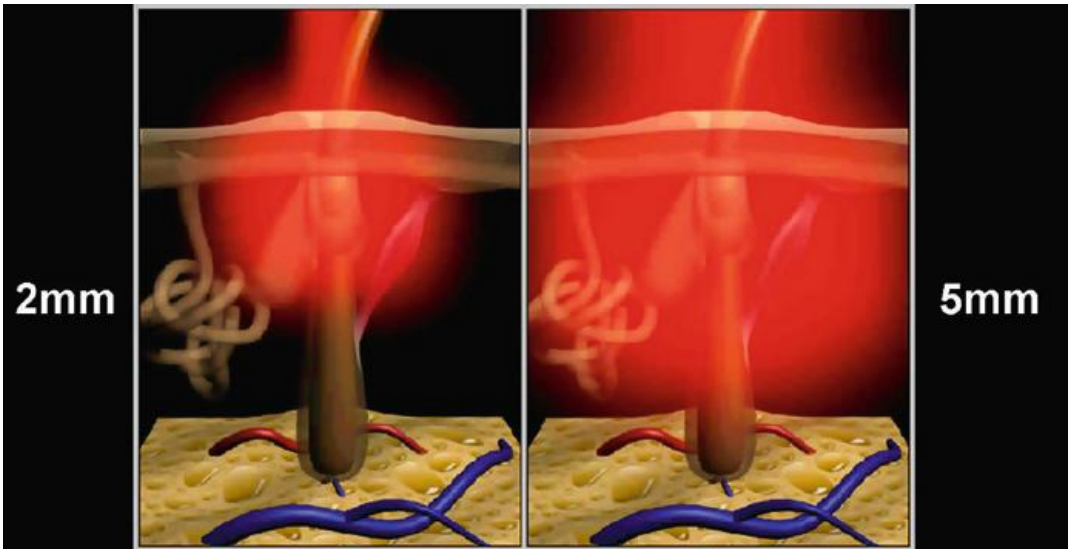

Figure 2.3 Association between spot size and depth of penetration (Fodor et al, 2011[129])

Pulse delay: represents the time interval in between pulses. This period is important as it allows the skin and blood vessels to cool down, while the heat is retained inside the target structure[129].

Shape of the pulse (spectral shape or output): the shape of the pulse is also an important parameter to be taken into consideration. The traditional IPL systems had variations in energy and spectral distribution of the beam during the pulse, producing a non-uniform pulse with the ends of the pulse more in the red/infrared spectrum and the middle of the pulse in the blue spectrum[137]. A significant amount of the energy discharged is wasted due to this uneven wavelength distribution[138]. Compared to the traditional IPL devices, the second generation IPL devices have a computer system that minimizes this so called "spectral jitter" and produces a "square pulse" (**Figure.2.4**)[113,137]. With a square pulse, the intensity does not reach peak levels and is constant over the entire pulse duration[137]. Therefore, a square pulse produces the lowest possible intensity for a given fluence and reduces the risk of side effects and may offer improved clinical efficacy[137].

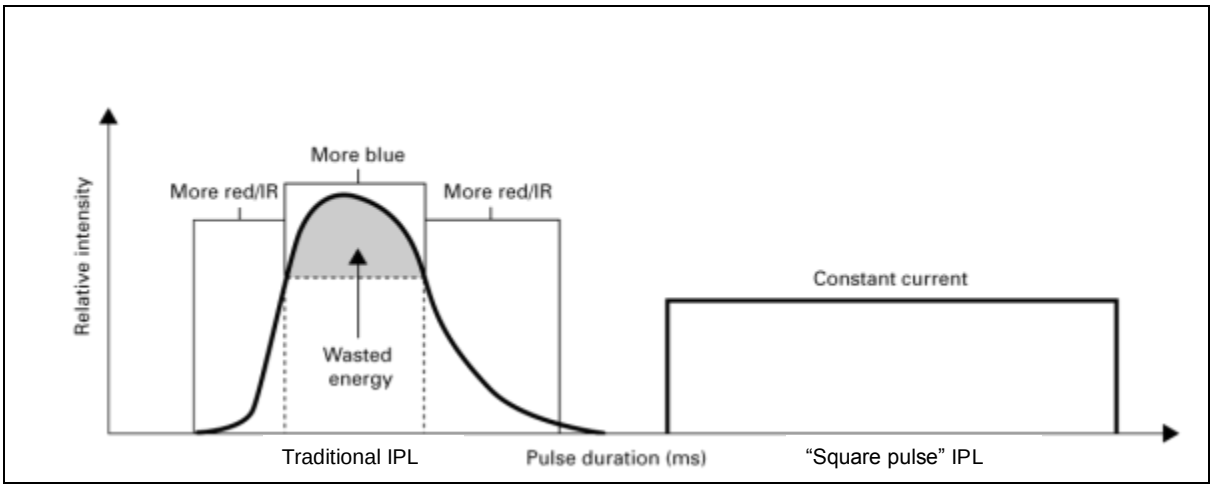

Figure 2.4 Differences in spectral output of Traditional vs. Modern IPL systems. (Town et al[138], 2007)

Cooling technology: Skin cooling during and/or after treatment helps to protect the epidermis from unwanted thermal injury and reduces the inadvertent transfer of heat to adjacent, non-target tissues[129,139]. Moreover, cooling enables the delivery of higher fluences and also reduces pain[139]. The cooling can be performed using cooling gels, ice gels, contact spray cooling, or cooling systems integrated into the handpiece[111,140].

Effects of light-skin interaction can be categorised into:

Photothermal – The light energy absorbed by skin chromophores is mostly converted into heat, which can result in thermal effects such as tissue coagulation or vaporization[129,132]. As the temperature is raised, various molecular changes take place as enlisted in **Table 2.1**[132].

Table 2.1 Photothermal effects of light-tissue interaction[131,132,135]

Temperature	Effect
42-50ºC	Hyperthermia and conformational changes
Above 60ºC	Protein and collagen denaturation; coagulation necrosis
Above 70ºC	DNA denaturation and membrane permeabilisation
Above 100ºC	Vaporization and ablation
Around 120ºC	Thrombosis of the blood vessels and necrotizing vasculitis

The temperature and duration of exposure influences the effects of heating on the target structure[129,132]. Photothermal effects are beneficial to the target tissue, but if allowed to diffuse into the surrounding tissue it may be detrimental[129,132]. The collagen and elastin-rich dermis is thermally more stable than the epidermis[129].

Selective photothermolysis – The concept of photothermolysis was introduced by Anderson and Parrish in 1983[131]. It is the site-specific, thermal injury of microscopic target tissue with minimal effect on the surrounding tissues by selectively absorbed pulses of radiation[131].

The three criteria necessary to produce selective photothermolysis include[131]:

- Absorption of a specific wavelength by the target structures
- An exposure time less than or equal to the time of cooling of the target structures (i.e. pulse duration ≤ TRT)
- Optimal fluence to generate sufficient temperature within the target structures to achieve the desired effect

Photochemical – Photo-excited molecules are likely to undergo chemical reactions by direct interaction with chemical bonds or by interaction with endogenous or exogenous photosensitizing agents[129]. Photodynamic therapy (PDT) utilizes this effect through the interaction of photosensitizing agents, light, and oxygen[132].

Photoimmunological – energy of photons when absorbed in cells or tissue may affect cellular metabolism and signalling pathways[141]. This may in turn modulate the level of cytokines and growth factors[142].

Photomechanical – light induced expansion or disruption of tissues[129,135].

The benefits and drawbacks of use of IPL devices are listed in **Table 2.2**

Table 2.2 Advantages and disadvantages of IPL[112,113,137]

Advantages	Disadvantages
Owing to its versatility, it can be adapted to different skin types and skin conditions. Broad-wavelength spectrum allows the three key skin chromophores (haemoglobin, melanin, water) to be activated with one single light exposure. The large spot size or footprints of IPL allows rapid treatment and more coverage. Lower cost and more robust technology compared to lasers. More eye-safe than lasers. However, eye protection is recommended for both operator and patient.	Emitted spectrum and fluence can be inconsistent from pulse to pulse (or during the pulse) Variations in fluence, wavelength ranges and spectral shapes and other treatment settings between different manufacturers and devices. The size and weight of the hand-piece limits its manoeuvrability Adequate experience is required to operate this device because of its different parameters and applications. Gel application and the direct skin contact with the hand-piece makes it difficult to observe the immediate local response. Risk of hair reduction due to the broad spectrum in patients who do not desire reduction of their hair.

2.2.2 IPL and acne vulgaris

In the past seven to eight years, a few studies have been published on the use of IPL monotherapy in the treatment of acne vulgaris. This section includes a brief overview of these studies[108-110,120-128].

Various IPL systems have been reported to treat inflammatory acne vulgaris[143]. The first IPL system to report benefits in acne was the ClearTouch™ system (Radiancy Inc. now known as the SkinStation®)[143]. Elman and Lask[120] evaluated the safety and efficacy of the LHE technology (combining pulsed light and heat energy, LHE) in the treatment of acne vulgaris. Nineteen patients (12 female and 7 males; aged 13 to 28 years) with mild to moderate acne underwent twice-weekly treatments for four weeks using the ClearTouch™ system (λ= 430-1100 nm, spot size: 22 x 55 mm, average fluence: 3.5 J/cm^2, and pulse width: 35 ms)[120]. Clinical assessment and acne severity grading were performed at baseline and at one and two months after the last session. Improvement in acne severity and lesion counts were observed as early as two weeks after commencement of treatment. Inflammatory lesions improved by 50% at the end of treatment, by 74% one month later and by 87% after 2 months[120]. Similarly, non-inflammatory lesions improved by 63%, 79% and 85%, respectively. No adverse effects were reported. The authors concluded that the ClearTouch pulsed light and heat energy (LHE) technology is effective and safe for the treatment of acne vulgaris[120]. However, this study did not specify the location of the acne lesions. This may affect the integrity of this study, taking into consideration the notion that different sites may respond differently to treatment.

Dierickx[121] investigated the safety and efficacy of a variable-filtration IPL system (EsteLux™, Palomar Medical, Inc., Burlington, MA) for facial and dorsal acne using a Lux V™ hand-piece (400-700 and 870-1200 nm). Fourteen patients with mild to moderate inflammatory acne vulgaris received five treatments (2 to 3 passes, average fluence: 10 J/cm^2) at two to four week intervals. At one month post-treatment, the acne clearance for the non-inflammatory and inflammatory lesions was 66% and 53%, respectively. This increased at six months to 72% and 73%, respectively. Post-inflammatory erythema and/or scarring that occurred secondary to acne also improved significantly. There was no recurrence or partial recurrence for 3-6 months after the last treatment. No adverse effects were noted during or after treatment. Thus, suggesting that multiple pass Lux V™ technique with a variable-filtration IPL system may be safe and effective for the treatment of acne vulgaris[121]. Nevertheless, this study was limited by its small sample size.

Santos and colleagues[122] designed a spilt-face clinical trial comparing the efficacy of ALA-PDT (ALA plus IPL) and IPL alone in acne vulgaris. Thirteen individuals with varying degrees of acne were treated with 20% topical ALA hydrochloride (DUSA Pharmaceuticals, Wilmington, MA, USA) on one half of the face, and after a 3-hour incubation period, the whole face was exposed to IPL (Quantum SR, Lumenis, Santa Clara, CA, USA; 560 nm cut-off filter, fluence: 26-34 J/cm^2, with a double pulse of 2.4 and 6.0 milliseconds, pulse delay: 25 ms, two sessions at 2 to 4 week intervals). The patients were clinically evaluated on the second, fourth, and eighth weeks. No improvement was observed at the second week on either sides of the face. By the fourth week, however, most of the patients experienced visible improvement of facial acne that was more significant on the ALA-treated side of the face. Ten of the subjects had a decrease in the formation of inflammatory acne on the ALA-treated side, and this improvement persisted until the eighth week post-treatment. Although patients perceived an improvement in facial appearance on both sides of the face, the facial half treated with IPL-only showed no significant improvement at the eighth week compared to baseline. All patients complained of a slight stinging and burning sensation on both sides of the face during the procedure. Post-procedure, the ALA-treated side showed oedematous erythema, crusting with exfoliation and slight darkening of the skin that resolved within 10 days. On the contrary, the facial half treated with IPL alone showed very minimal transient erythema immediately after the procedure. Although sebum production was not measured, an apparent reduction in sebum excretion was also noted on either side, which was more significant on the ALA side. This study reported that IPL alone was ineffective in the treatment of acne vulgaris, but along with ALA pre-treatment it produced a significant improvement in the lesions of acne vulgaris[122]. However, this study failed to report a statistical analysis of its results. In addition, there was no randomisation or blinded assessment. Therefore, questioning the reliability of these results.

On the contrary, Rojanamatin and Choawawanich[109] reported a beneficial effect of IPL monotherapy for inflammatory acne, although a greater improvement was obtained for IPL combined with topical ALA. They evaluated the efficacy and safety of short contact topical ALA plus IPL and IPL alone in the treatment of inflammatory facial acne. Fourteen patients (10 females and 4 males; aged: 16 to 27 years; Fitzpatrick skin phototypes III to V) with inflammatory facial acne vulgaris (≥ 10 active inflammatory

lesions) were treated with ALA-free placebo on the left side and 20% topical ALA (Biosynth, Staad, Switzerland) on the right side of the face for 30 minutes under occlusion. After removal of topical application, the entire face was treated with IPL (Quantum SR, ESC Medical Systems Ltd., Yokneam, Israel; λ = 560-590 nm, fluence: 25-30 J/cm^2, double-pulse mode: 1st pulse 2.4-3.6 ms and 2nd pulse 4-6 ms, with pulse delay: 20-40 ms). Treatments were administered for three sessions at three to four week intervals. Clinical photographs were taken and lesion counts were performed for evaluation. All the patients experienced a diminution in number of inflammatory acne lesions on both sides of the face. The reduction of acne lesion counts was noticed three weeks after the first treatment and continuously improved after second and third treatments. There was a statistically significant improvement in inflammatory facial acne lesion counts on both IPL (66.8% reduction; $p < 0.01$) and ALA-IPL (87.7% reduction; $p < 0.01$) treated sides at 12 weeks after the last treatment. The degree of improvement was much better on the ALA-IPL treated side than the IPL side ($p < 0.05$). Most patients developed transient erythema and minimal crusting. Mild oedema developed on the ALA-IPL treated side. Although IPL alone showed some beneficial effect in the treatment of inflammatory facial acne, the degree of improvement was more and lasted longer with the ALA-IPL treatment[109].

A prospective, randomised, single blind, split-face clinical trial was performed to evaluate the efficacy and safety of IPL alone and a combination of IPL and topical methyl aminolevulinate (MAL) in Chinese subjects with moderate facial acne vulgaris (> 10 inflammatory acne lesions)[123]. Thirty acne patients (skin phototypes IV or V) were randomly assigned to receive half-facial treatments with PDT (IPL plus 16% MAL cream), IPL alone, or as controls in the ratio of 1:2:1. All subjects applied adapalene 0.1% gel every night on the whole face until the last treatment session. The IPL (Ellipse Flex system, DDD, HØrsholm, Denmark; λ = 530-750 nm, fluence: 7.0 to 9.0 J/cm^2, spot size: 10 x 48 mm without overlapping, Single passes with double pulses, pulse delay: 10 ms and pulse duration: 2.5 ms) and MAL-IPL treatments were given four times at three-week intervals. Standardized photographs were taken for the assessment of acne lesions. Of the 30 patients, 23 completed the study. No significant improvement of inflammatory acne lesions were noted in the IPL or MAL-IPL treated sides 12 weeks after the last treatment, when compared with the control group. This could be attributed to the fact that all subjects applied adapalene gel on the entire face

throughout the treatment period. However, there was a delayed but statistically significant reduction of non-inflammatory lesions in the MAL-PDT (38%, $p = 0.05$) and IPL groups (43%, $p = 0.00$) 12 weeks after treatment. Whereas, the control group experienced a 15% increase in non-inflammatory lesions ($p = 0.36$) 12 weeks after treatment. IPL treatment was well tolerated, but 25% of the subjects in the PDT group withdrew from the study because of intolerance to treatment. Adverse effects included stinging, burning, erythema, and oedema, which resolved in 1-2 days. The results of this study showed no significant efficacy of IPL alone or IPL-MAL for moderate inflammatory acne vulgaris. However, this study is limited by the small sample size, reduced statistical power and use of adapalene by all the study participants, which may have confounded the results[123]. Robust prospective, randomised, controlled trials on a larger sample size and evaluating the long-term effects of IPL in acne are required.

In another split-face study, Chang et al[108] evaluated the efficacy of IPL for the treatment of inflammatory lesions of facial acne vulgaris. Thirty Korean patients (female; age: 23–32 years) with mild-to-moderate acne (Grade 2 of Korean acne grading system) were instructed to use topical benzoyl peroxide gel once a day on all facial lesions. One randomly selected side of the face was treated with IPL (Ellipse Flex, DDD; 530-750 nm filter, fluence: 7.5–8.0 J/cm^2, pulse duration: 2.5 ms, double pulse with pulse delay: 10 ms; three sessions at three week intervals). Baseline and post-treatment (three weeks after third session) lesion counts of papules and pustules were performed by two blinded investigators. Red macules were assessed with digital photography and a colorimeter. Irregular brownish pigmentation and skin tone were evaluated using photographs. All patients perceived a reduction in inflammatory lesions on both sides of the face. Three weeks after the final session, there was no significant difference in the mean lesion (papule plus pustule) counts between IPL-treated and untreated sides of the face (-3.2 vs.-3.1; $p > 0.05$). Nevertheless, improvement of red macules (63% vs. 33%) and irregular pigmentation and skin tone (63% vs. 16.7%) were observed on the IPL-treated side in comparison with the untreated side. No significant adverse effects were encountered. This study concluded that IPL effectively improved acne red macules, irregular pigmentation and skin tone, but had no effect on inflammatory acne in Asian skin[108]. However, the use of benzoyl peroxide gel on the entire face may have confounded the results of this study.

Sami et al[124] designed a study comparing the effectiveness of laser (pulsed dye laser, PDL) and non-laser light sources (IPL and light-emitting diode, LED) in moderate to severe acne vulgaris. Forty-five patients of skin type III and IV with moderate to severe acne (according to Burton classification) were randomly divided into three equal groups. Group 1 was treated with a 595 nm PDL (Vbeam®, Candela Corporation, Wayland, MA), group 2 was treated with IPL (EPI-C/plus®, Espansione Group, Bologna, Italy; 550-1200nm filter, fluence: 22 J/cm^2,spot size: 2.5 x 4.5 cm, and pulse duration: 30 ms), and group 3 was treated with a blue-red combination LED (Young Again®, Espansione Group). Unilateral weekly treatments were continued until a ≥ 90% clearance of inflammatory lesions was achieved. Clinical assessments were conducted at baseline, after 1 month, and after the final treatment session. The PDL group reached a ≥ 90% clearance after a mean of 4.1 +/- 1.39 sessions, whereas the IPL group required a mean of 6 +/- 2.05 sessions, and the LED group required a mean of 10 +/- 3.34 sessions. At one month, the reduction in acne lesions was ≥ 90% with PDL, 41.7% with IPL and 35.3% with LED. In comparison with IPL and LED, PDL required fewer sessions to achieve clearance. All treatments were well tolerated with minimal and usually self-limiting adverse events. The drawbacks of this study are its small sample size and short follow-up period. The authors suggested that laser and non-laser phototherapy may be beneficial for the treatment of moderate to severe acne, and that further studies are required to evaluate the different devices with a larger sample size and a longer follow-up period[124].

In a randomised, prospective, split-face clinical study, 20 Korean patients (4 men, 16 women; aged 18-30 years; Fitzpatrick skin type III or IV) with moderate to severe acne were randomised to receive either short incubation ALA plus IPL (30 minutes, n=9) or long incubation ALA plus IPL (3 hours, n=11) on one half of the face and IPL alone (n=20) on the other half[125]. After short or long contact ALA application to one side of the face, the whole face was exposed to IPL (BBL, Sciton Inc., Palo, Alto, CA; 590-nm cut-off filter, fluence: 12-15 J/cm^2, pulse duration: 30 ms, pulse delay: 20 ms). Patients underwent three sessions at four week intervals. Inflammatory lesion counts were performed at baseline and post-treatment, and a sebumeter was used to measure sebum secretion. Improvement in inflammatory acne lesions occurred in all subjects after three sessions of ALA-PDT (short or long incubation) or IPL alone ($p < 0.001$ in all groups). At four weeks after the last treatment, mean reduction of lesions was 84.4% in

the long incubation time group, 72.6% in the short incubation time group, and 65.9% in the IPL-only group. At twelve weeks after treatment, it was 89.5%, 83.0%, and 74.0%, respectively ($p < 0.001$ in all cases), suggesting that the therapeutic effect of PDT and IPL was sustained even 12 weeks after treatment completion. Improvement in inflammatory acne lesions was more pronounced in the long incubation group compared to the other groups. However, the difference in this reduction was statistically significant only between the long incubation and the IPL-only group ($p = 0.01$). Decreased sebum secretion was noted in all three groups after three sessions ($p < 0.001$ in all groups), but the between groups differences were not statistically significant. Transient erythema and mild oedema were the only adverse events reported for all treatment groups, and prolonged incubation time did not result in more adverse effects than short incubation time. In this study, significant improvement in inflammatory acne vulgaris was observed with ALA-PDT (short or long incubation) and IPL, and this effect was maintained even 12 weeks after completion of treatment[125]. Further studies with a larger sample size are required to validate these findings.

Most studies performed on IPL therapy for acne in Asian skin did not include blue light regions (400–500 nm) of the spectrum. Therefore, Kawana et al[126] designed a trial to evaluate the efficacy and safety of IPL with dominant wavelength ranges of 400 to 700 nm and 870 to 1,200 nm for the treatment of acne vulgaris in Asian skin. Twenty-five Japanese patients of skin phototypes II to V with moderate to severe acne were treated with five sessions of IPL (Medilux Plus with a LuxV handpiece, Palomar Medical Technologies, Burlington, MA; λ = 400–700 nm and 870–1,200 nm, fluence:13 J/cm^2) at weekly intervals. Non-inflammatory and inflammatory lesions counts and acne grade pre- and post-treatment were evaluated. After the first session, non-inflammatory and inflammatory acne lesions were reduced by 36.6% and 43.0%, respectively ($p < 0.05$). After the final session, they reduced to 12.9% and 11.7%, respectively, of their baseline values ($p < 0.01$). Also, acne grade improved significantly over the course of the treatment ($p < 0.01$). Most patients experienced transient erythema and burning or stinging, but no major adverse reactions were observed. This study demonstrated that broad-band smooth-pulsed IPL has a potent effect on both inflammatory and non-inflammatory acne lesions in Asian skin. The authors claim that the ability of the IPL system used in this study to deliver broad-band

pulsed light in one smooth pulse and its photon recycling effect (recapturing of scattered energy) make it more efficacious in the treatment of acne[126]. However, long-term follow-up is necessary to observe the course of acne after treatment completion and to determine the need for maintenance therapy.

Choi and group[110] carried out a split-face, single-blind, randomised trial comparing the therapeutic effects of PDL and IPL for the treatment of facial acne vulgaris. Twenty patients (1 male and 19 females; aged 20-37 yrs; Fitzpatrick skin phototypes III to V) with active inflammatory facial acne were randomised to non-overlapping pulses of 585-nm PDL (Cynergy; Cynosure, Inc. Chelmsford, MA, USA; parameters: λ = 585 nm, fluence: 8–10 J/cm^2, spot size: 10 mm, two passes, pulse duration: 40 ms) on one side of the face and IPL (Ellipse Flex System; DDD, Horsholm, Denmark; parameters: λ = 530-750 nm, fluence: 7.5-8.3 J/cm^2, pulse duration: 2.5 ms, triple light pulse with a 9.0 ms interval and two passes) on the contralateral side. Four sessions were administered at two-week intervals. Assessment of response to treatment included lesion counts, acne severity, patient subjective self-assessments of improvement, and histopathological examination. Both IPL and PDL produced improvements in inflammatory and non-inflammatory acne lesions. However, the course of improvement differed for the two treatments, particularly for inflammatory lesions. IPL treatment resulted in a rapid improvement (50% reduction compared to baseline after 1st session; $p < 0.05$ and 66% reduction compared to baseline after 4th session; $p < 0.05$). However, a slight rebound was observed eight weeks after the final treatment session. In contrast, PDL had a gradual but more sustained course of improvement (36% reduction after 1st session; $p < 0.05$ and 62% after 4th session; $p < 0.05$). Furthermore, significant improvements were observed at eight weeks (84% reduction; $p < 0.05$) after the final treatment session. Improvement in non-inflammatory lesions was also greater on the PDL-treated side. Acne severity grades improved with both treatments, but no significant difference was evident. Patients were satisfied with treatment results, and the satisfaction scores increased with time on both sides. Histopathologically, there was a reduction in the inflammatory reaction and an increase in transforming growth factor-β (TGF-β) expression with both treatments, which were more evident for the PDL-treated side. No significant adverse effects were observed. Both, PDL and IPL were found to be effective in treating acne, but PDL showed a more pronounced effect[110].

As previously discussed, adjustment of the various IPL parameters such as fluence, pulse duration, and pulse delay allow flexibility in treatment[127]. IPL devices discharge light in single- (fluence delivered in single shot) and burst-pulse (fluence is delivered in multiple pulses with a delay between the pulses) modes[127]. Kumaresan et al[127] compared the efficacy of burst-pulse mode with single-pulse mode IPL in the treatment of facial acne vulgaris. Ten patients (skin type IV; grade 1 to 4 facial acne vulgaris) were subjected to four sessions of IPL monotherapy (V care Medical Systems, Bangalore, Karnataka; parameters: 420 nm cut-off filter, fluence: 15-21 J/cm^2, auto mode, pulse width: 12 ms) at weekly intervals. The right side of the face was exposed to burst-pulse mode IPL (5 pulses; pulse delay: 6 ms), and the left side to single-pulse mode. Acne grading was done at baseline and at the end of the treatment period by a blinded investigator using Michelsons acne severity index. Clinical digital photographs were also obtained for evaluation. Results were statistically analysed using paired *t*-test. All patients experienced a reduction in acne lesions on both sides of the face. There was a 49.19% reduction in the acne severity score after four sessions of IPL. The burst-pulse mode treated side showed more improvement in acne severity compared to the single-pulse mode treated side (56.66% vs. 40.17% reduction after four sessions). No adverse effects were observed during treatment and post-treatment. IPL as a monotherapy showed significant beneficial effect in treatment of facial acne. Burst-pulse mode was demonstrated to be more efficacious than the single-pulse mode in treating acne. The authors suggested that combining IPL with other topical and systemic anti-acne treatments may further enhance the efficacy of IPL. This study was limited by a small sample size, lack of randomisation and lack of a control arm. Further studies with a robust design and large sample size are required to establish these findings.

More recently, Barikbin et al[128] compared two different pulse durations (55 ms and 101 ms) for IPL monotherapy (KE-Medical Hair & Skin IPL; λ = 572 nm and fluence: 35 J/cm^2) in fifteen female patients with facial acne vulgaris in a single-blind, split-face clinical study and found that although both parameters led to approximately 30% clearance of lesions there was no significant difference in IPL efficacy between the two pulse durations. Taking into consideration the greater risks associated with lower pulse duration, they recommended the use of longer pulse durations in darker skin types[128].

From the aforementioned studies it is clear that the degree of improvement of acne lesions with IPL is less convincing compared to lasers and PDT. Also, lasers and PDT have a more sustained effect than IPL. Nevertheless, IPL seems to have a better safety profile and was well-tolerated by the study participants. Also, its additional benefit of improving acne sequelae such as scarring, post-inflammatory pigmentation and erythema make it more appealing to patients.

Many of the studies mentioned here have a small sample size, making interpretation of these results ambiguous. Moreover, the differences in the IPL manufacturers, settings and parameters, and in the sample population make it difficult to compare the clinical outcomes of the various studies mentioned above. Therefore, further research is required to determine the optimal IPL device and parameters required to treat acne vulgaris and also to evaluate its long-term effects. In addition, it is necessary to delineate how IPL works in the treatment of acne vulgaris.

2.2.2.1 Potential mechanism for IPL in acne

Despite many studies investigating light treatment in acne, its underlying mechanism of action is not clear. There may be multiple targets for light in acne treatment. These targets are potentially considered to be *P. acnes*, sebaceous glands, infundibulum and the infra-infundibular components of the sebaceous follicle[144]. IPL sources probably target acne by photochemical, photothermal (selective photothermolysis or generalised water heating), photoimmunological (modulation of inflammatory response) or a combination of these tissue interactions[126,144]. There is no unique chromophore for acne lesions. Therefore, IPL devices may target various chromophores, such as endogenous porphyrin from *P. acnes*, dilated vessels, and oxyhemoglobin[126]. In addition, superficial desquamation of the epidermis may contribute to the improvement of non-inflammatory comedones by IPL[126,145]

Photochemical/photodynamic effect

Specific wavelengths of visible light have been proposed to produce highly reactive free radicals including singlet reactive oxygen species by activating endogenous porphyrins (such as coproporphyrin III and protoporphyrin IX) resulting in oxidative damage of the lipid layers in the cell membrane of *P. acnes* and damage of the cells of the sebaceous gland, and thereby leading to a reduction of *P. acnes* colonisation and improvement of acne lesions[112,146,147]. Theoretically the most effective visible wavelength for photoactivation of the major endogenous porphyrin component of *P. acnes* is in the blue light region, at approximately 410 or 415 nm (called the Soret band, the strongest porphyrin photoexcitation band), but blue light has poor depth of skin penetration[105,146]. Although photochemical reactions are most profound in this region, it is not limited to this region. Several weaker absorption peaks (Q bands; 505, 540, 580, and 630 nm) are present at longer wavelengths[126].

The main limitation of this mechanism is the ability of *P. acnes* to repopulate rapidly, despite reduction[148]. Therefore, treatment will be needed to be administered more frequently to maintain therapeutic effects, which is not a very practical solution[148]. Also, acne has been demonstrated to improve with light therapy even before bacterial reduction occurred[149], suggesting that additional mechanisms exist for their beneficial effect.

Photothermal effect

Photothermal effects may also play a role by utilizing the concepts of selective photothermolysis or generalised water heating[144,150]. The multitude of longer wavelengths of IPL can reach and thermally target multiple chromophores at different depths. Based on the principle of selective photothermolysis, IPL may reduce sebum secretion rate by targeting blood vessels that supply sebaceous glands[112]. The hypervascularity of inflamed acne lesions that occurs as a result of accumulation of numerous red blood cells in the dilated vessels supplying the sebaceous glands serves as a selective target for IPL treatment[108,109]. The resultant photothermal reaction in the hyperaemic acne lesions causes selective thermal damage of the sebaceous glands at

the sites of acne lesions leading to improvement and reduction of inflammatory acne lesions[109].

However, it appears that reduction of *P. acnes* and sebaceous gland activity alone are not sufficient for a therapeutic effect in inflammatory acne lesions[151,152]. Several laser and light treatments have shown therapeutic benefit without any reduction in *P. acnes* and sebum secretion, suggesting that alternative mechanisms exist for the therapeutic effect of light on inflammatory acne vulgaris[151,152]. Moreover, instead of targeting individual acne lesions, it would be interesting to determine whether IPL therapy with its broad-spectrum is able to interrupt acne pathogenesis and prevent the occurrence of new lesions[144].

Photoimmunological effect (modulation of immune responsiveness)

Although not conclusively proven, some studies have suggested that light-based techniques may be able to improve acne through the modulation of epidermal and dermal immune responses[110,148,152]. In comparison with blue light, the green, yellow, and red light regions are less effective at activating porphyrins, but penetrates more deeply into the skin and may have anti-inflammatory effects by inducing cytokine release from macrophages[142].

Boros-Gyevi and team[153] conducted a study to determine whether IPL therapy influences delayed type hypersensitivity reaction in photodamaged skin, and demonstrated a tendency in delayed type hypersensitivity reaction to be higher after IPL treatment. On histological analysis, the number of Langerhans cells also tended to be higher after IPL treatment. The authors concluded that IPL therapy may also affect the immunological functions of the skin[153].

Recently, a few *in vitro* and photorejuvenation studies have attempted to delineate the mechanisms underlying the clinical effects of IPL[140,145,154-158]. The general notion is that the photothermal injury induced may stimulate the dermal vasculature to initiate an inflammatory response and liberate cytokines that induce dermal remodelling via fibroblast proliferation and upregulation of collagen expression[159].

In photorejuvenation studies, IPL has been demonstrated to diminish the elastosis and stimulate neocollagenesis showing a “fibrillar” orientation, mainly at the level of the papillary and upper reticular dermis[145,155] Significant upregulation of collagen type I and type III have been observed[140,156]. Also, resolution of the superficial dermal inflammatory infiltrate have been noticed[155,160]. One study suggested that the aesthetic improvement of photodamaged skin following IPL treatment may be secondary to clearing of Demodex organisms and the reduction of associated lymphocytic infiltrate[160]

Furthermore, the heating of the dermal microvasculature is also considered to produce a heat shock response[157,161]. Exposure of the skin of rats to IPL (fluence: 34 J/cm^2, in triple pulses with pulse duration of 4, 5 and 6 ms, respectively, and pulse delays of 20 and 25 ms) induced the expression of heat shock protein-70 (HSP-70)[157]. HSP-70 immunoreactivity was observed on day 1 in the epidermal keratinocytes, sebaceous gland cells and endothelial cells. The staining peaked on day 7 and gradually weakened and disappeared by day 30. In another study, Preito et al[161] suggested that HSP-70 may induce neocollagenesis via thermal damage of collagen fibres[161]. They demonstrated that IPL irradiation (560 nm cut-off filter, fluence: 28-35 J/cm^2, spot size: 8 x 35 mm, pulse duration: 2.4/4.2 ms, pulse delay: 15 ms) of photodamged skin induced the expression of HSP-70 and procollagen 1 by dendritic cells that are scattered in the papillary and upper reticular dermis[161]. Heat-induced dermal activation of these cells may be the underlying mechanism of collagen deposition[161]. Further, HSP-70 may play a role in the expression of growth factors such as transforming growth factor beta (TGF-β)[162].

Apart from enhancing collagen production, IPL irradiation has also been demonstrated to impede collagen degradation by downregulating matrix metalloproteinases (MMPs)[163-165]. In an *in vivo* study, Luo et al[163] investigated the molecular effects of IPL on BALB/c mouse skin. The dorsal skin of BALB/c mice was exposed to two sessions of IPL (Lumenis One, USA; λ = 560-1200nm, fluence: 15 J/cm^2, spot size: 8 x 35 mm, pulse duration: 4 ms, and pulse delay: 30 ms) with a two week interval. The post-IPL sections showed dermal thickening, increased collagen (types I and III; $p < 0.05$) accompanied with improved organization, and the mRNA expression levels of the procollagen types I and III had also increased ($p < 0.05$). MMP-1 and MMP-2

mRNA levels progressively decreased after IPL irradiation ($P < 0.05$) in a time-dependent fashion[163]. Further, an *in vitro* study, investigated the effects of IPL on MMPs by irradiating human dermal fibroblasts cultured in contracted collagen lattices with IPL (PhotoDerm VL/PL system, Lumenis Ltd., Israel; 570-nm cut-off filter, fluence: 20, 50, and 75 J/cm^2, triple pulses of pulse duration: 7 ms and pulse delay: 70 ms)[164]. The protein and mRNA levels of MMP-2 were measured at 24, 48 and 72 hours following irradiation. After 24 hours, MMP-2 protein (reduced by 13%, 33% and 61% for 20, 50, and 75 J/cm^2, respectively) and mRNA levels (reduced by 11%, 18% and 40%, respectively) decreased in a dose-dependent manner. This inhibitory effect of IPL on MMP-2 was sustained up to at least 72 hours[164]. Subsequently, Chen et al[165] investigated whether IPL treatment alters the expression of MMP-1 and demonstrated that IPL irradiation (λ = 560-1200 nm, fluence: 15 J/cm^2) of human skin fibroblasts decreased the MMP-1 expression level by 11.47-fold, when compared to the control. These investigators also suggested that IPL may downregulate the UVB-induced AP-1 expression by illustrating a decrease in expression of the AP-1 components, c-Jun and c-Fos following IPL irradiatiom[165]. Paradoxically, Gu et al[166] demonstrated an upregulation of MMP-1, MMP-3 and MMP-12 following IPL irradiation (Lovely II, Alma; λ = 570-950 nm, fluence: 15 J/cm^2, spot size: 8 x 35 mm, 2 pulses of pulse duration: 12 ms, and pulse delay: 30 ms) in human skin. Further *in vivo* studies are needed to determine precisely the effect of IPL on the expression of MMPs.

Several inflammatory mediators have been considered to be responsible for the therapeutic action of the various lasers and light sources. Some hypothesize that the photothermal injury induces a wound healing response via a complex network of inflammatory mediators[159,167]. TGF-β is one of the key cytokines involved in wound healing and in inflammatory responses. A few studies on laser and light devices have suggested a potential mechanistic role for TGF-β[110,152,154,158,168-170].

A recent study by Wang and colleagues[158] evaluated the effect of IPL on TGF-β1 mRNA expression in rat skin. Three regions of the skin of fifteen rats were exposed to IPL (Quantum SRTM; λ = 640nm, fluence: 34 J/cm^2; triple pulses for a pulse duration of 4, 5, and 6 ms; pulse delay = 20 or 25 ms). In situ hybridization was used to detect TGF-β1 mRNA expression in the skin biopsies obtained from the treated (1, 3, 5, 7, 15, and 30 days after exposure) and non-treated areas. In the IPL-irradiated skin, strong

TGF-β1 mRNA expression was detected in the epidermal keratinocytes and dermis (endothelial and inflammatory cells) on day 1, which gradually increased on days 3 and 5, and peaked on day 7. There was a gradual decrease in mainly the epidermal expression from day 15, and by day 30 there was only weak expression in the dermis. This expression pattern was similar to that observed in normal cutaneous wound healing. The non-exposed regions did not express TGF-β1 mRNA. In addition, increased expression of HSP-70 & TIMP-1 was also observed. This study demonstrated that IPL enhances cutaneous TGF-β1 mRNA expression both in the epidermis and dermis of rat skin. The authors concluded that TGF-β1 may be involved in IPL-induced dermal remodelling and photorejuvenation[158].

Using reverse transcriptase-polymerase chain reaction and enzyme linked immunosorbent assay, Byun and group[154] demonstrated an increase in the *in vitro* expression of IL-10 protein (up to 5.95 fold) and TGF-β1 mRNA (up to 1.17 fold) and protein (up to 1.5 fold) in cultured keratinocytes (HaCaT cells) following exposure to IPL (Ellipse FLEX®, VL-2; λ: 555-950 nm, fluence: 4, 8, and 12 J/cm^2). They suggested that the induction of IL-10 and TGF-β1 may contribute to the anti-inflammatory effect of IPL in inflammatory dermatoses[154].

In another study, Wong et al[168] investigated the effects of IPL on extracellular matrix (ECM) proteins and TGF-β1 by exposing human dermal fibroblasts cultured in contracted collagen lattices to IPL (PhotoDerm VL/PL system, Lumenis Ltd., Yoknem, Israel; 570-nm cut-off filter, fluence: 20, 50, and 75 J/cm^2, triple pulses of pulse duration: 7 ms and pulse delay: 70 ms). After 24 hours, mRNA and protein levels of extracellular matrix proteins and TGF-β1 were analysed using quantitative real-time PCR and ELISA, respectively. They found that the mRNA levels of collagen III (increased to 171%, 257%, and 272% of the control, corresponding to fluences of 25, 50, and 75 J/cm^2, respectively; $p = 0.02$) and the TGF-β1 mRNA (increased to 116%, 113%, and 145, respectively; $p = 0.02$) and protein levels (increased to 102%, 109%, and 134%, respectively; $p = 0.04$) in dermal fibroblasts were upregulated. Taking into consideration the results of this study and that of a previous study that showed down-regulated MMP expression following IPL treatment[164], the authors provided a potential explanation for the cutaneous effects of IPL. They suggested that it involves the inhibition of extracellular matrix destruction by reducing MMP expression directly by

IPL irradiation and indirectly via IPL-induced TGF-β1 expression and also involves enhanced extracellular matrix construction by upregulation of collagen III and TGF-β1[168].

Interestingly, in a recent *in vitro* study[169], depending on the fluence setting there was a bidirectional influence of IPL (Miracle Laser Ltd., China; λ = 560-1200 nm, fluence: 0, 10,18, 27, 36, 72 J/cm^2, spot size: 8 x 34 mm, triple pulse of pulse duration: 4.2 ms and pulse delay: 40 ms) on the secretion of MMP-1 (only enhanced secretion at 10 J/cm^2) and TGF-β1 (inhibited at fluence $\leq$ 36 J/cm^2, but enhanced at 72 J/cm^2) by human skin fibroblasts[169].

As mentioned in the previous section, Choi et al[110] demonstrated an increase in TGF-β expression following IPL treatment in patients with facial acne vulgaris. However, the study by this group is the only one that has investigated the molecular effect of IPL in acne vulgaris[110]. Further studies are needed to corroborate these findings. On the basis of these facts, it would be interesting to understand the molecular biology and effects of TGF-β and to explore its association with the resolution of acne vulgaris.

At this point of time, data on the molecular effects of IPL is minimal and mostly anecdotal. Robust studies using similar IPL parameters, having adequate sample size and correlating clinical efficacy of IPL with its molecular effects are warranted to ensure that the interpretation of the post-treatment changes are more comparable and more reliable. Almost all the studies investigating its underlying mechanism are based on its photorejuvenation effect. Thus, emphasizing the requirement of future studies to look more closely at IPL's mechanism of action in the treatment of acne vulgaris.

Chapter 3: Transforming Growth Factor-Beta

3.1 Introduction

Transforming growth factor beta (TGF-β) belongs to a family of multifunctional peptide growth factors that comprise of the TGF-β subfamily, bone morphogenetic proteins (BMPs), activins, inhibins, nodals, mullerian inhibitory factor and various other structurally related members [171]. They regulate a myriad of biological functions such as growth and development (both adult and embryonic), repair and remodelling, and inflammation and host immunity[172,173]. TGF-β is ubiquitously expressed by nearly all cells and exerts a diverse and broad range of effects through a complex cell surface receptor system in a context-dependent manner[174].

Structurally, TGF-β is composed of several extended β-sheets stabilized by a common structural knot motif ("cysteine knot") composed of six cysteine residues that form three intrachain disulfide bonds[175,176]. Active TGF-β is a 25 kDa dimer stabilized by hydrophobic interactions and by an interchain disulfide bond formed by the seventh free cysteine of each monomeric unit[176].

Three structurally nearly identical (76-80% amino acid sequence homology) isoforms of TGF-β have been currently identified in mammals: TGF-β1, TGF-β2 and TGF-β3[177,178]. Among these isoforms, TGF-β1 is the one that has been predominantly researched in most tissues, including skin[179,180]. Despite a high level of sequence similarity and some overlapping functions, the three isoforms have been demonstrated to exert distinct biologic effects *in vivo*. The differences in the effects of the TGF-β isoforms are most clearly defined in data from studies conducted on transgenic knockout mouse models[181-184].

TGF-β1 knockout mice die by 3 to 4 weeks of age due to the development of a wasting syndrome and severe autoimmune-like multifocal inflammatory reaction resulting in multi-organ failure[181,182]. Targeted disruption of TGF-β2 resulted in perinatal mortality due to multiple developmental defects that are incompatible with life after birth[184]. These include cardiopulmonary, craniofacial, limb, spinal column, visual, auditory, neural and urogenital defects[184]. Mice lacking TGF-β3 had delayed pulmonary

development and cleft palate with no other craniofacial anomalies and died immediately after birth due to an inability to suckle effectively[183]. These differences in phenotypic expression suggest that these TGF-β isoforms have different roles in vivo.

Furthermore, the temporal-spatial expression of the TGF-β isoforms have been reported to be very different[185,186]. In normal skin, TGF-β1 expression in the epidermis was mild to moderate and was mainly localised to the upper differentiated layers, the stratum granulosum and stratum corneum [185,186]. Among the appendageal structures, it was present only in the inner keratinizing layers of the hair follicles and was not detected in the sweat glands or sebaceous glands[185]. TGF-β1 was absent in all dermal mesenchymal structures[185]. In contrast, TGF-β2 and, to a lesser extent, TGF-β3 were expressed throughout the epidermis and in both the outer and inner root sheaths of hair follicles[185,186]. Sebaceous glands showed immunoreactivity that was particularly strong for TGF-β3. Neither TGF-β2 nor TGF-β3 was present in sweat glands. TGF-β2 and TGF-β3 immunoreactivity were observed in smooth muscle cells of dermal arteries and arrector pili muscles[185]. TGF-β3 immunoreactivity was more extensive in the dermis with strong immunostaining in most dermal fibroblasts[185]. In addition, TGF-β isoforms have been shown to be differentially expressed (both spatially and temporally) during embryogenesis, differentiation, tissue repair, and in disease states[185-192]. This indicates that the TGF-β isoform expression in human skin is differentially regulated, and their distribution is varied and complex. The relative roles of different TGF-β isoforms in vivo may be influenced by their local availability and/or the regulation of their conversion from latent into active form[193].

3.2 Latent TGF-β (LTGF-β)

TGF-β is secreted from cells in a latent form (LTGF-β) that is composed of 390-414 amino acids[171]. The liberation of TGF-β from this latent state is critical for signalling (**Figure 3.1**)[194].

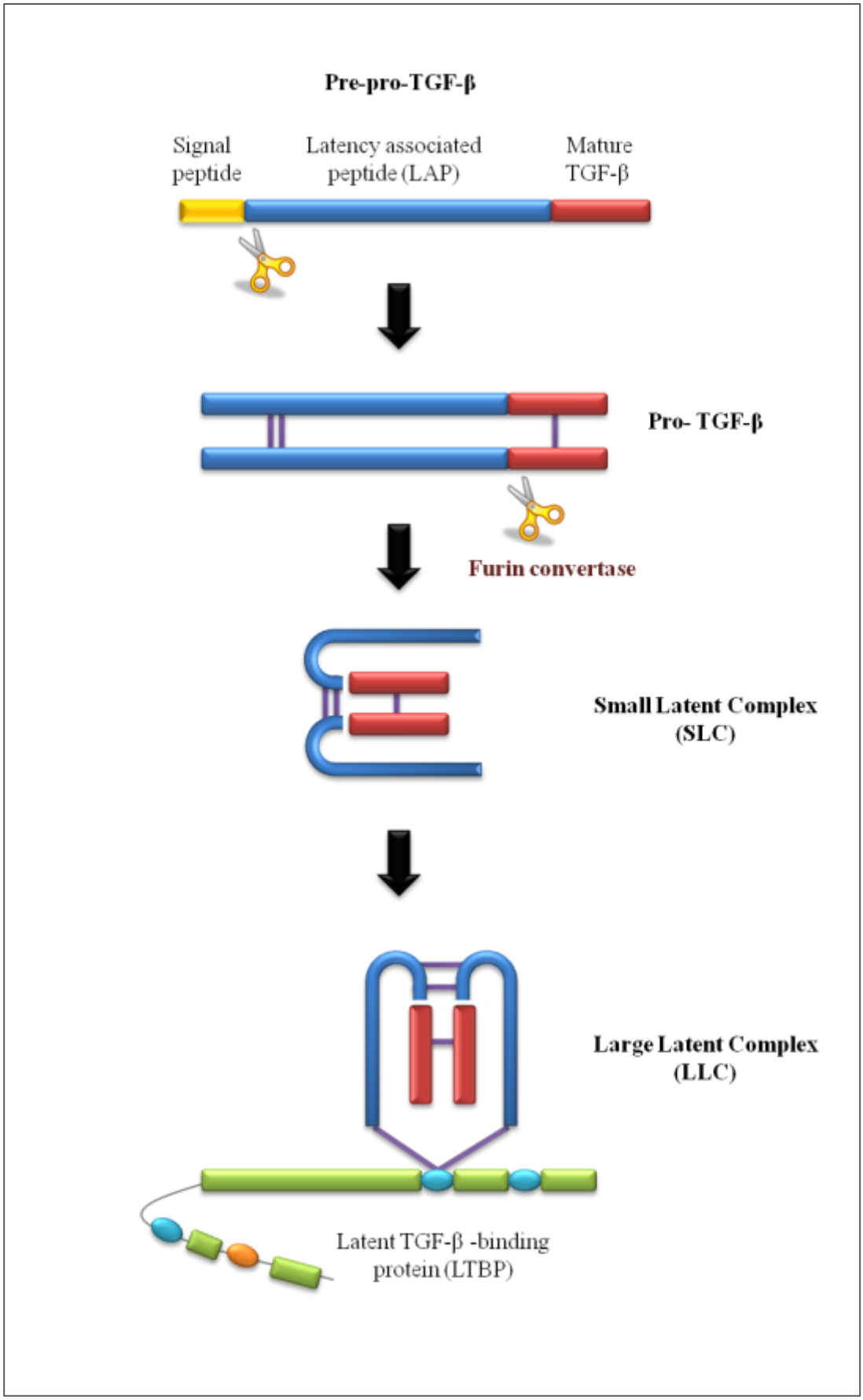

Figure 3.1 Processing of Latent TGF-β to form small and large latent complexes

The precursor form is converted to pro-TGF-β by cleavage of its amino terminal hydrophobic peptide signal region[171,195]. This pro-TGF-β undergoes cleavage between amino acids 278 and 279 in the trans-golgi by furin convertase to form a pro-peptide and mature TGF-β[196]. However, the propeptide remains non-covalently associated with the mature TGF-β, and this interaction maintains the latency [194,195]. This latent form cannot interact with its receptors and requires activation for biological activity[197]. There are two forms of latent TGF-β (**Figure 3.1**), which are:

- Small latent complexes (SLC), in which the active TGF-β dimer is noncovalently associated with a propeptide, the dimeric latency-associated peptide (LAP).
- Large latent complexes (LLC), which are composed of the active TGF-β dimer, LAP, and the latent TGF-β -binding protein (LTBP), which is disulphide bonded to the LAP.

3.2.1 Latency-associated peptide (LAP)

The propeptide that is noncovalently associated to TGF-β at the N-terminal is referred to as the latency associated peptide or LAP[195]. It is a homodimer that confers latency to TGF-β, and also ensures proper folding and secretion of TGF-β[198]. LAP consists of three N-glycosylated asparagine residues, of which two have mannose-6 phosphate groups that can interact with cell surface mannose-6-phosphate/insulin-like growth factor II receptors[195]. They have 3 cysteine residues at positions 33, 223 and 225 [199]. Cys^{33} is involved in binding to the LTBP, whereas Cys^{223} and Cys^{225} are required for dimerisation of LAP monomers[199]. Brunner et al[199] demonstrated that substitution of cysteine with serine at positions 223 and 225 of the LAP resulted in the release of bioactive TGF-β1, suggesting that dimerisation of LAP may be necessary for latency. Mutations in the region of these cysteines of LAP cause an autosomal dominant disorder called Camurati-Engelmann disease that is characterised by hyperosteosis and sclerosis of the diaphysis of long bones[200]. All these mutations affect the dimerisation of LAP, consequently impairing its ability to keep TGF-β in its latent form[200].

LAP also imparts latency to TGF-β by concealing the type II receptor binding site on the mature TGF-β dimer[201].There are 3 key regions in the LAP that contribute to the assembly and stability of latent TGF-β, which are the N-terminus TGF-β binding site, the LTBP binding site and the point of LAP dimerisation at the C-terminal[201]. In a recent study, Walton and co-workers[201] used *in vitro* mutagenesis and functional analyses and identified the residues in these key regions of the LAP (**Table 3.1**).

Table 3.1 Residues at the key LAP regions that facilitate assembly of LLC

Key LAP regions	**Residues**
TGF-β binding site	Ile^{53}-Leu^{59}
LTBP binding epitope	Arg^{45}, Arg^{50}, Lys^{56} and Arg^{58}
LAP dimerisation interphase	Trp^{195}-Cys^{225}

(Adapted from Walton et al[201], 2010)

In an animal model for scleroderma, Zhang et al[202] demonstrated that LAP could prevent TGF-β1 induced fibrosis. This effect could be due to the fact that association with LAP will keep TGF-β1 in the latent form and prevent TGF-β signalling. However, the immunoregulatory effect of TGF-β1 was not inhibited by LAP as opposed to anti-TGF-β1 antibodies, which abrogate both the pro-fibrotic as well as the immunoregulatory activities of TGF-β1[202]. The authors attributed this unperturbed immunomodulatory effect to previously synthesized TGF-β1 that is present bound to the tissue matrix, and suggested that LAP may not have a role in immunoregulation.

On the contrary, subsequent studies demonstrated that LAP does have an immunomodulatory effect[203-205]. Gandhi and co-workers[203] demonstrated that immature dendritic cells may inhibit T-cell activation by surface expression of LAP, in a TGF-β-dependent manner. More recently, this group identified a novel population of regulatory T-cells that express LAP ($CD4^+$ LAP^+ T-cells) and exhibit *in vitro* TGF-β and IL-10-dependent suppressive activity[204].

Moreover, Ali and colleagues[205] suggested that LAP has immunomodulatory activity independent of TGF-β1, which was demonstrated by TGF-β1-independent monocyte

chemotaxis both *in vitro* and *in vivo*, and suppression of delayed type hypersensitivity reaction in vivo.

3.2.2 Latent TGF-β -binding protein (LTBP)

Latent TGF-β is usually secreted as a large tripartite complex, which is formed by the linking of the small latent complex (TGF-β and LAP) to the latent TGF-β -binding protein (LTBP)[195]. LTBP does not confer latency, but serves to bind TGF-β to the extracellular matrix and to enable its proteolytic activation[177].

LTBPs are high molecular weight glycoproteins that share structural homology with fibrillins, and together they comprise the LTBP/fibrillin protein family[206]. So far, four isoforms of LTBP (LTBPs 1-4) and several splice variants have been identified[206]. Based on their ability to bind to LAP, LTBP-1, LTBP-3 and LTBP-4 form a subgroup within this family[207]. Fibrillins and LTBP-2 do not bind to LAP[207]. LTBP-1 & -3 bind to the SLC of all 3 TGF-β isoforms, whereas LTBP-4 binds weakly only to the SLC of TGF-β1[207].

LTBPs are characterized by multiple EGF-like repeats and 8-Cys residues/domains[208]. The structure of all LTBPs is composed of four parts, the N-terminal region, the hinge domain, central core of epidermal growth factor (EGF)-like repeats and the C-terminal region (**Figure 3.2**)[209].

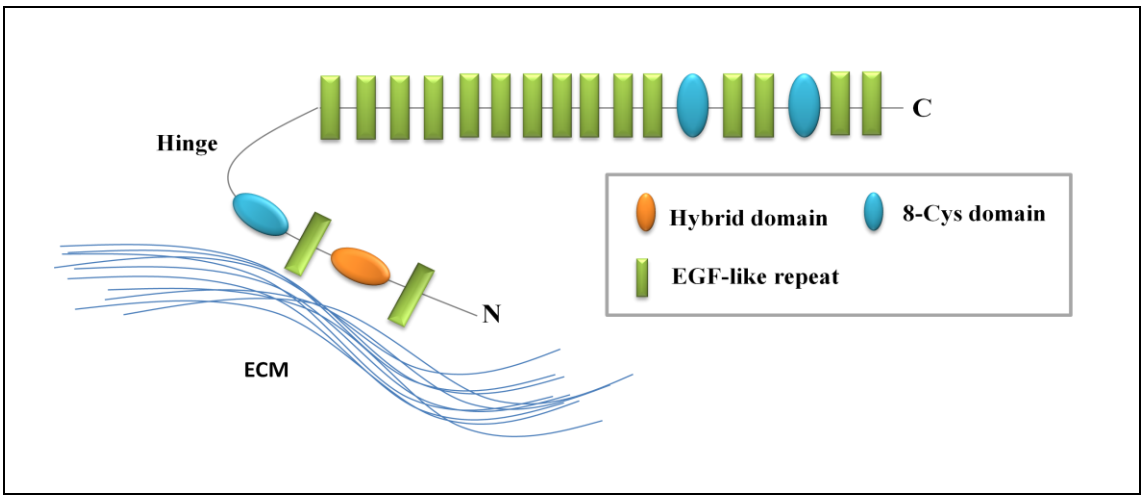

Figure 3.2 Schematic illustration of structure of the Latent TGF-β Binding Protein

The N-terminal region is composed of two to three EGF-like repeats and two 8-Cys domains, of which the first domain from the N-terminal end is called the hybrid domain (sequence similar to both EGF-like repeats and 8-Cys residues)[209,210]. This N-terminal region is mostly involved in interaction with the extracellular matrix as it contains transglutaminase substrate motifs, and transglutaminase is essential for extracellular matrix association of LTBP[209,210]. The third 8-Cys domain of LTBP-1,-3 and -4 has been identified as the LAP binding site[207]. The exact functions of the remaining 8-cys domains are not known. They probably facilitate the localisation of LTBPs to the extracellular matrix[206].

Chen and colleagues[211] using fluorescence resonance energy transfer analysis revealed that negatively charged amino acids surrounded the 2, 6 disulphide bond in the TGF-β-binding 8-cys domain, and these contributed to the electrostatic forces that initiate interaction of the SLC with the TGF-β-binding 8-cys domain. As previously mentioned, Walton et al[201] recently identified positively charged residues (**Table 3.1**) at the LTBP binding epitope of LAP corresponding to the negatively charged residues identified by Chen et al[211]. These findings suggest that initial electrostatic interactions between the LAP and LTBP precede covalent bonding between these two molecules.

The key role of LTBP is to target or localise latent TGF-β to the ECM[212]. Owing to the structural similarity with fibrillins, LTBPs are also thought to be a structural component of the ECM[194,212]. In addition, LTBPs interact with several ECM components such as collagen, fibrillin and fibronectin[213,214].

To evaluate the role of LTBP in TGF-β regulation, Yoshinaga and colleagues[194] bred mutant mice in which the cysteine residue (Cys^{33}) that binds TGF-β1-LAP to LTBP was substituted with serine, thereby preventing formation of the LLC. These mutant mice had multiorgan inflammation, lack of skin Langerhans cells, a reduced lifespan, and tumours of the stomach, rectum, and anus. The observed phenotype was consistent with decreased TGF-β1 levels, but was not as severe as with TGF-β1($^{-/-}$) null mice[194]. The findings of this study suggest that association with LTBP is important for efficient TGF-β1 functioning.

In the absence of LTBP, the SLC is secreted slowly and is misfolded due to aberrant covalent bond formation between the Cys^{33} of the LAP and a cysteine in mature TGF-β[215]. Therefore, LTBPs are considered to enhance the secretion of SLC and ensures its proper folding by correct disulphide bonding[215].

Interestingly, a considerable amount of LTBPs are secreted by cells without TGF-β, indicating that LTBPs may have some other functions that are independent of TGF-β[216]. Moreover, LTBP-2 does not bind to the SLC suggesting that it has a role unrelated to TGF-β regulation[216].

In brief, LTBPs may function as localisers of TGF- β to the ECM, structural components of the ECM, enhancers of SLC secretion, and as regulators of TGF-β availability.

3.2.3 Activation of latent TGF-β

Active TGF-β is essential for TGF-β signalling to take place. Therefore, activation of TGF-β plays an important role in controlling its biological activity[197]. Activation of the latent forms of TGF-β can occur either by cleavage of the LAP (by various proteases,

such as plasmin, thrombin, plasma transglutaminase, or endoglycosylases) or by conformational changes of the latent TGF-β complex (by physical interactions of LAP with proteins, such as thrombospondin-1) that exposes the TGF-β receptor binding site[177,197,217]. This allows release of bioactive TGF-β and its subsequent interaction with specific receptors. *In vitro*, the latent forms of TGF-β can be activated by various mechanisms such as increases in temperature, extremes of pH, chaotropic agents (urea, guandine hydrochloride), and detergents[197,218].

Barcellos-Hoff et al[219] demonstrated that production of reactive oxygen species either *in vitro* (using ionizing radiation or metal ion-catalyzed ascorbate reaction) or *in vivo* (by exposure to ionizing radiation) activated latent TGF-β. This type of activation probably involves site specific oxidation of certain amino acids (cysteine or methionine) residues in the LAP, which elicits a conformational change and releases free active TGF-β[219].

Several other agents have also been implicated as activators of TGF-β, such as retinoids, glycosidases, vitamin D3 derivatives, and glucocorticoids[195,208,220,221]. All these data suggest that multiple mechanisms exist for latent TGF-β activation. Decoding the mechanism of activation of latent TGF-β is essential for a better understanding of the action of this cytokine. The proper regulation of latency and activation of TGF-β is vital for the functioning of this cytokine, as any dysregulation may lead to dire pathological consequences[222]. Therefore, understanding latency, targeting and activation of this molecule is important.

3.3 TGF-β receptor

TGF-β elicits its cellular responses by binding to a family of transmembrane cell surface receptors that have intrinsic serine/threonine kinase activity[223]. They form an exclusive ligand-receptor system, as the TGF-β receptor family is the only known signalling receptor for TGF-β and these receptors can be activated only by ligands of this family[208]. The TGF-β receptors are subdivided into type I (TGF-βRI) and type II (TGF-βRII) receptors[224]. Structurally, they are composed of a cysteine-rich extracellular ligand binding domain, a hydrophobic transmembrane region and an

intracellular serine/threonine kinase domain. A characteristic GS domain formed by the SGSGSG (glycine-serine repeat) sequence is present in the juxtamembrane domain of type I, but not in type II receptors[225]. TGF-βRI and II exist as homodimers on the cell surface, but on ligand binding they assemble together as a heteromeric complex[208].

TGF-βRII is a constitutively active kinase, whereas the kinase activity of TGF-βRI needs to be activated by TGF-βRII[224,225]. TGF-β1 and TGF-β3 binds with high affinity to TGF-βRII, whereas TGF-β2 does so weakly[226]. This differential isoform binding affinity of TGF-βRII was investigated by De Crescenzo et al[226], and they attributed the low affinity binding of TGF-β2 to three amino acid residues at the ligand-receptor interface.

In normal skin, TGF-βRI is abundantly expressed in the upper differentiated layers of the epidermis[227]. TGF-βRII is moderately expressed in all epidermal layers and in the follicular epithelium[192,228,229]. Weak expression of TGF-βRI and TGF-βRII was found in dermal fibroblasts and endothelial cells[228,230]. The ratio of TGF-βRII to TGF-βRI may also influence TGF-β-mediated responses, more of which will be discussed later on[231].

3.3.1 Accessory receptors

A third cell surface transmembrane receptor, betaglycan also known as TGF-βRIII is a highly glycosylated protein consisting of a large extracellular region and a short cytoplasmic tail that does not have any kinase activity[208]. Betaglycan enhances TGF-β-mediated signalling by allowing high-affinity binding of TGF-β to TGF-βRII[232]. However, it has no intrinsic signalling activity. It enhances the receptor binding affinity of TGF-β2, which otherwise binds to TGF-βRII with low affinity. Betaglycan possibly alters the conformation of TGF-β2 to facilitate this interaction[232].

This function of betaglycan was further supported by the findings of a study conducted by Stenvers and colleagues[233], in which primary fibroblasts generated from TGF-βRIII-null mice embryos revealed significantly reduced responsiveness to TGF-β2, in terms of reduction in growth inhibition, reporter gene activation, and Smad2 nuclear

localisation. In contrast, the response to the other TGF-β isoforms was not significantly altered[233].

In contrast to the abovementioned membrane-bound form of betaglycan, the soluble form (formed by release of the extracellular domain from the cell surface) has been shown to sequester and inhibit TGF-β function in cell cultures[234]. Adding more ambiguity to the issue, another study showed that membrane-bound betaglycans that have undergone glycosaminoglycan modifications, can inhibit TGF-β signalling in epithelial cells by preventing the association between TGF-βRI and TGF-βRII[235]. It is evident from the contradictory findings of these studies that the role of betaglycan still remains to be clarified. It is also possible that betaglycan functions in a context dependant fashion either as an enhancer or inhibitor of TGF-β signalling.

Endoglin is a homodimeric glycoprotein that is highly expressed in endothelial cells that binds to TGF-βRI and TGF-βRII. It is structurally related to betaglycan but lacks glycosaminoglycan chains. The exact function of endoglin in TGF-β signalling is not known, but it is considered to have a role in vascular development, remodelling and homeostasis[236]. Furthermore, in mouse skin carcinogenesis, endoglin was shown to act as a suppressor of malignancy[236]. Several other accessory receptors such as glycosylphosphatidylinositol (GPI)-anchored proteins may also serve as accessory receptors for TGF-β.

3.4 TGF-β signalling

TGF-β initiates signalling by binding to the type II receptor (TGF-βRII), which in turn recruits, forms a complex with and phosphorylates the type I receptor (TGF-βRI)[176]. The activated TGF-βRI then phosphorylates downstream mediators, the Smad proteins[176,237].

Smads are intercellular mediators or signal transducers of the TGF-β signalling pathway. They are derived from the Sma and MAD (mothers against decapentaplegic) gene homologues in Caenorhabditis elegans and Drosophila melanogaster, respectively[238,239]. The Smads are transcription factors that in the basal state are mostly

localized in the cytoplasm[237,240]. Eight Smads have been identified in the mammalian genome[208].

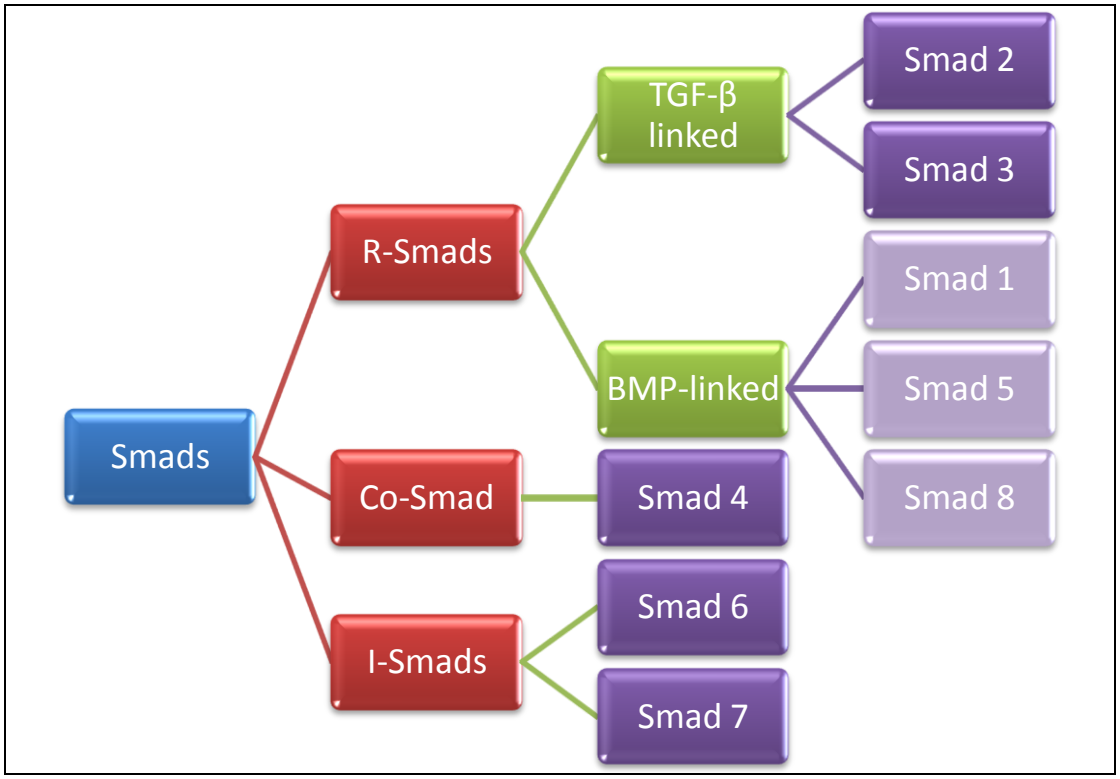

Figure 3.3 Classification of mammalian Smad proteins

Smad proteins are classified into three functional groups (**Figure 3.3**)[176,177,217]:

- Receptor-associated Smads (R-Smads), which directly interact with activated type I receptors in a ligand-specific manner (SMAD1, 2, 3, 5, and 8). Based on the type of receptor they are phosphorylated by, R-Smads are further subdivided into BMP-Smads (Smad1, Smad5 and Smad8 are phosphorylated by BMP type I receptors) and TGF-β-Smads (Smad2 and Smad3 are activated by TGF-β and activin type I receptors).
- Co-mediator Smad (Co-Smad), a common mediator of all TGF-β family members (Smad4), and
- Inhibitory Smads (I-Smads), SMADs (SMAD6, 7) that antagonise the signalling function of R-Smads & Co-Smad. Smad 6 inhibits BMP signalling, whereas Smad 7 can inhibit both TGF-β and BMP signalling.

Unlike the targeted disruptions of Smad2 and 4 that are embryonically lethal, the targeted disruption of Smad3 resulted in the birth of viable mutant mice[241,242]. The Smad3 null mice are smaller than their wild-type littermates and were demonstrated to have limb deformities[2]. These mutant mice develop a progressive wasting illness, with an onset around the time of weaning and typically die between one to eight months of age. The lethality of these Smad3 knock-out mice have been attributed massive inflammation due to leukocytosis, reduced T-cell responsiveness to TGF-β, and impaired mucosal immunity[241]. In addition, Smad3 disruption was also demonstrated to abrogate the antiproliferative effect of TGF-β[242]. Collectively, these findings suggest that Smad3 plays an important role in mediating the antiproliferative and immunomodulatory effects of TGF-β.

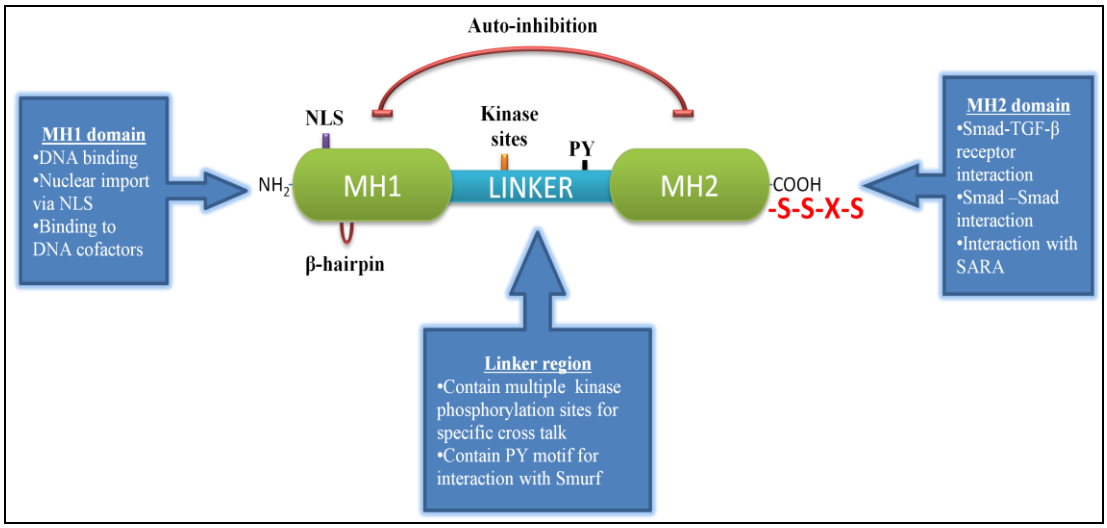

Figure 3.4 Schematic representation of the structure and functions of R-Smads

Smad proteins consist of two globular highly conserved MAD homology (MH) domains (**Figure 3.4**), referred to as MH1 (N-terminal) and MH2 (C-terminal)[243,244]. The MH1 domain is highly conserved among R-Smads and co-Smads[245]. In contrast, the N-terminal of I-Smads have only slight similarity to MH1 domains of the other Smads[245]. The MH1 domain of R-Smads and co-Smads have DNA-binding activity[244]. The crystal structure of the MH1 domain constitutes a compact globular fold, with four α helices, six short β strands, and five loops[244]. A DNA-binding motif, the β hairpin loop present in this structure interacts with the major groove in DNA[244]. One exception is Smad2, which has an insertion encoded by exon3 that does not allow DNA binding[246]. Deletion of exon3 from Smad2 enabled DNA binding. The MH1 domain

also interacts with various DNA-binding proteins and transcription factors[243]. Furthermore, in R-Smads and Smad4 this domain has a nuclear localisation signal (NLS) that may regulate nuclear import (translocation of the protein from the cytoplasm to the nucleus)[247,248].

The MH2 domain is highly conserved among all Smads[245]. The MH2 domain is responsible for protein-protein interactions (with cytoplasmic anchors, nuclear proteins and other Smads) and in the case of R-Smads for receptor binding[245]. The R-Smads have a characteristic Ser-Ser-X-Ser (SSXS) motif at their C-terminal end, the two most C-terminal serine residues of which are phosphorylated by the activated TGF-βRI[249,250]. The co-Smads cannot be phosphorylated by TGF-βRI as they lack the SSXS motif[208].

The MH1 and MH2 domains are connected by an intervening proline-rich linker region that is highly variable in its size and sequence[208]. Key regulatory peptide motifs exist in this region such as the proline-tyrosine (PY) motif (bind ubiquitin ligases of Smurf) and several phosphorylation sites for mitogen-activated protein kinases, Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) and protein kinase C (PKC)[245].

The R-smads remain inactive in the basal state due to a mutual autoihibitory interaction between the MH1 and MH2 domains[171,251]. They are made accessible to the TGF-βRI for phosphorylation by a protein known as the Smad anchor for receptor activation (SARA) [208]. Microtubules also serve as a cytoplasmic anchor for inactive Smads[245]. Phosphorylation of the serine residues at the C-terminal of the R-smads leads to activation of R-Smad and also a change in its conformation, which ultimately leads to its dissociation from TGF-βRI and from its cytoplasmic anchors (SARA, microtubular network)[217]. In addition, receptor-mediated phosphorylation exposes various epitopes on the Smad surface such as those involved in nuclear import and transcription regulation, and enhances the affinity of R-Smads for Smad 4[245]. Smad oligomerisation is also thought to occur following phosphorylation[245]. The phosporylated R-Smads then recruit the Co-Smad, Smad 4 to form a complex with it that facilitates nuclear translocation[243]. Unphosphorylated R-Smads are considered to exist as monomers[245]. However, the stoichiometry of the R-Smad-Co-Smad complexes is controversial and needs to be further clarified with structural studies[245].

In the nucleus this Smad complex interacts with various co-activators or corepressors and associates with the DNA-binding co-factor to bind to the DNA, and ultimately lead to target gene transcription. The TGF-β signalling pathway is schematically illustrated in **Figure 3.5**.

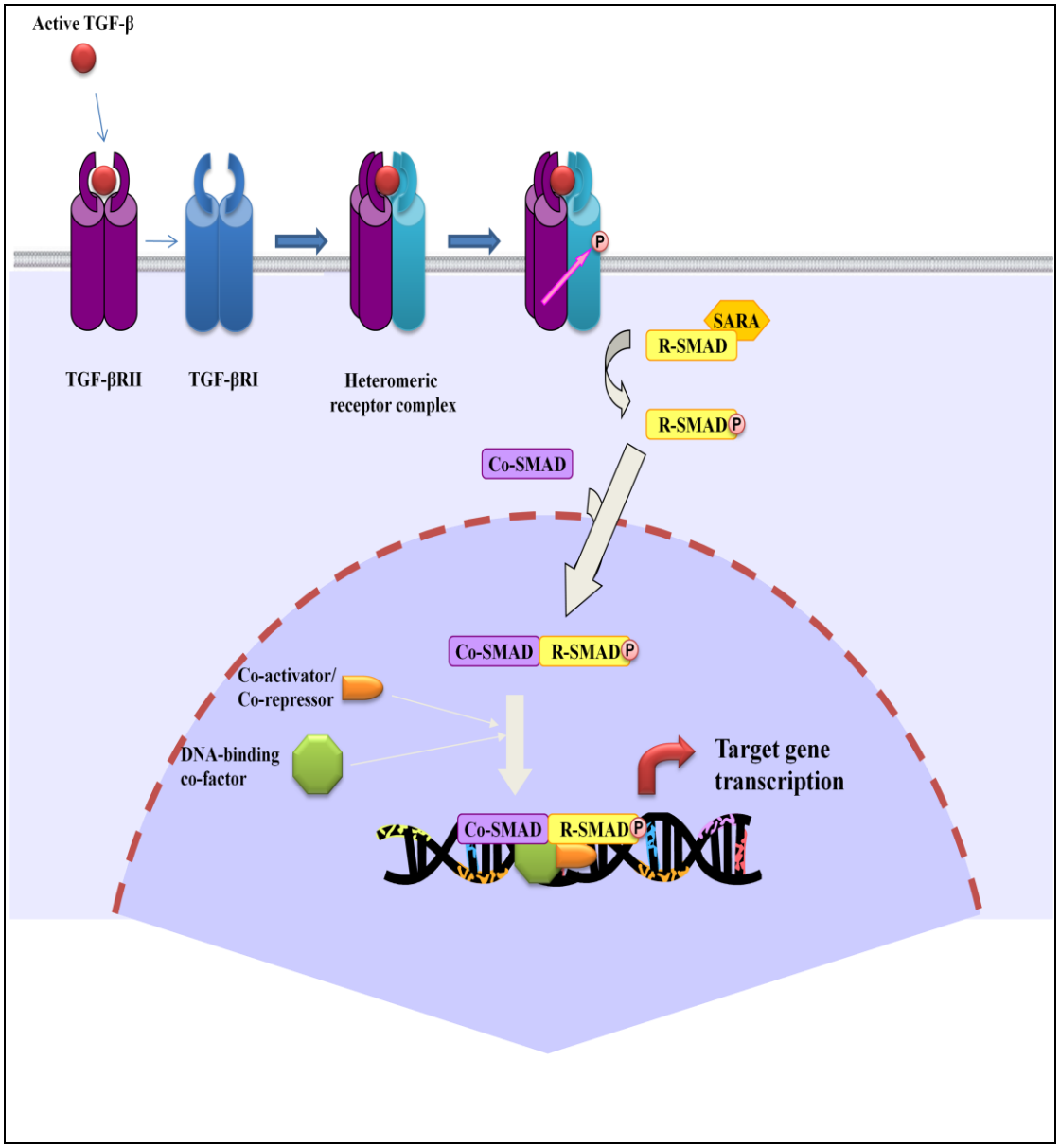

Figure 3.5 TGF-β/Smad signalling pathway

Depending on the cellular context and type, the activated Smad complex may positively or negatively regulate the expression of various target genes[208]. As previously discussed, the β-hairpin loop of the MH1 domain of R-Smads (with the

exception of long form of Smad2) and Smad4 possess intrinsic DNA-binding activity[244]. This β-hairpin loop was demonstrated to interact through the incorporation of hydrogen bonds with a specific DNA sequence, 5′-GTCT-3′, or its complement 5′-AGAC-3′, called the Smad-binding element (SBE)[244,252]. Smads have also been described to bind to GC-rich sequences in promoters of certain genes[253]. Owing to their relatively low DNA binding affinity, Smads must interact with other DNA binding co-factors/transcription factors[208]. The interaction of Smads with these transcription factors facilitates both high-affinity DNA binding and localisation of the Smad complex to a precise promoter region to elicit specific transcriptional responses[208]. Numerous transcription factors have been demonstrated to interact with Smads[243]. The diversity of the Smad-interacting transcription factors and their availability in different tissues or cells may in part be responsible for the varied response to TGF-β in each cell type and in different contexts[243]. Additionally, the Smad transcriptional complexes regulate transcriptional activity by recruiting co-activators or co-repressors to the promoter region[224]. Co-activators facilitate Smad transcription by bringing sequence-specific transcription factors in close proximity to the RNA polymerase II complex[208,254]. Some of these transcriptional co-activators such as p300 and CBP possess intrinsic histone acetyltransferase (HAT) activity that facilitates target gene transcription by acetylation of histones and chromatin remodelling[208,224]. On the other hand, co-repressors repress Smad transcription[224]. Some transcriptional co-repressors (e.g., c-Ski, SnoN, and TGIF) recruit histone deacetylases (HDAC) to Smad complexes, thereby inhibiting Smad transcription[255,256]. Additionally, they can also repress transcription by competing with CBP/p300 for Smad interaction[255,256].Another corepressor c-Myc directly associates with Smad2 and Smad3 at the promoter region and physically represses transcription[208]. These corepressors may also control the magnitude and duration of TGF-β signalling[257]. In the basal state, Ski and SnoN are associated with Smad3 and upon TGF-β stimulation they are rapidly degraded. However, their expression is rapidly induced by TGF-β, possibly contributing to the negative feedback loop[257].

The I-Smads act in an opposing manner to R-Smads in that they antagonise TGF-β signalling[208]. Although they have a conserved MH2 domain they differ structurally from the other Smads in that their N-terminal region only shows weak sequence homology with the MH1 domain of R-Smads and Co-Smads, and they lack the SSXS

motif[208]. They appear to exert their antagonistic action by various mechanisms. I-Smads compete with R-Smads for binding to activated type I receptors and thus inhibit phosphorylation of R-Smads[258]. In addition to functioning as competitive inhibitors, they promote TGF-β receptor ubiquitination and degradation by recruiting E3-ubiquitin ligases, known as Smad ubiquitination regulatory factor 1 (Smurf1) and Smurf2, to the activated type-I receptor, resulting in termination of signalling[259,260]. Recently, Shi and co-workers[261] reported that Smad7 recruits a complex of GADD34 (a regulatory subunit of the protein phosphatase 1 holoenzyme) and the catalytic subunit of protein phosphatase 1 (PP1c) to the activated TGF-βRI to dephosphorylate and inactivate it. In the basal state, I-Smads are predominantly nuclear in location and nuclear export of I-Smads occurs on ligand stimulation[262]. Further, ligand stimulation also induces I-Smad mRNA transcription, suggesting that they may act as autoregulatory negative-feedback signals in TGF-β signalling[263].

3.4.1 Termination of signalling

Duration and intensity are important determinants for the signalling specificity of TGF-β[264]. Taking into consideration the numerous functions of TGF-β, tight regulation of this pathway is essential for achieving normal cellular responses and for maintaining homeostasis[265]. Therefore, it is important to find out how TGF-β signalling is attenuated and terminated[264]. As previously discussed, inhibitory Smads (Smad6 and 7) and transcriptional co-repressors (c-Ski and SnoN) play an important role in the termination of TGF-β signalling. They are stimulated by TGF-β signalling and participate in a negative feedback mechanism so as to fine tune the duration and intensity of signalling[265]. The potential mechanisms considered to terminate Smad signalling in the nucleus are phosphatase-mediated dephosphorylation and ubiquitination- mediated proteosomal degradation of R-Smads[265]. Dephosphorylation is an important mechanism of Smad inactivation as phosphorylation plays a key role in the signalling pathway[208]. PPM1A (a metal ion-dependent protein phosphatase) was recently identified as a nuclear R-Smad phosphatase that directly dephosphorylates C-terminal phosphorylated Smad1, 2 and 3[264]. Whether other Smad phosphatases exist, remain to be determined. R-Smads have been found to be ubiquitinated by various classes of E3 ubiquitin ligases known as Smad ubiquitination regulatory factor

(Smurf)[264]. The ubiquitinated proteins are then targeted for proteasomal degradation[264]. Smurf in association with I-Smads also mediates ubiquitination of activated TGF-β receptors, leading to their preoteosomal degradation[208].

Apart from the canonical Smad signalling pathway several non-Smad pathways are also considered to be involved in TGF-β signal transduction, such as the MAP kinase pathway, Rho-like GTPase signalling pathway, and phosphatidylinositol-3-kinase/AKT pathway[208]. Although the knowledge on the Smad-independent pathways is limited at this point of time, it has been suggested that the disruption of the canonical Smad signalling pathway may unmask the stimulatory effects of TGF-β via the non-Smad pathways[266].

3.5 Biological effects of TGF-β

TGF-β is a pleiotropic cytokine that has a profound effect on various cellular processes including extracellular matrix formation, cell growth, apoptosis, differentiation, angiogenesis, and immune responses[208,267].

3.5.1 Effects of TGF-β on extracellular matrix

The extracellular matrix (ECM) is a complex macromolecular structural network of proteins that serves as a structural scaffold for cells in tissues[208]. TGF-β is a key regulator of ECM remodelling and also a potent fibrogenic factor[268]. It regulates ECM composition by controlling matrix production and degradation and also regulates the adhesive interactions between cells and the ECM[208]. It controls matrix production through contextual regulation of the expression of ECM proteins[269-271].

TGF-β is the major regulator of extracellular matrix synthesis in human skin[272]. It is known to stimulate dermal fibroblast proliferation and causes fibroblasts and keratinocytes to increase production of the extracellular matrix components such as collagen, elastin, fibrillin, fibronectin, laminin, and integrins, while inhibiting extracellular matrix degradation[187,270,271,273-278].

TGF-β1 and TGF-β2 are potent inducers of collagen gene expression and they regulate type I procollagen gene (COL1A2) transcription via the canonical Smad signalling pathway as well as alternate non-Smad pathways[275,276]. TGF-β1 and TGF-β2 also stimulates the expression of collagen VII, a major structural component of the cutaneous anchoring fibrils[271,277]. Furthermore, TGF-β1 and TGF-β2 may be involved in regulation of elastin expression during foetal development and tissue repair, as well as in pathological conditions[270].

Besides playing a role in matrix production, TGF-β also influences matrix degradation by downregulating the expression of enzymes that degrade the matrix such as interstitial collagenases and plasminogen activator, and by increasing production of protease inhibitors such as PAI-1 (plasminogen activator inhibitor type 1), TIMP-1 (tissue inhibitor of metalloproteinases-1) and TIMP-3[279-282]. Elevated matrix degrading activity was observed in transgenic mice with genetic disruption of TGF-β signalling, underscoring the role of TGF-β signalling in the negative regulation of matrix degrading enzymes[283]. This was further established in primary cultures of human dermal fibroblasts, where TGF-β was demonstrated to abrogate MMP-1 activity[266]. The repression of MMP-1 activity by TGF-β has been demonstrated to be mediated via Smad3[266]. Therefore, the pathological matrix degradation that is characteristic of several diseases may be attributed to aberrant or impeded TGF-β/Smad3 signalling.

TGF-β can also modulate the expression of cell adhesion proteins and their receptors that mediate the interaction of the cells with ECM proteins[284]. As a result of its effects on ECM composition and cell-ECM adhesion, TGF-β plays a role in cell migration, invasion, wound healing and fibrosis[208].

3.5.2 Effects of TGF-β on cell proliferation

The effect of TGF-β on cell proliferation is a significant and considerably researched area, particularly in epithelial cells. TGF-β is a potent regulator of cell proliferation, and its effects depend on the type of the target cells[285]. It inhibits proliferation of

epithelial, endothelial and hematopoietic cells, whereas it stimulates the growth of some mesenchymal cells, such as fibroblasts[285-287]. In epithelial cells, TGF-β exerts its cytostatic effects by downregulation of c-*myc* and/or the induction of cyclin dependent kinase inhibitors, resulting in cell cycle arrest at the G1 phase[288,289]. This growth inhibition has been demonstrated to be Smad3 dependent in epithelial cells[290].

Selective association of TGF-β1 with nonproliferating keratinocytes in the suprabasal layers of the epidermis and its exclusion from the proliferating keratinocytes in the basal layer suggest that it may be a physiological regulator of keratinocyte proliferation[291]. Moreover, keratinocytes in TGF-β1-deficient mice showed a higher proliferative index than the corresponding wild-type cells[292]. Sellheyer et al[293] demonstrated that transgenic mice with expression of TGF-β1 targeted to the epidermis had taut, shiny skin, they were rigid with restricted movement and breathing, and they died within 24 hours of birth. This skin phenotype demonstrates that TGF-β1 overexpression leads to inhibition of both normal skin development and epithelial cell proliferation[293].

In skin keratinocytes, TGF-β1 has been demonstrated to rapidly inhibit c-myc transcription[286,287]. Further, antisense c-myc oligonucleotides inhibit keratinocyte proliferation as effectively as TGF-β1, suggesting that repression of c-myc expression at the level of transcriptional initiation may be essential for TGF-β-induced growth inhibition[3,287,294-296]. It has been demonstrated that TGF-β1 induced growth inhibition involves synthesis or modification of a protein that may interact with the c-myc gene, resulting in inhibition of transcriptional initiation[287]. A few studies have suggested that the protein product of the retinoblastoma gene (pRB), or related proteins may be essential for TGF-β1 suppression of c-myc transcription[286,296,297]. TGF-β1 induced growth inhibition leads to a G1 growth arrest, and this inhibition is reversible[287,296,298]. TGF-β1 arrested growth of normal human keratinocytes within 52 hours and 79% of the growth-arrested cells were in the G0/G1-phase of the cell cycle, a situation that approaches that of the normal epidermis[299].

TGF-β1 also mediates cell cycle arrest through the induction of cyclin dependent kinase (CDK) inhibitors[288]. TGF-β1 treatment or Smad3 over-expression in mouse keratinocytes induced the expression of two CDK inhibitors, p16(ink4a) and

p19(ARF), whereas Smad3 depletion or Smad7 over-expression blocked their induction[288]. Thus, indicating that Smad3 is essential for the induction of $p16^{ink4a}$ and $p19^{ARF}$ by TGFβ1. Furthermore, inactivation of the genetic cdkn2a (ink4a/arf) tumor suppressor locus reduced sensitivity to TGF-β1 mediated cell cycle arrest, suggesting that the loss of TGF-β1 sensitivity may promote tumour development[288].

TGF-β markedly inhibited the growth of keratinocytes in the same manner under low and high Ca^{2+} conditions, suggesting that it is a strong growth inhibitor in both low and high Ca^{2+} environments[295]. These data indicate that growth inhibition of human keratinocytes by TGF-β is direct and not induced by differentiation[295].

In contrast, TGF-β exerts a differential proliferative effect on human dermal fibroblasts, which was found to be concentration-dependent, but isoform-independent[300]. TGF-β regulates the proliferation of normal human skin fibroblasts depending on their developmental origin[285,300]. It strongly inhibits foetal fibroblast proliferation, whereas it stimulates the proliferation of adult fibroblasts[285,300]. Curcumin, a natural product used for wound healing was found to completely abrogate the inhibitory effect of TGF-β1 on human foetal skin fibroblasts, without affecting the stimulatory action on fibroblasts from adult donors, suggesting that the response of foetal and adult normal human skin fibroblasts to TGF-β may be regulated by distinct signalling pathways[285,300]. Moreover, the differential proliferative response of foetal and adult human skin fibroblasts to TGF-β, possibly mirrors the differences in their wound healing responses[300].

Interestingly, TGF-β1 can stimulate proliferation of fibroblasts at low concentration, but inhibit cell proliferation at high concentrations of TGF-β1[301]. The exact mechanism underlying this bi-directional modulation of TGF-β1 in fibroblast proliferation is not known, but c-Ski a major co-repressor of TGF-β signalling is considered to play a role[301]. c-Ski expression decreased at high concentrations of TGF-β1, but increased at low concentrations of TGF-β1[301]. In addition, knockdown of c-Ski abolished the bi-directional role of TGF-β1 on fibroblast proliferation[301].

The growth regulatory effect of TGF-β is vital for tissue homeostasis, and escape from this response is characteristic of many tumour cells and hyperproliferative disorders[291,302].

3.5.3 Effects of TGF-β on apoptosis (programmed cell death)

Depending on the cell type, state of differentiation and cellular context, TGF-β can exert either pro-apoptotic or anti-apoptotic effects. However, in most cases the response is pro-apoptotic[208]. TGF-β induced apoptosis is vital in the removal of damaged or abnormal cells from normal tissues [303].

TGF-β potently induces apoptosis of epithelial cells, including interfollicular and follicular epithelium[304]. TGF-β2 appears to initiate catagen (regression) phase of the mammalian hair cycle via induction of apoptosis in anagen hair follicles [304-307]. It can induce apoptosis in several other cell types as well, and this effect is often accompanied with growth inhibition[308].

3.5.4 Effects of TGF-β on cell differentiation

In addition to being a potent inhibitor of keratinocyte growth, TGF-β can alter the differentiation pathway undertaken by keratinocytes[309]. Many studies have extensively investigated the growth inhibitory effect of TGF-β on keratinocytes. However, its role in regulating keratinocyte differentiation is still poorly defined[310].

Keratinocyte differentiation involves a complex sequence of morphological and biochemical modifications that ultimately terminally differentiate to form the stratum corneum[190]. The existence of a calcium gradient is key to normal epidermal differentiation[311]. The effect of TGF-β on human keratinocyte differentiation is Ca^{2+} dependent, as it enhances keratinocyte differentiation under high Ca^{2+} conditions, but inhibits it under low Ca^{2+} conditions[295]. There were isoforms-specific differences in keratinocyte differentiation[190]. TGF-β1 expression increased during calcium-induced differentiation, whereas TGF-β2 and TGF-β3 expression decreased during

differentiation suggesting that TGF-β1 may be associated with a more differentiated state[190].

Buschke et al[310] demonstrated that impaired Smad signalling impeded epidermal differentiation, resulted in epidermal-to-mesenchymal transition and switched to an alternative differentiation program. Therefore, active Smad signalling is essential for terminal epidermal differentiation[310].

In addition, TGF-β signalling plays an important role in mesenchymal differentiation, and in redirecting epithelial cells into mesenchymal differentiation (Epithelial-Mesenchymal transition, EMT)[312]. TGF-β can either induce or repress differentiation of a cell by switching on or switching off the expression of differentiation marker genes in various cell types[208]. EMT is essential for normal embryonic development and can also be linked with pathological conditions such as cancer and fibrosis in adults[312].

The balance between cell growth and maturation is key to maintaining epidermal homeostasis[310,313]. Hence, TGF-β signalling plays an important role in maintaining homeostasis of the epidermis[299,309].

3.5.5 Effects of TGF-β on the immune system

TGF-β1 is the predominant isoform within the immune system, and is a potent regulator of the immune response[314,315]. In the skin, TGF-β1 is produced by keratinocytes, fibroblasts and resident and infiltrating cells of the immune system[316]. As emphasized in studies on TGF-β knockout mice, TGF-β1 plays a key role in the maintenance of immune homeostasis[182,317]. It can modulate the proliferation, differentiation, and function of most classes of immune cells such as lymphocytes, macrophages, and dendritic cells[224]. It is predominantly considered to be an immunosuppressive molecule[224]. However, it exerts both positive and negative effects on inflammation and immune responses, depending on the cell type, the state of differentiation of the cells and the cytokine mileu[172].

The bipolar effect of TGF-β on cells of the immune system is rather perplexing. At the early stages of an immune response TGF-β is released from local platelet stores and acts as a potent chemoattractant[316]. Subsequently, it also plays a role in resolution of inflammation by downregulating the inflammatory response through the inhibition of proliferation and inhibition of cytokine production by activated cells and by promoting apoptosis[267]. TGF-β inhibits T-cell proliferation by inhibiting IL-2 production via Smad3 and by downregulating the expression of cell cycle regulators such as cdk and c-myc[267,318,319]. TGF-β also antagonises IL-1 dependent T-lymphocyte proliferation and possibly exerts profound immunosuppressive effects on lymphocyte function in healthy human skin[320,321]. Furthermore, TGF-β inhibits differentiation of effector T-cells by inhibiting the expression of transcription factors T-bet and GATA-3[322,323]. TGF-β also inhibits B-cell proliferation and differentiation[315]. Apart from inhibiting the function of inflammatory cells, TGF-β also suppresses immune responses by promoting the function of regulatory T-cells (Treg cells, also known as suppressor T-cells) by inducing the expression of Foxp3 (forkhead box P3)[324,325]. Foxp3 suppresses the function of activated T-cells by inhibiting the expression of pro-inflammatory cytokines such as IL-2, IFN-γ, and IL-4[326]. In addition, to inhibiting pro-inflammatory cytokines it also promotes the production of the anti-inflammatory cytokine, IL-10[327].

In contrast, TGF-β in combination with IL-6/IL-21 induces the differentiation of pro-inflammatory Th17 cells via Smad2[328]. However, the differentiation of Th17 cells is highly context dependent as it is impeded by several cytokines[315]. It has also been suggested that TGF-β is not directly required for Th17 cell differentiation, and that its inhibition of Th1 and Th2 differentiation indirectly promotes the differentiation of Th17 cells[329]. Recent studies on human cells indicate that murine Th17 cell differentiation differs from that in human Th17 cells[330,331]. These studies demonstrated that the cytokine combination essential for the priming of human Th17 cells differ from those needed to prime murine Th17 cells[330,331]. In humans, TGF-β was shown to inhibit Th17, Th1 and Th2 differentiation[331]. Further studies are required to clarify the role of TGF-β in Th17 cell differentiation.

As with the TGF-β knock-out models, the Smad3 knock-out mice have also demonstrated immune dysregulation[241]. The T-cells in Smad3 knock-out mice were demonstrated to have an activated phenotype and a reduced responsiveness to TGF-

β[241]. Furthermore, in a murine model of contact dermatitis, Smad3 deficiency enhanced the expression of pro-inflammatory Th2 and Th17 type cytokines[332]. These findings suggest that the immunosuppressive effects of TGF-β may be mediated via Smad3.

3.6 Role of TGF-β in wound healing

From the abovementioned data it is clear that TGF-β plays an important role in maintenance of epidermal homeostasis. Wound healing is one of the major homeostatic mechanisms that attempts to restore the structural and functional integrity of tissues[285]. In collaboration with several other growth factors and cytokines, TGF-β is involved in the different stages of wound healing and affects all participating cell types[333,334]. Following injury, TGF-β1 is released within wound tissues by keratinocytes, platelets, monocytes, macrophages and fibroblasts, and exerts many important functions, including reepithelialisation, fibroblast proliferation, wound contraction, inflammation, angiogenesis, and ECM deposition and remodelling[185,191,301,333-335]. TGF-β1 is also essential for initiating granulation tissue formation[334].

Of the three isoforms, TGF-β1 and TGF-β2 contribute to the healing process by improving tensile strength through an increase in synthesis of collagen and other matrix proteins, whereas TGF-β3 has been shown to reduce connective tissue deposition[189,334]. Consequently, TGF-β1 and TGF-β2 may induce cutaneous scarring, whereas TGF-β3 may prevent scarring[189]. There is a strong induction of TGF-β3 expression at the later stages of wound healing after completion of the proliferative phase, which may be responsible for limiting the fibrotic response[191]. Increased expression of TGF-β3 during the early phase of wound healing could lead to inhibition of epithelial cell proliferation and delay in reepithelialization[191]. Smad3 may impede wound healing as it is involved in the inhibition of re-epithelialization[241,336]. In contrast, Smad4-deficient wounds had delayed wound closure and remodelling[337].

Profound differences exist between foetal and adult wound repair strategies. Foetal skin wounds heal without contraction and scarring, and with a minimal inflammatory response and normal collagen architecture[187,338]. TGF-β1 and β2 play a more prominent role in adult wound healing, whereas TGF-β3 is elevated in foetal

wounds[339]. The mechanism of scarless repair is not completely understood but is considered to involve differential expression of TGF-β isoforms, receptors and signalling mediators, and also involves coordinated keratinocyte-fibroblast cross-talk[187,340,341].

Mucosal wound healing is similar to foetal wound healing in that both are characterised by rapid healing and a lack of scarring[342]. Comparable to foetal wound healing, altered levels of TGF-β1 and -β3 play a key role in the healing of the oral mucosa[342]. Reduced TGF-β1 expression along with an increase in the TGF-β3 to TGF-β1 ratio is observed in oral wounds[342]. Therefore, the TGF-β3 to -β1 ratio appears to determine healing outcomes, with higher ratios leading to scar formation and lower ratios leading to scar reduction[189].

3.7 TGF-β in skin disorders

Despite its potential involvement in the regulation of various physiological processes, TGF-β has also been linked to various pathological conditions. The dysfunction of TGF-β regulation has been demonstrated in developmental disorders, fibroproliferative diseases, cancer, various inflammatory diseases and in autoimmune diseases[343]. Increased or decreased TGF-β activity, due to alterations in the expression or mutations in the genes for the TGF-β isoforms or its signalling components are considered responsible for these conditions.

3.7.1 Cancer

As previously discussed, TGF-β is a potent inhibitor of proliferation of epithelial cells and is responsible for apoptosis and differentiation. Owing to these functions, the elements of the TGF-β signalling pathway play a pivotal role in tumour suppression[236,288,344-346]. Consequently, a deficiency of TGF-β or a lack of responsiveness to TGF-β is associated with hyperproliferation and an increased risk for malignant conversion[292]. Paradoxically, many tumours over-express TGF-β[347]. The general assumption is that TGF-β acts as a tumour suppressor at the early stages of carcinogenesis, but switches to a tumour promoter mode at the late stages of carcinogenesis[344]. This role reversal from a tumour suppressor to a tumour promoter may be attributed to a cellular insensitivity to TGF-β-induced growth inhibition in the later stages[348-350]. Resistance of malignant cells to TGF-β has been linked to aberrant expression or mutations in the receptors, signalling proteins and/or transcription factors involved in the TGF-β pathway[345,346,348,350-352]. After escaping from growth inhibitory constraints, TGF-β facilitates tumour progression through increased angiogenesis, epithelial-mesenchymal transition and by evading immune surveillance[344,347,348,353].

Several reports have shown that TGF-β receptors are downregulated in malignant skin tumours. A decrease in the expression of TGF-βRII and to a lesser extent TGF-βRI has been demonstrated in squamous cell carcinomas (SCC) [230,354-356]. This may be associated with a loss of responsiveness to TGF-β, suggesting that aberrant TGF-βRII expression is a contributing factor to the pathogenesis of SCC. The expression of TGF-βRI and TGF-βRII also correlated with the level of differentiation of SCC, as the expression decreases when the tumours become more aggressive and less differentiated[230,354]. Similar alterations in TGF-β receptors have also been described in other malignant skin tumours such as basal cell carcinoma, dermatofibrosarcoma, cutaneous T-cell lymphoma and Kaposi's sarcoma [350,357-359]. In addition to the TGF-β receptors, mutations or alterations in the expression of the Smads and other downstream mediators of TGF-β have also been identified in skin tumours[346,359]. In contrast with the above mentioned tumours, no aberrations in the TGF-β signalling mediators have been identified in melanoma that could explain their resistance to the growth inhibitory activity of TGF-β[347,360]. One potential explanation for specific escape from the antiproliferative activity of TGF-β is that melanoma cells express high

levels of both c-Ski and SnoN, which are negative regulators in the TGF-β signalling pathway [351,361].

The dual role of TGF-β in carcinogenesis is still not completely understood. Its effects are complex and may depend on several factors such as the type and the stage of the tumour, the genetic makeup of the cells, and the tumour microenvironment[355,362]. But the general paradigm is that in early tumour stages, transformed epithelial cells are usually sensitive to TGF-β-mediated growth inhibition and in later stages the epithelial cells frequently escape from TGF-β growth control and TGF-β may act as a tumour progression promoter[362].

3.7.2 Scarring and Fibrosis

Both positive and negative influences of TGF-β1 on wound healing have been reported[333]. Excessive and prolonged TGF-β1 at the wound site does not benefit wound healing, but may lead to dermal scarring and fibrosis[333,335]. TGF-β1 and -β2 are considered to be profibrotic, whereas TGF-β3 is thought to reduce scarring by promoting ordered dermal remodelling[189,363].

TGF-β has been linked to fibrotic diseases such as keloids, hypertrophic scarring, and scleroderma. Overproduction of profibrotic TGF- β1 and -β2 can result in excessive deposition of scar tissue and fibrosis, whereas TGF-β3 is thought to reduce scarring[189]. The TGF-β signalling components (TGF-β isoform, receptor, Smad) and collagen type I have increased expression with increasing gestational age in keratinocytes[187].

A number of studies have reported that the expression of TGF-β1 and TGF-β2 are elevated in keloid fibroblasts, while the expression of TGF-β3 was downregulated [188,364-366]. In addition to the ligands, TGF-β receptors (types I and II) and Smad3 phosphorylation were increased and Smad7 expression was decreased in keloids, suggesting that increased TGF-β signalling has a potential profibrotic role in keloid pathogenesis[364,367]. Also, TGF-βRI/TGF-βRII ratio was increased in keloid fibroblasts and these keloid fibroblasts show a unique sensitivity to TGF-β ligand stimulation[366]. In hypertrophic scars, the expression of TGF-β1, TGF-β2, TGF-βRI and TIMP-1 were

increased, whereas expression of TGF-β3 and TGF-βRII were decreased when compared with normal skin[368-370]. These findings suggest that TGF-β signalling has a pivotal role in the pathogenesis of keloids and hypertrophic scars. However, genetic studies performed to date have not found any association between TGF-β polymorphisms and the risk of keloid and hypertrophic scar formation[371,372].

TGF-β has also been linked to the pathogenesis of scleroderma, a generalized or localized connective tissue disorder characterized by inflammation and fibrosis of the skin and/or other target organs[373-375]. Mast cell counts, particularly degranulating mast cells which secrete TGF-β are elevated in the skin of patients with scleroderma [376,377]. The expression of TGF-β, particularly TGF-β2, and TGF-β receptors are increased and also p300 expression is elevated in lesional tissue suggesting that dysregulated TGF-β signalling may be involved in the pathologic fibrotic process of scleroderma[373,376,378-382].

3.7.3 Impaired wound healing

In contrast, low expression of TGF-β or its mediators may impede the healing process. TGF-β signalling is deranged in the setting of chronic, nonhealing ulcers[383]. TGF-β1 expression is essential for re-epithelialization of human skin wounds[227]. Lack of TGF-β1 expression in chronic wounds may be linked with their delayed or impaired healing response[227,384]. The elevated TGF-β1 expression observed in acute wound healing is absent in chronic non-healing ulcers, whereas TGF-β3 expression is enhanced in chronic ulcers[384]. This may partly contribute to the chronicity of such wounds[384]. Chronicity may also occur as a result of reduced expression of TGF-β receptors (TGF-β1 and TGF-βRII) in chronic ulcers, rendering these ulcers unresponsive to TGF-β1[385,386].

Further, the wound healing defect seen in glucocorticoid-treated animals has been attributed to the reduced expression of TGF-β1, TGF-β2, and TGF-βRII and increased expression of TGF-β3 and TGF-βRI[191]. Therefore, an imbalance in the levels of the elements of the TGF-β pathway may contribute to impaired wound healing responses.

3.7.4 Psoriasis

Psoriasis vulgaris is a chronic inflammatory skin disorder characterized by hyperproliferation of keratinocytes[387]. TGF-β is considered to play a role in the pathogenesis of psoriasis, and this is supported by the fact that TGF-β regulates both keratinocyte growth as well as differentiation[291,388,389]. Apart from affecting keratinocyte growth and differentiation, several other functions of TGF-β may also be modulated by the pathomechanisms of psoriasis. TGF-β1 can impede adhesiveness of T lymphocytes to dermal microvascular endothelial cells, so reduction of its signalling may lead to lymphocyte infiltration into psoriatic plaques[390]. Also, TGF-β inhibits the acanthotic and degenerative effects of TGF-α and downregulates proliferative IL-2 signalling[387,389,391]. Moreover, calcipotriol, UV-B treatment and peptide T used in the treatment of psoriasis, induce the expression of TGF-β[392-394]. Thereby, suggesting that the antiproliferative and anti-inflammatory effects of TGF-β may partly contribute to the resolution of psoriatic lesions by these treatment modalities[392-394].

Dysregulation of TGF-β signalling in psoriasis has been reported in many studies[180,190,192,291,387,392,395-397]. The expression of TGF-β isoforms, Smads and particularly TGF-β receptors (TGF-βRI and II) have been reported to be decreased in the psoriatic epidermis[190,192,392,396,397]. Thus, suggesting that attenuated TGF-β-signalling contributes to development of psoriasis. Contrastingly, a few reports reported an increase or no change in TGF-β1 expression in psoriatic skin compared to normal skin[180,291,387,395]. Further, Baran et al[398] reported that there is no association between TGF-β1 polymorphisms and psoriasis susceptibility.

Increased serum TGF-β1 concentration was observed in patients with psoriasis, which correlated significantly with psoriasis area and severity index (PASI)[389,399]. In contrast, TGF-β1 concentration in scales decreased with the degree of the disease severity[389]. Transgenic mice that over-express human TGF-β1 in basal keratinocytes (K5.hTGF-β1 transgenic mice) have been reported as having a psoriasis-like disease, suggesting that TGF-β1 over-expression may play a pathological role in psoriasis[395]. However, the inter-species differences and the fact that the inflammatory skin condition in K5.TGF-β1 transgenic mice is T-cell independent, limits their suitability as a model for the immunopathogenesis of human psoriasis[400,401].

The available data are insufficient to make a definitive conclusion on the role of TGF-β1 in the pathogenesis of psoriasis. Further studies are needed to decide whether or not the TGF-β-Smad signalling pathway is directly involved in the development of psoriasis.

3.7.5 Atopic dermatitis

Atopic dermatitis is a chronic, pruritic and eczematous inflammatory skin disorder[402]. A few studies have shown that low expression of TGF-β is implicated in atopic dermatitis pathophysiology[402-406]. Deficiency of TGF-β1 in the skin possibly leads to a cutaneous immune response characteristic of this condition[403]. Atopic dermatitis is linked with a TGF-β1 genotype known to be associated with lower production of this cytokine[403]. In addition, the mRNA expression of TGF-β was significantly lower in peripheral blood mononuclear cells of atopic dermatitis patients in comparison to controls[404]. Gambichler et al[405] observed significantly decreased levels of Smad3 and Smad4 in skin of atopic dermatitis patients, when compared to healthy skin of controls. Interestingly, the Smad3/4 levels significantly increased after narrow band UVB phototherapy and this correlated with a significant improvement of the skin lesions[405]. Furthermore, an immunohistochemical study demonstrated reduced TGF-β staining in lesions of atopic dermatitis compared to that of healthy controls[406]. Treatment of these lesions with tacrolimus, increased the expression of TGF-β to levels observed in the normal skin[406]. In contrast to the abovementioned reports, one study showed that TGF-β and its receptors were significantly expressed in lesional skin of atopic dermatitis[407]. However, a majority of the studies have demonstrated a lower expression profile for TGF-β in lesions of atopic dermatitis. These findings were further supported by studies that suggested that the TGF-β/Smad pathway may play a role in the resolution of atopic dermatitis[402,406,408-410]. More on this aspect will be discussed in the therapeutics section of this chapter.

3.7.6 Photoaging

Skin aging results from the disintegration of cutaneous collagenous networks, chiefly by reduction in the expression of type I collagen, the most abundant protein in the dermis [411,412]. Photoaging in the skin is caused by chronic ultraviolet (UV) irradiation from the sun[413]. UV irradiation reduces production of type I procollagen (COLI)[414]. TGF-β/Smad pathway is the major regulator of type I collagen synthesis in human skin[412,414]. UV irradiation attenuates TGF-β/Smad pathway in human skin by downregulation of TGF-βRII and Smad3/4 and by upregulation of Smad7[229,272,414,415]. This prevents downstream signalling and consequently reduces expression of type I procollagen[229,272,414,415]. These findings suggest that there is a lower responsiveness to TGF-β in photoaged skin.

3.7.7 Hair disorders

TGF-β signalling to a certain extent contributes to male pattern baldness[416]. Male pattern baldness is the result of premature entry of hair follicles into catagen due to androgens[416]. This involves dihydrotestosterone (DHT) induced synthesis of TGF-β2 in dermal papilla cells, which in turn suppresses epithelial cell proliferation and promotes activation of the intrinsic caspase network, resulting in apoptosis of the epithelial cells [416]. Thus, contributing to the shortening of the human hair cycle[416].

Retinoid-induced telogen effluvium is one of the most frequent adverse effects of treatment with systemic retinoids (isotretinoin, acitretin)[417]. TGF-β may act as a mediator of retinoid-induced hair growth inhibition[417]. All-trans retinoic acid (ATRA) can induce a catagen-like stage in human hair follicle and this involves premature upregulation of TGF-β2 in the dermal papilla[417]. TGF-β2 neutralizing antibody partially abrogated the hair growth-inhibitory effects of ATRA[417].

Alterations/ dysregulation in TGF-β signalling has been reported in several other skin conditions as well, a few of which are rhinophyma, acquired reactive perforating collagenosis, leprosy, discoid lupus erythematosus and aplasia cutis congenita[418-422]. In all these conditions a disruption in the TGF-β signalling pathway was involved, underscoring the importance of this pathway in maintaining the homeostasis of the skin.

3.8 TGF-β based treatment approaches

Taking into consideration the fact that the components of the TGF-β pathway are altered in disease states, several studies looked into their role as potential therapeutic targets. In conditions where there was insufficient TGF-β activity, exogenous TGF-β or treatments that enhance its signalling were looked into, and in conditions of TGF-β over-activity, blocking agents for this pathway were investigated. This section will look at both novel and currently used drugs targeting this pathway.

3.8.1 Therapeutic agents that antagonize TGF-β activity

Fibroproliferative diseases such as keloids, scarring and scleroderma that express excessive TGF-β and several late stage tumours that over-express TGF-β are potential candidates for TGF-β blocking therapies.

Several TGF-β antagonising drugs such as neutralizing antibodies, soluble TGF-β receptors, antisense oligonucleotides, small interfering RNA (siRNA), serine/threonine kinase inhibitors and inhibitors of Smad transcriptional activation are currently being developed to treat these conditions[189,287,423-425]. Encouraging results have been obtained for these agents in animal models and *in vitro* studies [189,287,423-425]. In addition, current treatments such as UVA phototherapy used in the treatment of fibrotic skin conditions resulted in significant downregulation of TGF-β[272,426].

Some studies have described the effects of peroxisome proliferator-activated receptor-γ (PPAR-γ) on connective tissue homeostasis and suggested that they may have a beneficial effect on dermal scarring and fibrosis[427-430]. Troglitazone, a PPAR-γ agonist significantly decreased the expression of connective tissue growth factor (CTGF), TGF-β1 and collagen I in skin fibroblasts[428,429]. Therefore activation of PPAR-γ may represent a novel therapeutic approach to target profibrotic responses of TGF-β by abrogating TGF-β-induced stimulation of collagen gene expression[427,430].

Similarly, in patients who have TGF-β-producing tumours, blocking TGF-β activity may induce an immune response and impede metastasis[178].

As previously highlighted, suppression of TGF-β2 activity is considered to inhibit the transition from anagen to catagen and to prolong the anagen phase[417,431]. Taking this into consideration, Sasajima et al[431] investigated the effect of trans-3,4'-Dimethyl-3-hydroxyflavanone (t-Flavanone), a hair growth enhancing compound on TGF-β levels in cocultures of human hair papilla cells and human keratinocytes. They demonstrated that t-flavanone suppresses TGF-β2 activation and therefore suggested that it is likely to be beneficial in alopecia[431].

3.8.2 Therapeutic agents that enhance TGF-β activity

The potential of TGF-β to promote healing and its potent immunosuppressive effects provides rationale for the use of TGF-β ligands or agents that enhance its activity in the management of several diseases. Diseases associated with insufficient TGF-β activity, such as impaired wound healing, some inflammatory diseases and autoimmune diseases may be amenable to these therapeutic strategies.

Exogenous TGF-β application improves the rate of healing and wound strength in animal models of impaired healing, such as glucocorticoid-treated animals[191]. In addition, administering treatments that enhance TGF-β activity such as human placental extracts or aloe vera improved the wound healing response[432,433]. In contrast, the topical application of TGF-β1 on the healing of chronic ulcers was ineffective[385]. Reduced responsiveness due to the disruption of TGF-β receptors or signalling mediators may be responsible for the inefficacy of exogenous TGF-β in chronic wounds[385,23].

As opposed to PPAR-γ, PPAR-δ induces TGF-β1 expression[434]. PPAR-δ plays an important role in cutaneous wound healing by accelerating ECM-induced cellular interactions via TGF-β1/Smad3 signalling-dependent or -independent pathways[434]. In a mouse model, administration of a PPAR-δ ligand promoted wound closure and

significantly increased the expression of collagen types I and III, phosphorylated Smad3 and TGF-β1[434].

TGF-β3 is a key regulator of the scar-free healing of foetal and mucosal wounds[341,342]. In a murine model of skin wounding, localized intradermal transduction of active TGF-β3 reduced scar tissue formation by reducing the re-epithelialization density and myofibroblast transdifferentiation within the wound area[363]. Moreover, intradermal injection of exogenous TGF-β3 following cutaneous wounding in adult rats promoted regeneration of normal skin and reduced scarring[189]. The scar-reducing potential of avotermin (Juvista; Renovo, Manchester, UK) a recombinant, active, human TGF-β3 has been demonstrated in pre-clinical and human Phase I and II clinical trials[435,436]. It restores the dermal architecture to a state that more or less resembles normal unwounded skin[435,436]. The treatment was safe and well tolerated in humans[436]. It is currently being evaluated in Phase III trials[437].

The potent immunosuppressive effects of TGF-β make it a potential therapeutic agent in the treatment of inflammatory (e.g. psoriasis and atopic dermatitis) and autoimmune diseases (e.g. DLE) with low TGF-β activity[420]. Interestingly, standard treatments such as calcipotriol and UVB phototherapy and others like peptide T that have been shown to improve psoriasis, induce the expression of TGF-β[392-394]. Studies that correlate the activity of TGF-β to the clinically observed improvement in response to these treatments are required to determine its exact role in psoriasis.

Sumiyoshi et al[408] demonstrated that in HaCaT cells, TGF-β1 inhibited the production of IFN-γ and TARC/CCL17 (thymus and activation regulated chemokine), a TNF-α-induced Th2 chemokine which is known to be upregulated in lesional atopic dermatitis skin. This effect was mimicked by the overexpression of Smad2/3, suggesting that the TGF-β/Smad signalling pathway may play a role in the resolution of atopic dermatitis. Furthermore, in a mouse model of atopic dermatitis, subcutaneous injection of recombinant TGF-β1 suppressed eczematous skin lesions with attendant reduction of serum immunoglobulin E (IgE) levels[402]. Histological analysis showed that TGF-β1 significantly inhibited the infiltration of mast cells and eosinophils into the skin of these mice[402]. These results suggest that TGF-β1 may have a therapeutic potential in

atopic dermatitis[402]. Studies on humans are needed to confirm these findings and rule out interspecies differences.

In addition, the findings from several studies have suggested that the upregulation of TGF-β1 probably contributes to the therapeutic efficacy of tacrolimus ointment[406,409,410,438,439]. These studies have proposed that tacrolimus-induced TGF-β1 may act at several levels such as by producing Treg cells, regulating langerhan cell development and function, and by inhibiting IgE-dependent mast cell activation and dampening mast cell-mediated inflammatory responses [406,409,410,438,439]. Further studies are warranted to validate these findings.

In addition to all of the above, TGF-β also has a role in photorejuvenation[411,440,441]. Therapeutic modalities used for photoaging such as superficial dermabrasion, α-Lipoic acid (α-LA) and topical creams incorporating TGF-β1 in them, enhances type I and type III collagen synthesis in the papillary dermis through the activation of Smad signalling by a TGF-βRI kinase-dependent pathway[411,440,441]. The resultant TGF-β1 induced remodelling improves the visual appearance of the skin[441].

3.9 Summary

The TGF-β signalling pathway is a tightly controlled pathway that plays a regulatory role in a multitude of biological effects. The various factors that may influence these effects have been depicted in **Figure 3.6**.

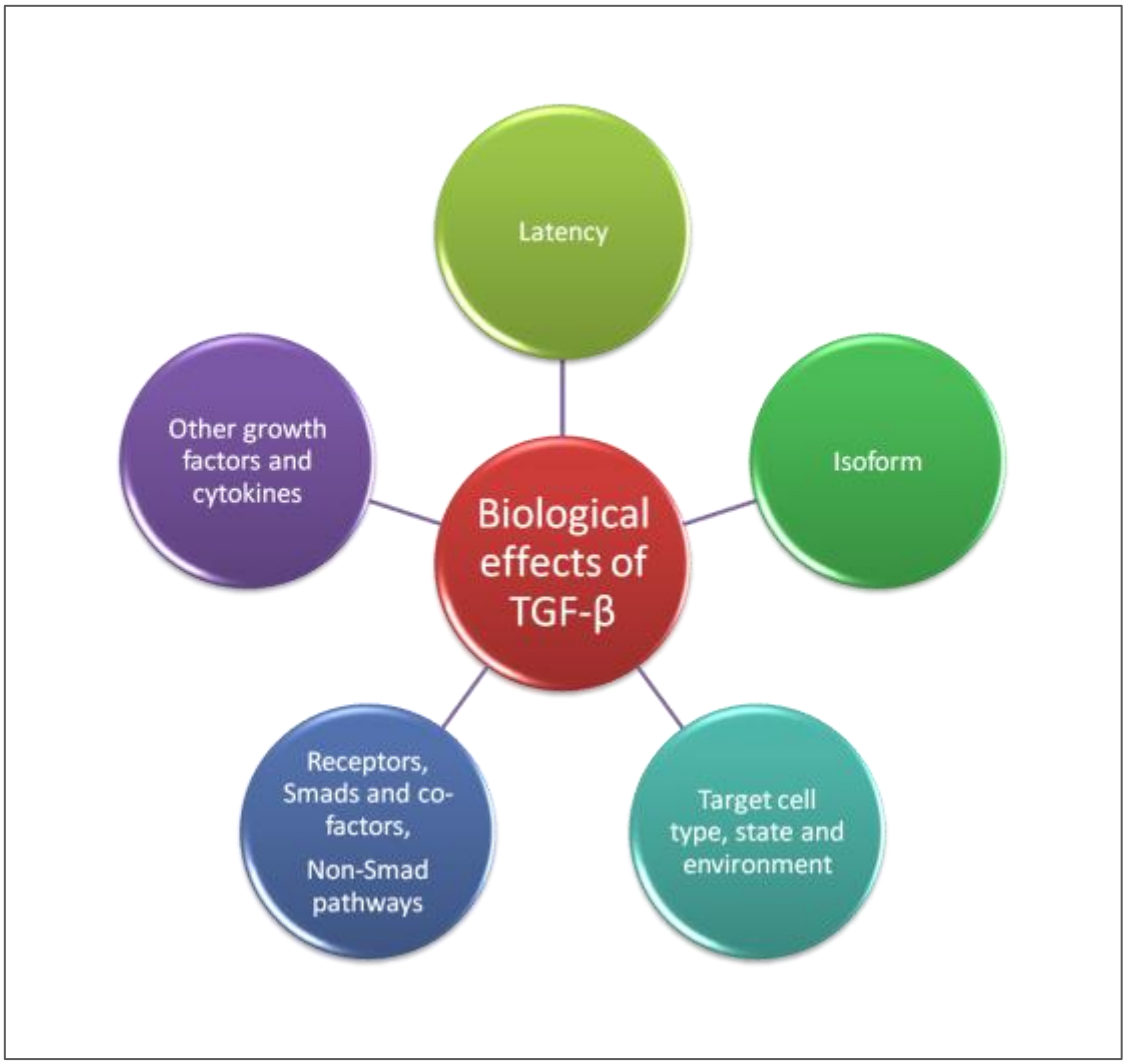

Figure 3.6 Factors influencing the biological effects of TGF-β

Disruption in the TGF-β signalling pathway in the various pathological skin conditions underscores the importance of this pathway in maintaining the homeostasis of the skin. Several new and promising treatments targeting this pathway are currently being developed. Although, these treatments may alleviate the pathology of the concerned diseases, considering the TGF-β pathway as a therapeutic target is questionable in view of its diverse biological role in a variety of cellular functions. Blocking or enhancing

TGF-β to contain the detrimental effect, may in turn disrupt the other normal homeostatic actions of TGF-β. The surrounding normal cells or tissues may also be affected by these therapeutic agents leading to undesirable consequences. Local, as opposed to systemic treatment is more favourable in this context. However, localizing the desired effect to only the pathological tissue or cell type is challenging and warrants the development of targeted drug delivery systems. As a result of these complex issues, the use of TGF-β based therapeutic strategies should be cautiously approached and their long term effects should be thoroughly evaluated.

This chapter attempted to summarise the TGF-β biology and its association with skin diseases so as to have a better understanding of this molecule and its correlation with the skin.

Chapter 4: TGF-β and Acne Vulgaris

A few studies have suggested that the upregulation of TGF-β may be linked to the resolution of inflammatory acne lesions. TGF-β may contribute to acne resolution by reducing inflammation and by inhibiting keratinocyte proliferation[110].

Furthermore, Downie et al[442] conducted an *in vitro* study to determine the effects of TGF-β1, TGF-β2 and TGF-β3 on the proliferation and differentiation of human sebaceous glands[442]. They demonstrated that TGF-β2 and TGF-β3 significantly inhibited sebaceous gland function by inhibition of lipogenesis and cell proliferation in sebaceous gland organ cultures[442]. Based on these findings, the authors suggested that TGF-β may mediate the effects of anti-acne agents such as retinoic acid on sebaceous glands[442]. This section explores the purported association between TGF-β signalling and some of the currently used treatments and other potential agents used in the treatment of acne vulgaris.

4.1 Retinoids and TGF-β in the skin

Retinoids as systemic or topical agents are clinically important therapeutic modalities for several dermatologic disorders, such as acne vulgaris, psoriasis, ichthyosis, and palmoplantar keratoderma[417,443]. It is also used for the chemoprophylaxis of skin cancers[444]. In the skin, retinoids have been shown to suppress sebum production, sebocyte proliferation, and keratinization, and to enhance keratinocyte differentiation and ECM production, and to exert anti-inflammatory effects[417,444]. The underlying mechanisms through which retinoic acid exerts its effects on the abovementioned skin disorders are unknown[445].

Retinoids and glucocorticoids are known to have a potential to modulate the expression of TGF-β[444]. Several studies have shown a notable overlap in the biological actions of retinoic acid and TGF-β, particularly on epithelial cell proliferation and ECM regulation[446]. Thus, raising the possibility of significant interactions between these two molecules[446].

The first evidence for the interesting link between retinoic acid and TGF-β in the skin was uncovered by Glick et al in 1978[447]. This group explored the interaction between topical retinoic acid and TGF-β in a mouse model, and found that topical retinoic acid induced expression of TGF-β2 mRNA and protein in cultured mouse keratinocytes (*in vitro*) and in intact mouse epidermis (*in vivo*). The upregulated TGF-β2 was in the biologically active form, suggesting that the TGF-β induced by retinoic acid *in vivo* is functional. Blocking antibodies to TGF-β2 partially reversed (~30%) the ability of retinoic acid to inhibit keratinocyte DNA synthesis in cultured keratinocytes[447]. Nuclear run-on transcription experiments showed that the retinoic acid-induced increase in TGF-β2 peptide and transcripts occurred through a posttranscriptional mechanism[447]. The authors concluded that the antiproliferative effect of topical retinoic acid is at least partially mediated through the induction of active TGF-β2, and that retinoids may utilize this mechanism in the control of proliferative skin diseases and in the prevention of cancer[447]. Extending these findings to human tissue, a subsequent study showed that all-trans retinoic acid enhances the inhibitory effect of TGF-β on DNA synthesis and cell growth in human epidermal keratinocyte cultures as well[448].

Using the vitamin A-deficient rat as a model, Glick and colleagues[449] immunohistochemically analysed the expression of different TGF-β isoforms under conditions of vitamin A deficiency and also with systemic administration of retinoic acid. Expression of all three isoforms was minimal in the vitamin A-deficient rat[449]. However, treatment with retinoic acid induced a rapid and transient increase in the expression of TGF-β1, TGF-β2 and TGF-β3 in the epidermis[449]. The specificity of the staining was confirmed by using blocking peptides to each of the isoforms[449]. The increase in expression of TGF-β isoforms was mostly limited to the epidermis with negligible change in the dermis[449]. In comparison to the initial study by Glick and colleagues[447], increase in the expression of the TGF-β1 isoform was observed in this study, which may be attributed to either a species specific response or a difference in the route of drug delivery[449].

Subsequently, Fisher and co-researchers[450] investigated the effects of topical retinoic acid on the expression of TGF-β1 and TGF-β2 in human skin and in cultured human keratinocytes. Human subjects were treated with Retin-A cream (0.1% retinoic acid) for four days under occlusion. Immunoreactivity and mRNA levels for TGF-β1 and TGF-β2 were measured[445,450]. Immunohistochemistry revealed increased expression of TGF-β1 in retinoic acid-treated skin compared to vehicle-treated skin[450]. Epidermal TGF-β1 expression was most marked in the suprabasal layers of the epidermis[450]. No changes were detected in its mRNA level, suggesting that a post-transcriptional mechanism may be responsible for the enhanced expression of TGF-β1 protein[450]. Treatment of cultured adult human keratinocytes with retinoic acid resulted in a 50% increase in TGF-β1 expression[450]. Contrary to the results obtained in the study by Glick et al[447] that used a mouse model, no detectable change in the expression of the TGF-β2 isoform was noticed in retinoic acid-treated human skin or cultured adult human keratinocytes[450]. This could probably be attributed to interspecies variation[450]. The TGF-β1 and TGF-β2 expression patterns observed in retinoic acid-treated skin were also observed in skin treated with the irritant sodium lauryl sulphate, suggesting that the TGF-β1 modulation induced by retinoic acid was non-specific and may be related to topical irritation[450]. However, mucin deposition, which is TGF-β-induced, was specifically elevated in retinoic acid-treated skin, but not in sodium lauryl sulphate-treated skin[450].

More recently, Leivo et al[444] examined the effect of oral isotretinoin on the expression of TGF-β1 and TGF-β2, in suction blister fluid and serum obtained from acne patients. A statistically significant 19% increase ($p = 0.037$) in suction blister fluid TGF-β1 was observed after six weeks of isotretinoin treatment[444]. In contrast, betamethasone-17-valerate treatment caused a statistically significant 17% decrease in suction blister fluid TGF-β1[444]. Isotretinoin had no affect on the serum TGF-β1 and TGF-β2 levels[444]. Increase in suction blister fluid TGF-β1 with no change in serum TGF-β1 after isotretinoin treatment, suggests that isotretinoin probably mediates its cutaneous effects via local interstitial fluid TGF-β1 modulation[444]. Future studies need to determine whether the locally induced TGF-β has a role in isotretinoin-mediated acne resolution by correlating its levels with the clinical manifestations.

Increased FGFR2 signalling has been linked with the development of acne in Apert syndrome and unilateral acneiform nevus and has recently been hypothesized to be involved in acne pathogenesis[29]. In the scenario that this signalling pathway plays a role in acne, agents that attenuate FGFR2 signalling may be effective in treating acne, and anti-acne drugs that have been found to be effective may potentially act in this manner. TGF-β signalling has been demonstrated to suppress downstream FGFR-signalling in fibroblast cultures by inducing the regulatory protein *Sprouty*, an important FGFR antagonist[451]. Moreover, all-trans-retinoic acid is also reported to attenuate increased FGFR2b signalling via upregulation of Sprouty[452]. It would be interesting to investigate whether the retinoic acid-induced *Sprouty* upregulation is mediated via TGF-β. Further research on the role of FGFR2 signalling in acne pathogenesis and treatment is needed to validate these findings.

As mentioned in the previous chapter, all-trans retinoic acid via a premature upregulation of TGF-β2 in the dermal papilla can induce a catagen-like stage in human hair follicles[417]. Furthermore, topical retinoic acid enhances collagen gene expression in photodamaged skin[453]. In the epidermis of UVB-irradiated hairless mice, retinoic acid induced an increase in TGF-β1 expression, and to a lesser extent TGF-β2 expression without any associated changes in mRNA levels[453]. Retinoids also rectify steroid-impaired healing by restoring TGF-β and IGF-I levels, which consequently stimulates collagen production[454]. Therefore, the effect of retinoids on photodamaged skin or dermal repair may be mediated through TGF-β produced by the epidermal cells, which in turn stimulates dermal fibroblasts to synthesize collagen[453,454]. In addition to enhancing collagen biosynthesis, retinoids can correct impaired homeostasis of dermal tissue by interacting synergistically with TGF-β to stimulate the production of tissue inhibitor of metalloproteinases and to a lesser extent by downregulating collagenase production in human skin[455,456]. Neutralising antibodies to TGF-β can block these responses[456].

Moreover, retinoic acid enhances TGF-β/Smad3 signalling and this induces Foxp3 (Forkhead box p3) expression, thus promoting the conversion of naive $CD4^+$ T-cells into potent suppressive $Foxp3^+$ Treg cells[457-460]. Further, retinoic acid signalling through RAR receptors in the T-cell suppresses the inhibitory effects of pro-inflammatory cytokines, such as IL-6/IL-21/IL-23, on the TGF-β mediated Foxp3

induction[457,460]. Thus, retinoic acid suppresses pathogenic IL-6/IL-21/IL-23-driven Th17 signalling and enhances suppressive Treg cells by inducing TGF-β/Smad3 signalling[460].

Retinoids have also shown potential as chemoprophylactic agents[461]. A synthetic retinoid, N-4-hydroxyphenyl retinamide (4-HPR) induced apoptosis in immortalised human epidermal keratinocytes, whereas this effect diminished in the more aggressive tumour cell lines[462]. Interestingly, TGF-β-induced growth inhibition was also reduced in the more aggressive cell lines[462]. 4-HPR was also demonstrated to increase the amount of active TGF-β in the culture medium[462]. Blocking of TGF-β signalling attenuated 4-HPR-induced apoptosis, whereas addition of TGF-β1 and TGF-β2 enhanced 4-HPR-induced apoptosis and growth inhibition[462]. Thus suggesting that the chemopreventive action of 4-HPR is modulated by TGF-β[462]. Furthermore, using an in vitro model of cancer progression (human keratinocytes immortalized by HPV16 DNA, HKc/HPV16), it was demonstrated that retinoic acid treatment of HKc/HPV16 resulted in a dose-and time-dependent induction (up to 3-fold) of TGF-β[461]. Similar to the previous study, the loss of growth inhibition by retinoic acid paralleled the loss of TGF-β sensitivity in this study as well[461]. In addition, retinoic acid-treated tumours were demonstrated to express higher levels of TGF-β1[463]. These studies suggest that retinoic acid may prevent tumour progression via TGF-β induction, but tumours resistant to retinoic acid may have altered sensitivity to TGF-β signalling[446,461-464].

All these findings suggest that TGF-β may be a local mediator of retinoid action in the skin. However, the mechanism of interaction between these two regulatory molecules is largely unknown[454,465]. Retinoids could regulate TGF-β at several levels of its signalling, including modulation of TGF-β receptor expression or sensitivity, activation of latent TGF-β, phosporylation of Smad3 or by enhancing mRNA stability or processing[446,454,465-467]. The lack of an identifiable retinoid response element in the promoter region of the TGF-β genes, supports the interpretation gathered from the results of nuclear run-on experiments that this interaction is post-transcriptional rather than transcriptional[417,447,465]. Hence, the elevated TGF-β expression may result from an increased half-life of the TGF-β mRNAs and from increased translational efficiency[454]. Furthermore, a significant proportion of the TGF-β induced by retinoids is in the biologically active form, which has a comparatively shorter half life than its latent

form, suggesting that the retinoid-induced TGF-β may be limited to local action at the site of production[220,221,466]. In certain cases, this can be correlated with the ability of retinoids to increase the expression of transglutaminase, one of the elements involved in the activation of latent TGF-β[454,468].

Although TGF-β isoforms have been reported to be induced by retinoids in the skin (*in vitro* and *in vivo*) of animal and human subjects[445,447,449,450], these molecules have several independent effects as well[313,469]. Downregulation of TGF-β2 has been reported in mouse embryos with retinoic acid-induced malformations[443]. Furthermore, in some cases retinoids were shown to decrease or not induce TGF-β at all in keratinocyte cultures[469,470]. These discrepancies could be explained by the differences in species, cell type, culture condition or the type of retinoid used in these studies. Further studies are required to determine the effects and the mechanism for the complex interactions between TGF-β and retinoids in skin.

Up to now only the study by Leivo et al[444] has examined the association between retinoids and TGF-β in acne patients. Further research concentrating on its role in acne is warranted.

In addition, future studies should clarify whether the induction of TGF-β is a non-specific effect of retinoid treatment or whether it actually mediates the therapeutic effects of retinoids in acne vulgaris.

4.2 TGF-β and other acne treatment modalities

A randomised, prospective, split-face, double-blind, vehicle-controlled trial was recently conducted to evaluate the clinical efficacy and safety of 1% nadifloxacin cream (a fluoroquinolone with broad-spectrum antibacterial activity) in the treatment of mild to moderate facial acne in thirty-four Korean patients[471]. In addition, the histopathological changes (haematoxylin and eosin staining; IL-8 and TGF-β immunostaining) after nadifloxacin treatment were evaluated[471]. All participants were treated with 1% nadifloxacin cream on one-half of the face and vehicle cream on the contralateral side, twice daily for eight weeks[471]. At the end of the treatment period, inflammatory lesions were reduced by 70% on the nadifloxacin-treated skin compared

to the 13.5% increase in the vehicle-treated skin. Non-inflammatory acne lesions were reduced by 48.1% in nadifloxacin-treated skin and by 10.1% in vehicle treated skin[471]. In comparison with the vehicle-treated skin, the nadifloxacin-treated skin demonstrated significant reductions in inflammatory reaction and the expression of IL-8[471]. These findings corresponded with the treatment response[471]. However, no statistically significant changes were detected in TGF-β expression after eight weeks of treatment. In keeping with the early and transient action of TGF-β, its upregulation may have been initiated during the first few weeks of nadifloxacin treatment[471]. However, in this study the post-treatment biopsy was obtained after eight weeks of commencement of treatment, which may have been too late to observe TGF-β changes[471].

Inhibitors of dipeptidyl peptidase IV (DP IV) and aminopeptidase N (APN), a promising therapeutic strategy for acne was shown by Thielitz and co-researchers[472] to suppress T-cell proliferation and IL-2 production and enhance the expression of the TGF-β1 in *P. acnes*-stimulated peripheral blood mononuclear cells (PBMC). TGF-β/Smad3 signalling is known to inhibit T-cell proliferation by inhibiting IL-2 production and by downregulating the expression of cell cycle regulators[267,318,319]. Therefore, it would be interesting to investigate whether the anti-proliferative and immunosuppressive effects of DP IV and APN inhibitors are mediated via TGF-β. Future *in vivo* studies are warranted to corroborate these findings.

Non-ablative laser and light therapy used for the purpose of photorejuvenation was shown to increase cutaneous TGF-β1 expression[473,474]. They have been reported to induce dermal remodelling and neocollagenesis[152]. In addition to their use in photorejuvenation and scarring, non-ablative laser therapies have been increasingly used in recent years for the treatment of inflammatory acne vulgaris [475-477]. The mechanism for their therapeutic effect is unknown, but has been proposed to occur secondary to damage of *P. acnes* and the sebaceous gland[170].

A study by Seaton et al[152] explored the in vivo effects of a short pulse duration non-ablative pulsed-dye laser (NA-PDL) on cytokine production, *P. acnes* colonization density (using a scrub-wash technique and culture at 0 and 24 h) and sebum excretion rate (using absorptive tape at 0, 2, 4, 8 and 12 weeks)[152]. NA-PDL (NliteV; Chromogenex Light Technologies, U.K.) had no effect on *P. acnes* or sebum excretion

rate. Hence, destruction of *P. acnes* or sebaceous glands may not be the explanation for the therapeutic effect of this device in acne vulgaris[152]. To assess the effect on cytokine expression reverse transcription-polymerase chain reaction (RT-PCR) was performed on biopsies obtained from the gluteal skin of eight subjects at 0, 3 and 24 hours following laser therapy[152]. A rapid and significant increase in TGF-β1 mRNA levels was observed in all subjects. An increase in levels of TGF-β1 mRNA was apparent as early as 3 hours after laser therapy, however this was not statistically significant (p = 0·093). A fivefold increase (p = 0.012) was observed after 24 hours with a 15-fold increase in two subjects[152]. TGF-β is known to be a potent stimulus for neocollagenesis and a potent immunosuppressive cytokine[152]. Therefore, the upregulation of TGF-β by NA-PDL may stimulate dermal remodelling and collagen synthesis and may also be involved in the inhibition of inflammation in acne[152]. This study not only offered a potential explanation for the action of laser therapy in inflammatory acne vulgaris, but also provided a foundation for conducting similar studies using other non-ablative laser and light devices. However, the results of this study should be interpreted with caution as this study was conducted on a small sample and the biopsies were taken from an area not normally involved in acne[152]. Moreover, there was no clinical correlation of the obtained results as the study was conducted in healthy adult volunteers[152]. Further studies with different non-ablative systems, larger sample size, in subjects with inflammatory acne lesions and on skin sites involved in acne need to be conducted to corroborate these results.

More recently, Jung and co-workers[170] conducted a 12-week, double-blind, randomised, prospective, split-face trial that compared the efficacy and safety of PDL with combined 585/1,064-nm laser therapy in sixteen patients with mild to moderate facial acne vulgaris. The patients underwent three treatment sessions at 0, 2 and 4 weeks, and were followed up at 8 and 12 weeks[170]. Haematoxylin and eosin staining and immunohistochemical staining for IL-8 and TGF-β were performed on 2-mm punch biopsies taken from treated areas at baseline and at the final visit[170]. Both treatments were found to be effective in treating inflammatory and non-inflammatory acne lesions[170]. On histopathological analysis, the inflammatory reaction and IL-8 immunostaining were found to be significantly reduced (mean staining at baseline=3.00; PDL=2.25, p=0.03; 585/1,064-nm laser =2.00, p=0.01) and TGF-β expression was significantly increased (mean staining at baseline = 1.67; PDL = 2.33,

p = 0.01; 585/1,064-nm laser = 2.08, p = 0.005) following treatment with either of the two approaches. The histopathological findings corresponded with the clinical improvements. Both treatments were demonstrated to be safe and effective in the treatment of inflammatory and non-inflammatory acne lesions of acne vulgaris, with the combined 585/1,064-nm laser being significantly better than PDL in treating non-inflammatory acne. The authors concluded that inflammatory lesions showed greater and more rapid improvements than non-inflammatory lesions for either treatment. However, this study failed to mention which isoform of TGF-β was upregulated. Further research is required to identify the down-stream effects of laser induced TGF-β in the treatment of acne vulgaris.

More recently, Choi et al[110] carried out a randomized split-face, single-blind trial to compare the safety and therapeutic efficacy of PDL and IPL for the treatment of acne vulgaris[110]. Twenty patients with facial acne were randomized to receive a series of four treatment sessions of 585-nm PDL (Cynergy; Cynosure, USA; parameters: 585-nm wavelength, 10-mm spot size, 40 ms pulse duration, 8–10 J/cm^2 energy fluence and two passes) on one side of face and IPL (Ellipse Flex System; DDD, Denmark; parameters: photorejuvenation filter 530–750 nm, 7.5–8.3 J/cm^2 energy fluence, 2.5 ms pulse duration, triple light pulse with a 9.0-ms interval and two passes) on the contralateral side at 2-week intervals[110]. Assessment of lesion counts, acne severity, and subjective scoring of improvement were performed to determine therapeutic efficacy[110]. In addition, a 2 mm punch biopsy of inflammatory lesion was obtained from all patients at baseline and final visits[110]. H&E staining and TGF-β immunostaining were performed on these biopsies[110]. TGF-β staining intensities were graded from 0 (not stained) to 4 (intensively stained)[110]. Both treatments produced clinical improvements by reducing inflammatory and non-inflammatory lesions[110]. For inflammatory lesions, IPL treatment resulted in a rapid and marked improvement, but a rebound aggravation of acne was observed 8 weeks following the final treatment session[110]. In contrast, PDL produced a gradual improvement and this improvement was sustained even at 8 weeks after the final treatment[110]. Reduction of inflammatory reactions and an increase in TGF-β expression was observed with both treatments on histopathological examination[110]. These changes were more prominent for PDL-treated sides (TGF-β immunostaining intensity at 8 weeks after treatment: IPL = 2.5, PDL = 2.9 vs. baseline intensity of 2.2). However, the differences were not statistically

significant[110]. The greater induction of TGF-β with PDL may be attributed to a more pronounced heat shock response via a greater photothermal effect[110]. Both PDL and IPL were found be effective and safe for the treatment of facial acne vulgaris[110]. Overall, PDL produced better results and showed a more sustained effect[110]. The authors suggested that the observed clinical improvement and decrease in inflammatory reaction after either treatment may be a result of the immunomodulatory effect of the photothermally induced TGF-β[110].

4.3 Role of TGF-β in acne resolution

All these findings based on some of the therapeutic modalities used in acne, suggest that TGF-β may have a potential role in the resolution of acne lesions. Further, its inhibitory effect on keratinocyte proliferation may hamper microcomedo formation which occurs as a result of keratinocyte hyperproliferation.

Moreover, TGF-β is a potent immunosuppressive cytokine that inhibits inflammatory cytokine production and promotes resolution of inflammation. This inhibitory action is abrogated in Smad3 deficiency suggesting that the immunosuppressive effect of TGF-β is Smad3-mediated. Smad3 signalling has been shown to play an important role in mediating the antiproliferative effects of TGF-β on keratinocytes and also in mediating its stimulatory effects on ECM by dermal fibroblasts[478-480]. It has also been demonstrated to be critical in mediating the immunomodulatory effects of TGF-β[241,242,336]. In Smad3 deficiency states, immune cells become insensitive to TGF-β mediated inhibition of pro-inflammatory cytokines and chemokines[241,242,481]. Targeted deletion of Smad3 results in a viable mouse that ultimately dies from a progressive illness involving massive inflammation and impaired mucosal immunity at 1–6 months of age[241,242]. These Smad3-knockout mice exhibit immune dysregulation, altered mucosal defence mechanisms, and reduced ECM production[241,336,478]. In a murine model of contact hypersensitivity, Smad3 deficiency resulted in increased pro-inflammatory, Th2 and Th17 type response in the skin and increased neutrophil infiltration into the skin[332,482].

In addition to inflammatory cytokines such as interleukin (IL)-8, matrix degrading enzymes (MMPs) also play a key role in the development of inflammatory lesions of acne vulgaris[81]. Enhanced production of matrix degrading enzymes, particularly MMP-1 and MMP-3 have been implicated in acne pathogenesis[81]. TGF-β has been demonstrated to regulate metalloproteinase and tissue inhibitors of metalloproteinase (TIMP) expression[483]. It is a potent inhibitor of MMP expression and a stimulator of TIMP in mesenchymal cells, particularly dermal fibroblasts[266,455,484,485]. TGF-β suppresses inflammatory cytokine-induced expression of MMP-1 and MMP-3[266,375,455,484-487].

Consistent with the aforementioned findings, Yuan and Varga[266] reported that TGF-β prevented IL-1β -induced MMP-1 gene and protein expression, and ectopic expression of Smad3 or Smad4 reproduced this response in dermal fibroblasts. However, Smad1 or Smad2 were not involved in MMP-1 repression[266]. Furthermore, the inhibitory Smad7 and dominant negative mutants of Smad3 or Smad4 blocked the TGF-β-induced repression of MMP-1 transcription[266]. In Smad3-deficient murine embryonic fibroblasts, TGF-β potentiated MMP-1 expression instead of suppressing it[266]. Overall, these results demonstrate that the TGF-β-induced negative regulation of MMP-1 is mediated through Smad3 and Smad4 in dermal fibroblasts[266]. Thus, TGF-β/Smad3-mediated repression of MMP-1 expression may be important for preventing excessive matrix degradation induced by inflammatory cytokines in acne.

As TGF-β has been demonstrated to inhibit MMP-1 gene expression and inflammatory cytokine production through a Smad3-mediated signalling pathway it would be reasonable to investigate whether IPL used for acne treatment induces TGF-β/Smad3 signalling and whether it has a potential role in acne resolution.

Chapter 5: Materials and Methods

5.1 Background:

Current evidence suggests that inflammation plays a key role in the pathogenesis of acne vulgaris[5,77,81,488,489]. Inflammatory cytokines such as interleukin (IL-8) and matrix degrading enzymes (MMPs) have been shown to be prominently upregulated in inflammatory acne lesions[5,81]. Curbing the production of these inflammatory mediators may assist the resolution of acne. Improvement of lesions of acne vulgaris with the use of various intense pulsed light (IPL) sources has been demonstrated in a few studies[109,110,120,121,124-128]. However, its mechanism of action has not been elucidated. Recent studies have suggested that IPL therapy may affect the immunological functions of the skin[153]. Animal and *in vitro* studies have attributed the therapeutic effect of IPL to transforming growth factor beta (TGF-β)[154,158,168]. Furthermore, when used for photorejuvenation IPL enhances the expression of TGF-β1[473,474]. Retinoic acid, one of the main modalities of treatment in acne vulgaris, has been demonstrated to induce the cutaneous expression of TGF-β1, TGF-β2 and/or TGF-β3[445,447-450,453]. In addition, non-ablative laser therapy for acne vulgaris was also shown to increase cutaneous TGF-β1 expression[152]. TGF-β is a key anti-inflammatory and immunomodulatory cytokine[172]. It has also been shown to arrest keratinocyte growth and may therefore interfere with microcomedo formation[287]. TGF-β has been demonstrated to prevent cytokine-induced MMP-1 gene expression and inflammatory cytokine production through a Smad3-mediated signalling pathway[266,332]. Taking these facts into consideration, we carried out a study to determine whether intense pulsed light used for the treatment of inflammatory acne vulgaris alters the *in vivo* expression of the TGF-β isoforms, Smad3, MMP-1, and IL-8.

5.2 Objective:

This study aims to determine whether TGF-β is involved in the mechanism of action of intense pulsed light in the treatment of acne vulgaris by examining changes in the *in vivo* expression of the TGF-β isoforms (TGF-β1, β2 & β3), translocation of the

transcription factor Smad3 to the nucleus, and changes in the expression of two potential downstream targets, IL-8 and MMP-1.

More specifically:

1. To determine whether IPL upregulates TGF-β expression.
2. To determine whether IPL activates TGF-β signalling via Smad3 by evaluating the percentage of Smad3-stained nuclei.
3. To determine whether IPL impedes matrix destruction by downregulating the matrix degrading enzyme, MMP-1.
4. To determine whether IPL downregulates the expression of IL-8, a pro-inflammatory cytokine.
5. To correlate the results of these laboratory experiments with the clinical results obtained from the study conducted by Dr. Marisa Taylor.

5.3 Materials and methods

5.3.1 Tissue

This study was a continuation of a previous clinical study (conducted by Dr. Marisa Taylor, Department of Dermatology), wherein 28 patients with mild to moderate acne vulgaris were treated with four sessions of IPL (VPL Energist Ultra®; 530-950 nm Settings: 40 J/cm^2, 2 passes, 15 pulses, 5 ms duration, 20 ms delay) at two week intervals. Clinically, the patients experienced an overall reduction in the number of inflammatory lesions after IPL irradiation (28.04 % reduction; p = 0.002). To compare the histological effects of IPL treatment, punch biopsies (4 mm) of skin were obtained from the upper back of patients (n=29) at baseline (B1), 48 hours after the first IPL treatment (B2) and 1 week after the final treatment (B3). To avoid the practical difficulties involved in timing an acne lesion throughout its evolution, the biopsies provided were taken from an area adjacent to the inflammatory lesions.

5.3.2 Sample size and power calculation

Due to the longitudinal nature of this study, it was derived that a sample size of 20 would enable the detection of a shift of 0.63 times the standard deviation of within-subject differences with a power of 80% at the conventional 5% alpha level. This calculation could apply to any of the inflammatory markers studied.

5.3.3 Processing

In immunohistochemical analysis, antigen preservation is superior in frozen sections when compared to paraffin sections[490]. In addition, the processing of the biopsies is simple and more rapid[491]. Also, as fixation is done later on with cryostat sections, the fixative used can be optimised according to the antigen of interest[490]. Although the morphological detailing is inferior to paraffin embedded sections, it is within acceptable standards[491]. Moreover, from past experience with immunohistochemistry in our laboratory, it was noted that the antigen retrieval step employed for paraffin embedded sections severely damaged the morphology[492].

Therefore, the punch biopsies were embedded in Tissue-tek OCT (Optimal Cutting Temperature embedding Matrix; RA. Lamb, East Sussex, UK) and then snap frozen in a liquid hexane bath (Fisher Scientific, Loughborough, UK) cooled on dry ice. These frozen biopsies were then stored in liquid nitrogen at the Dermatology Research Laboratory (Cardiff University). Serial sections (6 μm) of these biopsies were obtained from the frozen tissue blocks using a Thermo Scientific Cryotome FSE cryostat. The sections were then mounted on SuperFrost® Plus glass slides (catalog no. J1800AMNZ; Menzel GmbH, Braunschweig, Germany). The SuperFrost® Plus glass slides are made by a process wherein a permanent positive charge exists on standard microscope slides, which aids in the electrostatic attraction of frozen tissue sections on to the slide[493]. These slides were left to air dry for 30 minutes. After drying, they were labelled and wrapped back to back in aluminium foil and then stored in the freezer at -80˚C until the time of staining.

5.3.4 Immunohistochemistry

Immunohistochemistry is a procedure that is used for the detection and localisation of a cellular protein or other antigen within cells or tissues using an antibody specific for the desired antigen[491]. Immunohistochemical techniques are broadly classified as direct or indirect[491]. The direct method is a simple one-step staining procedure that involves direct interaction of the antigen with a specific labelled primary antibody[490]. However, this method can lack sensitivity[490]. Whereas, indirect immunohistochemistry involves using a labelled secondary antibody (must be raised against an immunoglobulin of the same type and species as the primary antibody) or a secondary antibody and labelled tertiary compound, that binds to the unlabelled primary antibody-antigen complex[491]. This indirect approach is more sensitive as it generates an amplified signal[490]. In order to further amplify the signal, various three-step methods have been developed such as the peroxidase anti-peroxidase, alkaline phosphatase-anti-alkaline phosphatase and avidin-biotin based methods[491]. In this study the avidin-biotin based method has been utilised. In this method, the first layer is an unlabelled primary antibody, the second layer is a biotinylated secondary antibody and the third layer is either a complex of avidin-biotin peroxidase (ABC method) or an enzyme-streptavidin conjugates (Streptavidin method)[491].

Cryostat sections from twenty cases were analysed by immunohistochemistry using the primary antibodies specified in **Table 5.1**. The antibodies specific for the markers of interest were identified by researching the available literature for immunohistochemical studies of the respective markers conducted on frozen sections of human tissue. The individual antibodies were selected on the basis of the quality of the staining produced in the published pictures. The protocols used in these studies[92,229,494,495] were then tested on normal skin sections and modified according to the results obtained until good positive staining was detected and all the non-specific staining and background were minimised.

Table 5.1 Antibody panel and immunostaining conditions used in this study

Antibody (Cat. No)	*Host*	*Source. (Lot. No)*	*Block*	*Diluent*	*Pri Ab dilution (incubation period)*	*Sec Ab (1:200)*	*Blocking peptide*	*Tertiary*
TGF-β1 (sc-146)	Rabbit polyclonal	Santa Cruz biotech (H0509)	10% donkey serum Avidin/Biotin blocking	1% BSA & 1% marvel in PBS-TX (0.1% Triton X-100 in 1x PBS)	1:200 (1 hour)	Anti-rabbit biotinylated (from donkey)	sc-146P	ABC kit
TGF-β2 (sc-90)	Rabbit polyclonal	Santa Cruz biotech (E0509)	10% donkey serum Avidin/Biotin blocking	1% BSA & 1% marvel in PBS-TX	1:800 (1 hour)	Anti-rabbit biotinylated (from donkey)	sc-90P	ABC kit
TGF-β3 (sc-82)	Rabbit polyclonal	Santa Cruz biotech (D2709)	10% donkey serum Avidin/Biotin blocking	1% BSA & 1% marvel in PBS-TX	1:500 (1 hour)	Anti-rabbit biotinylated (from donkey)	sc-82P	ABC kit
Smad3 (51-1500)	Rabbit polyclonal	Invitrogen (609945A)	10% donkey serum Avidin/Biotin blocking	1% BSA in 1x PBS	1:150 (1 hour)	Anti-rabbit biotinylated (from donkey)	_	ABC kit
IL-8 (BMS-136)	Mouse monoclonal	BenderMed Systems (51169000)	10% sheep serum Avidin/Biotin blocking	1% BSA & 1% marvel in 1x PBS	1:200 (Overnight at 4ºC)	Anti-mouse biotinylated (from sheep)	_	Streptavidin (1:100)
MMP-1 (MAB3307 clone 41-1E5)	Mouse monoclonal	Millipore (NG1735413)	10% sheep serum Avidin/Biotin blocking	1% BSA & 1% marvel in 1x PBS	1:250 (Overnight at 4ºC)	Anti-mouse biotinylated (from sheep)	_	ABC kit

5.3.4.1 Preparation of buffer

Five litres stock of 10 x PBS solution (phosphate buffered saline, pH 7.2) was prepared and stored in bottles (**Table 5.2**).

Table 5.2 Constituents for preparation of 10 x Phosphate Buffered Saline solution

Constituents	**Amount (in grams) required for 1 Litre (L)**	**Amount (in grams) required for 5L**
Sodium chloride, NaCl	80	400
Disodium hydrogen phosphate (dodecahydrate), $Na_2HPO_4.12H_2O$	35.8	179
Potassium dihydrogen phosphate (anhydrous), KH_2PO_4	2.4	12
Potassium chloride, KCl	2	10
Distilled water	To make up to 1L	To make up to 5L

A 1x PBS buffer solution was prepared from this stock for washes and for preparing the diluent. PBS with 0.1% Triton-X-100 (1ml of Triton-X-100 in 1000 ml of 1 x PBS, PBS-TX) was used as the buffer solution in the TGF-β1, -β2 and -β3 staining protocols, whereas, 1 x PBS without detergent was used for Smad3, MMP-1 and IL-8. The diluent (intended for dilution of the primary antibody, secondary antibody and the tertiary reagent, and also used as a negative control) was prepared by adding 0.25 g of BSA (bovine serum albumin) and/or 0.25 g of Marvel (non-fat milk protein) to 25 ml of the buffer. BSA and Marvel were added to prevent non-specific binding of the primary antibody (reduces the background by minimising non-specific interactions between the primary antibody and non-target cellular proteins).

5.3.4.2 Fixation

Slides were taken out of the freezer and allowed to reach room temperature, before removing the foil. The slides were then labelled and immersed for 15 minutes in a fixative (dry acetone for MMP-1 and IL-8 or 4% paraformaldehyde in PBS for TGF-β1, -β2, -β3 and Smad3). The acetone-fixed slides were air dried for 15 minutes to allow evaporation of

excess acetone followed by three five-minute washes in the buffer solution (1x PBS/PBS-TX). The paraformaldehyde-fixed slides were directly rinsed in the buffer solution.

5.3.4.3 Blocking

Each of the sections on the slides were encircled with a water-repelling wax pen (Dako Pen, Glostrup, Denmark) and placed in a humidified chamber (a shallow plastic box with a moistened paper towel placed at the bottom). To reduce non-specific binding of the secondary antibody, the slides were incubated with 10% normal serum (from the same species, in which the secondary antibody was raised) for an hour at room temperature. This was followed by three five-minute washes in the buffer solution. To suppress endogenous biotin activity it is necessary to block it with avidin and then block the unoccupied biotin binding-sites of avidin with biotin. Therefore, an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) was used. The slides were incubated with avidin for fifteen minutes followed by fifteen minutes incubation with biotin with brief rinses (x 3) in the buffer solution in between.

5.3.4.4 Primary antibody

The sections were incubated with an optimum dilution of the primary antibody or blocking peptide-antibody mix (blocking peptides were available for TGF-β1, -β2 and -β3 and were preincubated with the antibody for 30 minutes) for an hour at room temperature (TGF-β1, -β2, -β3 and Smad3) or overnight at 4˚C (MMP-1 and IL-8). Slides incubated with plain diluent without any primary antibody were run in parallel as a negative control for each case and time point. This was followed by three five-minute washes in the buffer solution.

5.3.4.5 Secondary antibody and tertiary immunoperoxidase step

The sections were then incubated with a relevant biotinylated secondary antibody for 30 minutes, followed by incubation with either streptavidin biotinylated horseradish peroxidase complex (RPN 1051; GE Healthcare UK Limited, Buckinghamshire, UK) (IL-8) or reagents of the avidin-biotinylated enzyme complex kit (Vectastain ABC kit, Vector

Laboratories Inc.) for a further 30 minutes. The ABC reagent was prepared at least thirty minutes before being used by adding 1 drop of reagent A and 1 drop of reagent B to 2.5 ml of the diluent. The sections were then washed in the buffer solution three times and developed with 0.25% horse radish peroxidase substrate solution (0.5 ml of 3, 3' Diaminobenzidine, 4.5 ml of PBS and 6 µl of H_20_2) for 10 minutes to produce colorimetric immunoprecipitates.

5.3.4.6 Counterstaining and mounting

The sections were then rinsed in tap water and counterstained with haematoxylin for 5 minutes and washed again in tap water. Finally, all sections were dehydrated through a graded series of alcohol solutions (1 x 70%, 1 x 90%, and 3 x 100% industrial methylated spirit; Genta Medica, York, UK) for 5 minutes each, and cleared in three changes of xylene (Genta Medica, York, UK) for 5 minutes each. The slides were then carefully mounted with a cover slip using DPX mounting medium (a mixture of distyrene, a plasticizer, and xylene; RA Lamb, East Sussex, UK) and then left to dry overnight.

5.3.4.7 Visualisation and photography

The following day, the slides were observed under a Nikon optiphot microscope for detection of positive staining. Positive staining (or antibody binding) was visualised as brown staining. Digital images were captured with an *Axiocam* camera system (Zeiss) and Axiovision software (Zeiss) under different magnifications using three objectives (10x, 20x, and 40x). Four fields of view were captured per section. Standardised settings were used on the microscope and image capturing software to allow accurate comparisons.
The steps for the immunohistochemical analysis carried out are summarised in **Figure 5.1**.

Figure 5.1 General simplified workflow for immunohistochemistry procedure.

5.3.4.8 Image analysis

The digital images that were captured were semi-quantitatively assessed using Image Pro Plus image analysis software (IPP version 6.0; Media Cybernetics, Bethesda, MD, USA). To quantify the immunostaining (for TGF-β isoforms, MMP-1 and IL-8), integrated optical density (IOD) of the visible staining was obtained for the epidermis and dermis. The integrated optical density (IOD) refers to the total amount of antigen present (as detected by the brown staining) and is equal to the mean optical density multiplied by the area of positive staining[496]. Also, the area and length of the epidermis and dermis were measured to normalise the data.

In order to quantify the staining, it is necessary to ensure that the images are calibrated. This involved capturing images of a stage micrometer scale with the same optical settings as for the data images for each objective (4x, 10x, 20x and 40x). These images were then loaded on to the image analysis software and the calibration settings were entered and saved for each objective. The image file was opened and the objective-specific calibration setting was applied. The image intensity format was converted from the default free form to standard optical density.

The area of interest (epidermis or dermis) was outlined or traced out using a wand tool. After selecting the area of interest, the measurement parameters were selected, which include IOD, area, and length. The pixels to be analysed were defined using the select colours tool. The count option was clicked on to obtain measurements of the selected parameters. These measurements were then exported to a Microsoft Excel spreadsheet.

ImageJ image-analysis software program (ImageJ v1.42q, National Institutes of Health, Bethesda, MD) was used to count Smad3-stained nuclei and total nuclei. These values were exported to a Microsoft Excel spreadsheet and the percentage of Smad3-stained nuclei was calculated.

5.3.4.9 Statistical analysis:

The results were collected, tabulated, normalised and statistically analysed using the SPSS 17.0 statistical software. All data were expressed as mean ± SEM (standard error of mean). Repeated-measures one-way analysis of variance (ANOVA) was used to determine if there was a difference in cytokine expression between any of the groups (B1, B2 & B3), and to determine which pair of groups were statistically different, a post hoc Bonferroni test was used. The differences were considered statistically significant at $p < 0.05$.

5.3.5 Western Blotting

Western blotting (also called immunoblotting) is a procedure that employs specific antibodies to identify proteins that have been separated from one another according to their size by gel electrophoresis. The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. As, cognate blocking peptides were not available for Smad3, IL-8 and MMP-1, western blotting was carried out to validate the specificity of the antibodies used for the detection of these three markers.

5.3.5.1 Electrophoretic separation of the proteins

The first step in a western blotting procedure is to separate the protein using polyacrylamide gel electrophoresis (PAGE). For this a resolving gel has to be prepared. The percentage of acrylamide to be used in the resolving gel depends upon the size of the protein of interest (large proteins require less concentrated gel and vice versa). The proteins we were examining were ranging in size from 10 to 60 KDa, therefore a 12% gel solution was prepared from the reagents listed in **Table 5.3** and loaded into two Novex disposable plastic cassettes (Catalogue no. NC2010; Invitrogen, Paisley, UK) up to a level of about 2 cm below the top of the cassette. The resolving gel was then carefully layered with water to ensure a flat top surface. While the resolving gel was allowed set, the solution for the stacking gel was prepared (**Table 5.4**).

Table 5.3 Constituents for preparation of resolving gel

Constituents	**Amount required for 12% gel**
1.5M Tris pH 8.8	3.75 ml
Acrylamide/Bis-acrylamide, 30% gel solution	6 ml
10% Sodium dodecyl sulphate	100 µl
Distilled water	5.1 ml
10% Ammonium persulphate*	75 µl
N,N,N′,N′-Tetramethylethylenediamine (TEMED)*	7.5 µl

* Should be added just prior to pouring gel into cassette

Table 5.4 Constituents for preparation of stacking gel

Constituents	**Amount required for 4% gel**
0.5M Tris pH 6.8	1.25 ml
Acrylamide/Bis-acrylamide, 30% gel solution	0.65 ml
10% Sodium dodecyl sulphate	50 µl
Distilled water	3.05 ml
10% Ammonium persulphate*	50 µl
TEMED*	10 µl

* Should be added just prior to pouring gel into cassette

Once the resolving gel was set, the water from the top was drained off and the stacking gel was loaded on to the cassette. A comb was immediately inserted into the liquid stacking gel. Once set, the comb was carefully removed and the wells were rinsed with distilled water. The white strip at the lower end of the cassettes was removed and the cassettes were placed in the electrophoresis cell (XCell SureLock™ Mini-Cell Electrophoresis System, Invitrogen, Paisley, UK) with the wells facing inwards and locked in place. Following this a 1x running buffer (prepared from the 10x stock solution described in **Table 5.5**) was loaded into the wells.

Table 5.5 Constituents for preparation of 10x running buffer

Constituents	Amount required
Tris (molecular weight 121.1)	30 g
Glycine	144 g
Sodium dodecyl sulphate (SDS)	10 g
Distilled water	To make up to 1L

Low-range prestained SDS-PAGE molecular weight marker (5 µl; Bio-Rad Laboratories, Richmond, CA) was loaded into the assigned wells. Extracts (15µl) from the MDA-MB-231 human breast cancer cell line (a kind gift from Dr. Afnan Bugis, PhD Student, Cancer and Genetics) and HaCaT keratinocyte cell line (CLS, Cell Lines Service, Eppelheim, Germany) were loaded in to the assigned wells. The chamber between the gels and the outer chamber of the apparatus were then filled with the 1x running buffer. The electrophoresis apparatus was then connected to a power pack and run at a starting voltage of 150V and a constant current of 50 mA (25mA per gel) until the tracking dye reached the bottom of the gel. The cassettes were unlocked and removed from the apparatus, after which they were carefully dismantled by breaking the seal around them using the supplied novex spatula. The stacking gel and the lower portion of the resolving gel were cut off.

5.3.5.2 Transfer of proteins to a membrane

Following electrophoresis, the protein must be transferred from the electrophoresis gel to a membrane. For this, western blotting buffer was prepared using a stock 25x blotting buffer (**Table 5.6** and **5.7**).

Table 5.6 Constituents for preparation of 25x blotting buffer

Constituents	**Amount required**
Tris (molecular weight 121.1)	18.2 g
Glycine	90 g
Distilled water	To make up to 500 ml

Table 5.7 Constituents for preparation of western blotting buffer

Constituents	**Amount required to make up 500 ml**
25 x blotting buffer	20 ml
Methanol	100 ml
Distilled water	380 ml

Four pieces of filter paper and two pieces of polyvinylidene difluoride (PVDF) membrane (Immobilon-P Transfer Membrane, Catalogue number: IPVH00010 Millipore Ltd, Watford, UK) were cut according to the dimensions of the gel (6 cm x 8 cm). The filter paper and 4 pads were allowed to soak in the western blotting buffer. The hydrophobic membrane was made hydrophilic by immersing it in methanol for 10 seconds followed by two five minute washes in distilled water. The gel membrane sandwich was assembled on the cathode plate of the blotting apparatus in the following order: pad, filter paper, gel, membrane, filter paper, pad, filter paper, gel, membrane, filter paper and the remaining two pads (**Figure 5.2**).

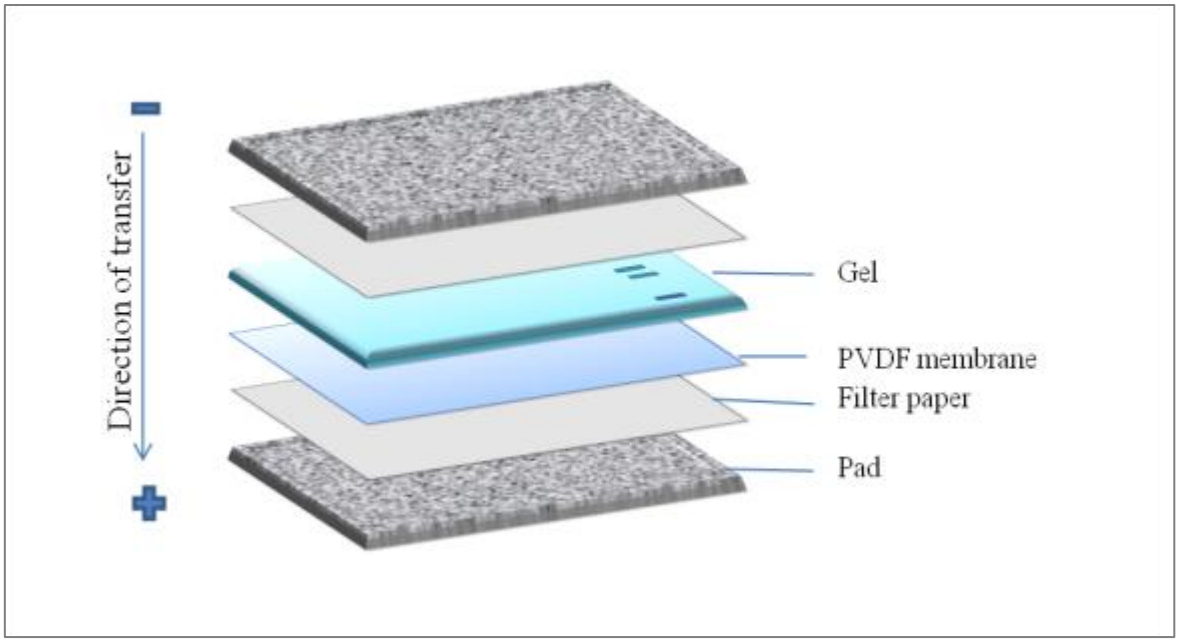

Figure 5.2 Illustration of a gel-membrane sandwich for a single gel

The other plate of the module was placed on top and closed. The assembled module (Xcell II Blot Module; Invitrogen, Paisley, UK) was then inserted into the tank of the blotting apparatus. The inner blotting chamber was filled with 1x western blotting buffer and distilled water was added into the outer chamber of the blotting apparatus for cooling during the electrophoresis. It was then run at 25 V constant voltage (around 100 mA starting current) for 2 hours.

5.3.5.3 Blocking and antibody incubation

After 2 hours, the membranes were carefully removed and placed in a Petri dish and washed once in PBS-T20 (**Table 5.8**) for 5 minutes. All the washing, blocking, and primary and secondary antibody incubation steps were performed on an orbital shaker to ensure proper contact with the membrane.

Table 5.8 Constituents for preparation of PBS-T20

Constituents	**Amount required to make up 500 ml**
PBS (10x)	50 ml
Tween-20	0.5 ml
Distilled water	449.5 ml

The PVDF membrane used in western blotting has a high affinity for proteins. Therefore, after the transfer of the proteins from the gel, it is important to block the remaining surface of the membrane to prevent any nonspecific binding of the antibodies to the membrane surface in subsequent steps. Therefore, the membranes were then placed in blocking buffer (5 g of Marvel in 100 ml of PBS-T20) for an hour.

Following this, the membranes were placed in 1:1000 dilution of the primary antibody (Smad3, MMP-1 or IL-8) diluted in 5% Marvel in PBS, overnight at 4° C. The next day the membranes were washed in PBS-T20 (3 times for 5 minutes) to remove unbound reagents and reduce background.

The membranes were placed in 10 ml of 1:1000 dilution (diluted with 5% Marvel in PBS) of the secondary antibody (species corresponding to the primary antibody) conjugated to horse radish peroxidase (Dako UK Ltd, Ely, UK) for one hour. This was followed by washes in PBS-T20 (twice for thirty seconds, once for 15 minutes and then thrice for 5 minutes).

5.3.5.4 Chemiluminescent detection

To visualise the protein signals, an enhanced chemiluminescence (ECL) technique was employed, which has to be performed in a dark room. The membranes were placed on cling film with the protein side up and a 1:1 mixture of solution I (**Table 5.9**) and solution II (**Table 5.10**) were added to the membranes (approximately 2 ml per membrane) and it was left for 5 minutes.

Table 5.9 Constituents for preparation of solution I

Constituents	Amount required
Luminol (250 mM in dimethyl sulfoxide, DMSO)	1 ml
Coumaric acid (90 mM in DMSO)	0.44 ml
Tris (pH 8.5)	10 ml
Distilled water	To make up to 100 ml

Table 5.10 Constituents for preparation of solution II

Constituents	Amount required
30 % Hydrogen peroxide	64 µl
Tris (pH 8.5)	10 ml
Distilled water	To make up to 100 ml

Following this the excess solution was drained off and the membranes were placed protein side down on fresh cling film, wrapped up and placed in an X-ray cassette with the protein side facing upwards. The lights in the dark room were switched off and an X-ray film (Amersham Hyperfilm ECL, catalogue number: 28-9068-35; GE Healthcare UK Limited, Buckinghamshire, UK) of appropriate dimension was placed over the wrapped membranes in the cassette. The exposure time ranged from 1 to 5 minutes. The film was removed from the cassette and placed in the developer solution till clear bands appeared, following which it was briefly immersed in the stop solution (water) and then in the fixer for around 30 seconds. The films were washed in distilled water for 15 minutes and then allowed to dry. Once dried the size of the bands on the film were determined by comparing it with the pre-stained molecular weight marker. The procedure followed for western blotting is illustrated in **Figure 5.3**.

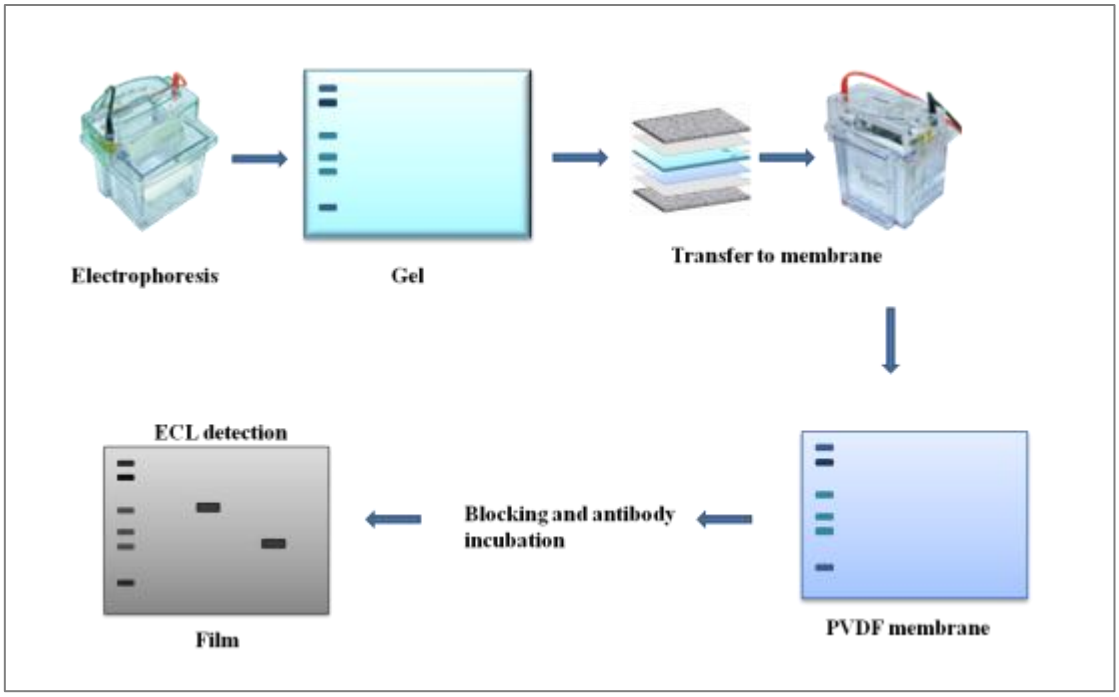

Figure 5.3 Simplified workflow for western blotting procedure

5.3.6 Real-time RT-PCR

Reverse transcription combined with polymerase chain reaction (RT-PCR) is a robust technique for quantitative estimation of gene expression[497]. Over the past few years, real-time PCR technology has been used to perform quantitative RT-PCR and due to its accuracy and sensitivity, real-time RT-PCR has become the preferred method for quantifying changes in gene expression[498]. Quantitative real-time PCR is based on the detection of a fluorescent signal produced proportionally during the exponential amplification phase[499]. Various methods of real-time PCR are currently available such as the TaqMan® method, the molecular beacon method and the SYBR® Green method[500]. The TaqMan® real-time PCR involves the use of a TaqMan probe in addition to the PCR primers[499]. This probe is an oligonucleotide with a fluorescent dye reporter attached to the 5' end and a non-fluorescent quencher attached to the 3' end[499,501]. They are designed to anneal to the target sequence between the forward and reverse primers[499]. In the intact probe, the proximity of the reporter dye to the quencher suppresses reporter fluorescence signal primarily by Förster resonance energy transfer (FRET)[500,501]. However, during PCR the *Taq* polymerase activity cleaves the probe, releasing the reporter dye and resulting in fluorescence[499]. The amount of fluorescence is proportional to the amount of PCR product generated in each cycle[499]. This probe-based real-time PCR technique increases the

specificity of detection as it does not detect non-specific amplifications and allows accurate measurement of the reporter dye fluorescence[500]. It is reported to be a rapid and reproducible method for quantification of cytokine and growth factor genes expressed at low levels in small samples[500].

Taking these facts into consideration, gene expression of TGF-β1, Smad3 and IL-8 was determined using a quantitative real-time-PCR assay based on TaqMan® Low Density Array technology (TLDA, Applied Biosystems) (**Figure 5.4**). The TLDA assay uses micro-fluidic cards that allows testing of many independent genes at the same time. Each TLDA card has eight separate loading ports and 384 wells. Each well contains primers and probes, capable of detecting a single gene and supplied by 8 ports in total. Reverse transcription and TLDA-based real-time PCR was carried out by Dr. Claudia Consoli at the Central Biotechnology Services (CBS) Facility, Cardiff University. The expression of candidate genes were measured using the TLDA Human Immune Panel (Format 96a, Part Number: 4370573, Applied Biosystems). This TLDA panel included 90 target genes and 6 housekeeping genes.

5.3.6.1 RNA extraction

The initial and key step is to obtain high quality, intact RNA. 750µl of Trizol (Invitrogen, Paisley, UK) was added to at least 600µm of cryosections from each biopsy in the fume hood. The samples were briefly vortexed and allowed to incubate for 5 min and then 200µl of chloroform was added to it and mixed vigorously by hand for 15 to 20 seconds and allowed to stand on the bench for 3 minutes. Samples were then centrifuged for 15 minutes at 12,000 x g relative centrifugal force (***RCF***) and at a temperature of 4°C. After centrifugation, the mixture separates into 3 phases: Red (phenol-chloroform) phase, interphase and the upper aqueous (colourless) phase. The colourless supernatant (containing the RNA) was carefully removed into an RNAase/DNAase free microcentrifuge tube and 200µg of glycogen was added as per the manufacturer's instructions. 500 µl of isopropyl alcohol (Fisher Scientific) was added to the solution which was then briefly vortexed. It was then allowed to precipitate at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA formed a tiny pellet on the side or bottom of the tube. The supernatant was then removed with a 1ml

pipette taking care not to disturb the pellet. The pellet was then washed with 1 ml of 75% ethanol (prepared with RNAase/DNAase free water) and centrifuged at 7500 x g for 5 min at 4° C. The supernatant was again discarded and washed with 70% ethanol and centrifuged at 7500 x g for 5 min at 4°C. Following this, all the remaining ethanol was removed and the pellet air-dried by leaving the tube open for about 5 minutes. The RNA pellet was then dissolved in 40 µl of RNAase-free water and stored at -80ºC

5.3.6.2 RNA analysis

All RNA samples were 'cleaned' using an RNeasy MinElute Cleanup Kit (Qiagen Ltd., Crawley, UK) to remove any impurities or enzymatic inhibitors that may affect target amplification efficiency. The RNA concentration was determined following analysis of the ratio of the optical densities at 260 and 280 nm, using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was assessed by the Agilent 2100 Bioanalyzer using the RNA Nano LabChip kits and the Eukaryote total RNA assay. RNA samples were stored at -80°C until further use.

5.3.6.3 Reverse transcription

Total RNA from each sample was reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit as per the manufacturer's protocol (Applied Biosystems, California, USA). A master mix was made up using 2 µl of 10x RT Buffer, 0.8 µl of 25x dNTP mix (deoxyribonucleotide triphosphate, 100mM), 2 µl of 10x RT Random Primers, 1 µl of MultiScribe Reverse Transcriptase, 1 µl of RNase Inhibitor and 3.2 µl of Nuclease-free H_2O. To this master mix 10 µl of RNA sample (300-500 ng) was added to make up a final volume of 20 µl. The tube was then placed in the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) and the reverse transcription reaction was performed according to the optimised conditions prescribed by the manufacturer: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and hold at 4°C. The cDNA was then aliquoted and stored at -20ºC.

5.3.6.4 Real-time PCR

Microcentrifuge tubes were labelled for each sample-specific PCR mix. cDNA was removed from the freezer and allowed to thaw. The cDNA was diluted (5x) and then gently vortexed and centrifuged. Sample-specific PCR mix was prepared by adding 10 µl of the specific diluted cDNA sample, 40 µL of RNase-free water and 50 µL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems) into the labelled microcentrifuge tube. The tube was gently vortexed for thorough mixing and then centrifuged to eliminate air bubbles. The TaqMan Array was carefully removed from its packaging and placed on the bench foil side down. 100 µl of the desired sample-specific PCR mix was loaded into the fill port (larger hole on the left arm of each fill reservoir) of the array with the pipette at an angled position. The arrays were then centrifuged twice (1200 rpm for 1 minute) to distribute the samples to the reaction wells. Following this the arrays were sealed and the fill reservoirs were cut off from the array. The plate document was then set up on the Sequence Detection System software.

The prepared array was placed in the instrument tray of the ABI 7900HT Real-Time PCR System (Applied Biosystems) and the PCR amplification was carried out under the following thermal cycler conditions: 2 min at 50°C, 10 min at 94.5°C, 30 s at 97°C and 1 min at 59.7°C for 40 cycles. Each sample was run in triplicate to ensure accuracy, precision and reproducibility of results.

5.3.6.5 Calculations and analysis of real time PCR data

The TLDA data were analysed using the RQ Manager 1.2 software of the ABI Sequence Detection System version 2.3 software package (Applied Biosystems). The threshold cycle (Ct) values calculated by the RQ Manager software were imported into a Microsoft Excel spreadsheet. The Ct value corresponds to the cycle number at which the amount of amplified target reaches a fixed threshold[502]. The relative quantification method ($2^{-\Delta\Delta CT}$ method) was used to analyse and calculate the PCR data. This method analyses the relative changes in gene expression from real-time quantitative PCR experiments by comparing the PCR signal of the target gene in the treatment group to that of another sample such as an untreated control[498]. The data are presented as fold change in gene

expression normalised to an endogenous control and relative to an untreated control or a sample at time zero in a time-course study[498]. An endogenous control is a housekeeping gene that is consistently expressed in all samples and used as an active reference for normalisation. The TLDA card included glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), human transferrin receptor (p90, CD71) (TFRC), human β-glucuronidase (GUSB), human phosphoglycerate kinase 1 (PGK1), and 18S ribosomal RNA as housekeeping genes. The Ct values were normalised using the most stable housekeeping gene to obtain the ΔCt values [ΔCt = Ct (target gene) – Ct (housekeeping gene)]. To determine the stability of the housekeeping genes, the geNORM Visual Basic application was used (Dr. Peter Giles, CBS, Cardiff University). Then, ΔΔCt values were calculated by subtracting the calibrator (untreated/baseline sample) from the mean ΔCt values of each target. The average "-fold" change in expression relative to baseline was then calculated using the formula $RQ = 2^{-\Delta\Delta Ct}$. The procedure followed for PCR is illustrated in **Figure 5.4**.

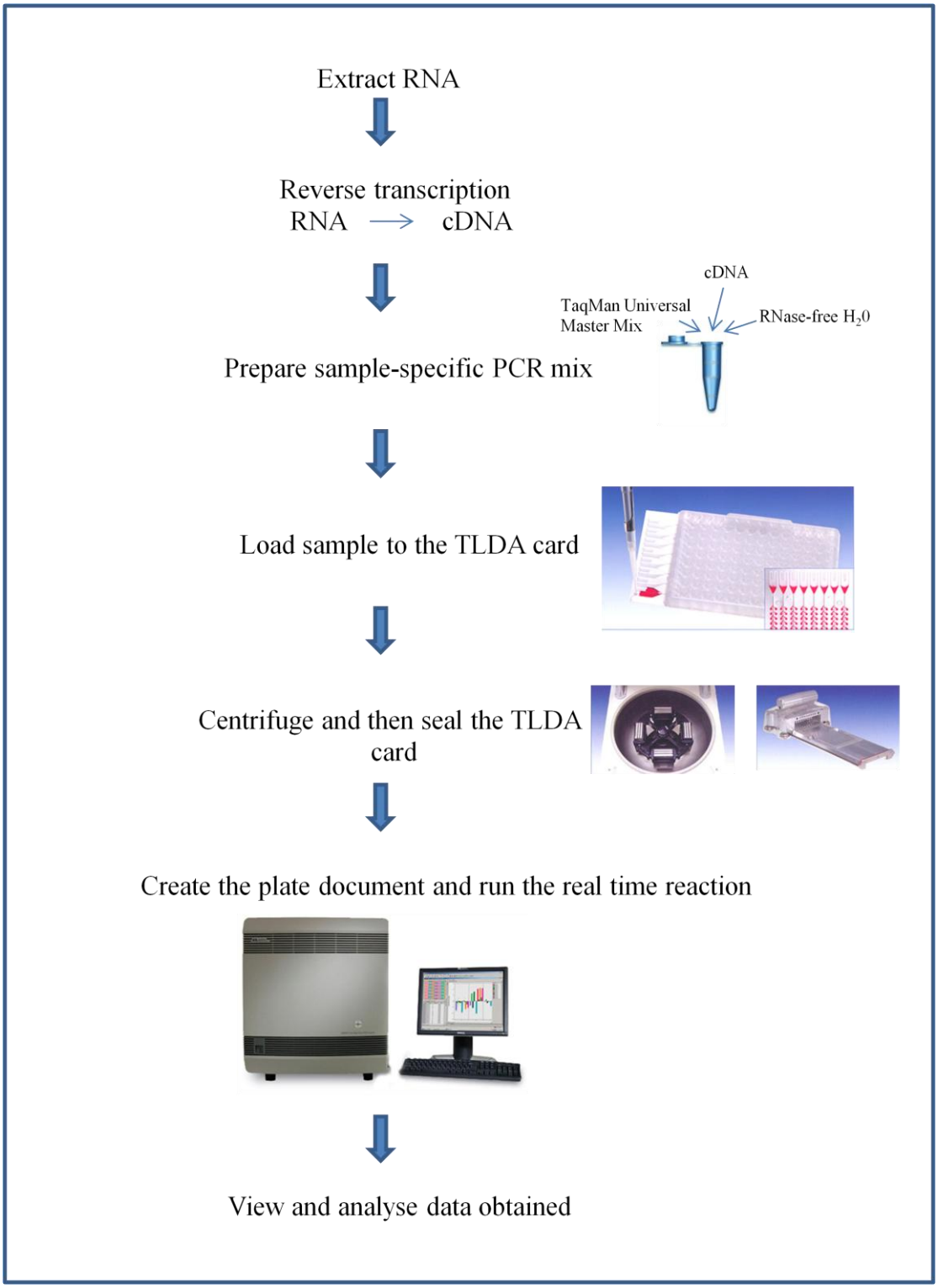

Figure 5.4 Workflow for real-time-PCR assay based on TaqMan® Low Density Array technology

Statistical analysis was carried out using the by repeated-measures ANOVA and the post-hoc bonferroni test to determine if there was a significant difference in gene expression between any of the groups (B1, B2 & B3). The differences were considered statistically

significant at $p < 0.05$. The presence of statistical significance in gene expression values signified differential expression of that particular gene (either upregulated or downregulated). The absence of statistical significance signified unchanged expression of that gene. All data were expressed as mean ± SEM.

In summary, the methods used for this study were:

- Immunohistochemistry and image analysis to determine if IPL induced any changes in protein expression
- Western blotting to verify the specificity of the antibodies for which there were no blocking peptides, and
- TLDA-based PCR to determine if there were any changes in gene expression

Chapter 6: Results

Effects of IPL on acne-prone skin

This study was carried out to investigate the in vivo effects of intense pulsed light (IPL) on acne-prone skin using an immunohistochemical approach. The effect of IPL was investigated on the expression of TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3), Smad3, IL-8 and MMP-1 in human skin *in vivo*. Three biopsies from each of the 20 patients (60 biopsies in total) were available for immunohistochemical analysis.

6.1. Effects on TGF-β/Smad3 signalling

6.1.1 TGF-β1

The expression of TGF-β1 was immunohistochemically analysed on frozen sections obtained from patients with mild to moderate acne vulgaris at baseline (B1), 48 hours after the first IPL treatment (B2) and 1 week after the final treatment (B3). The particular TGF-β1 antibody (sc-146) used was selected due to the availability of a cognate blocking peptide and based on its previous use for immunohistochemical analysis of frozen human skin sections as demonstrated in a study conducted by Quan et al[229].

An increase in TGF-β1 staining was observed in the post-treatment sections in the majority (17 out of 20 cases) of the cases (**Figure 6.1 and 6.2**). However, in a few cases (3 out of 20 cases) there was a decrease in staining post-IPL (**Figure 6.3**). The localisation of the staining is as described below.

Epidermis and its appendages

The epidermis showed mild to moderate TGF-β1 immunoreactivity that was cytoplasmic and most intense in the upper differentiated layers of the epidermis, the stratum granulosum and the stratum corneum (**Figure 6.1**). Appearing like a distinct brown band beneath and in the lower layers of the stratum corneum (**Figure 6.2**), this pattern of expression was consistent with previous reports on the spatial and temporal localisation of TGF-β1 in normal human skin[185,186]. There was low intensity immunostaining of hair follicles and sebaceous glands in some cases (**Figure 6.4**). The epidermal appendages were excluded from the analysis because they were not present in all sections.

Dermis

Immunoreactivity in the dermis was negligible.

Negative control and blocking peptide

The sections incubated in the absence of the TGF-β1 antibody did not show any immunoreactivity (**Figure 6.1**, top panel). In addition, preincubation of TGF-β1 antibody with the cognate blocking peptide (Santa Cruz Biotechnology, catalogue no. sc-146 P) resulted in no detectable staining (**Figure 6.1**, bottom panel). Thus, verifying the specificity of the TGF-β1 antibody used.

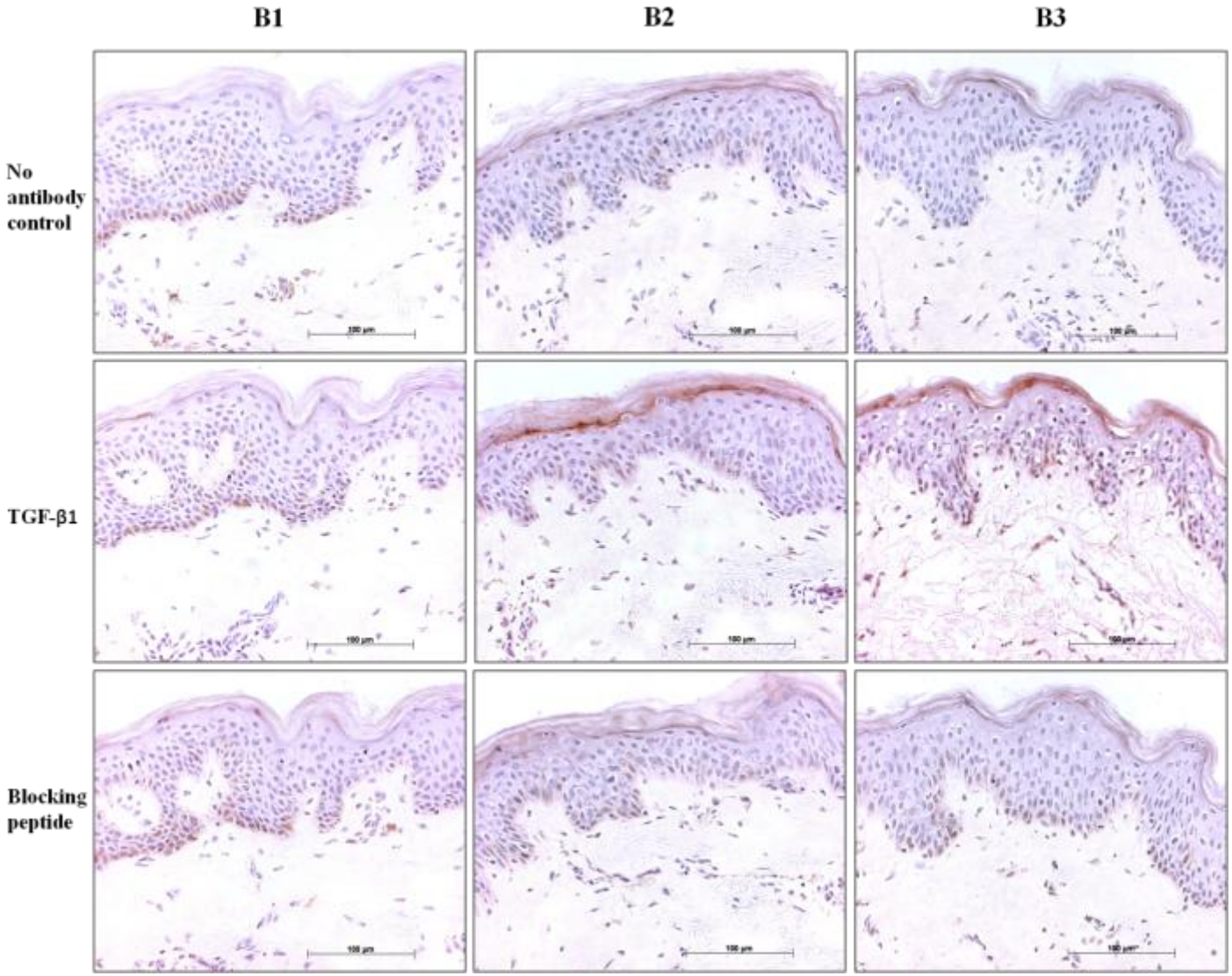

Figure 6.1 Immunohistochemical analysis of TGF-β1 showing increased staining in the post-IPL sections. Representative photomicrographs of TGF-β1 staining with antibody alone (middle panel), with blocking peptide (bottom panel) and without the primary antibody (top panel) at baseline (B1), 48 hours following 1st IPL session (B2), and 1 week after the 4th session of IPL treatment (B3).

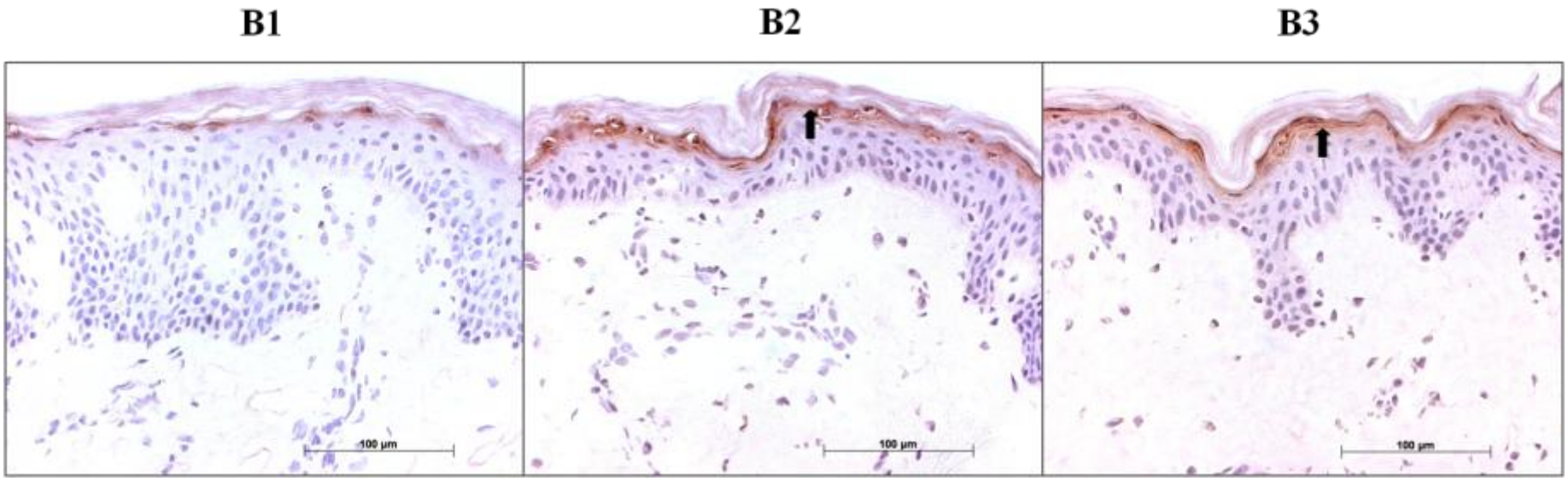

Figure 6.2 TGF-β1 immunoreactivity depicted by the brown band beneath and in the lower layers of the stratum corneum (as indicated by the arrows)**.**

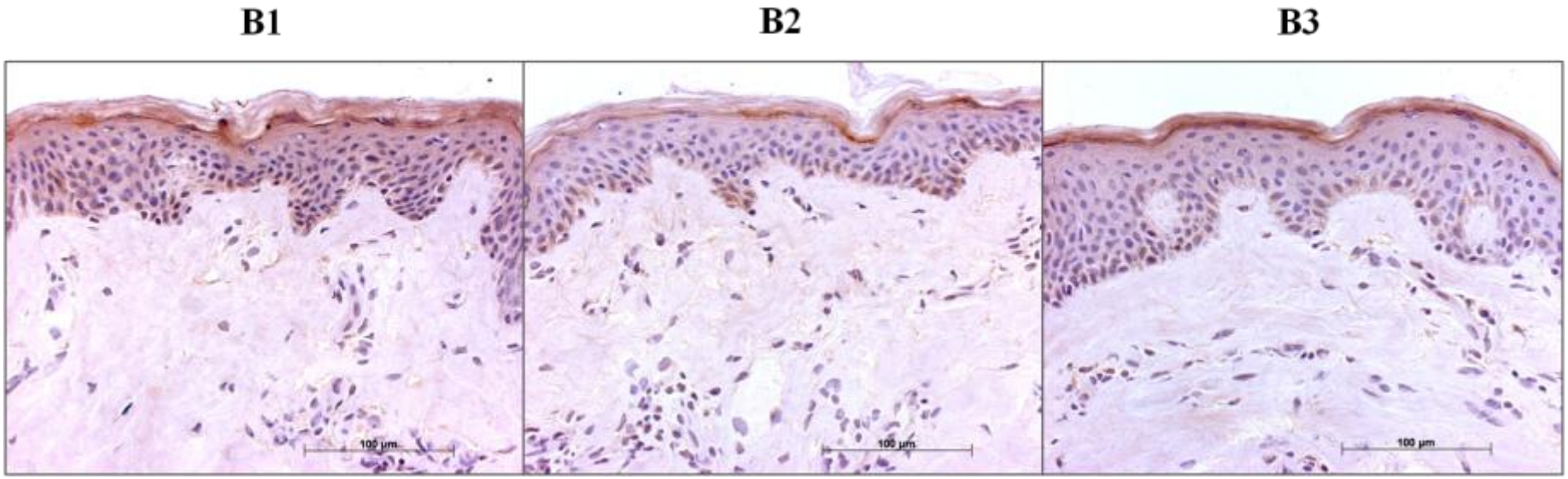

Figure 6.3 Immunohistochemical analysis of TGF-β1 showing decreased staining in the post-IPL sections. Photomicrographs of one of the three cases wherein there was a downregulation of TGF-β1 expression post-IPL.

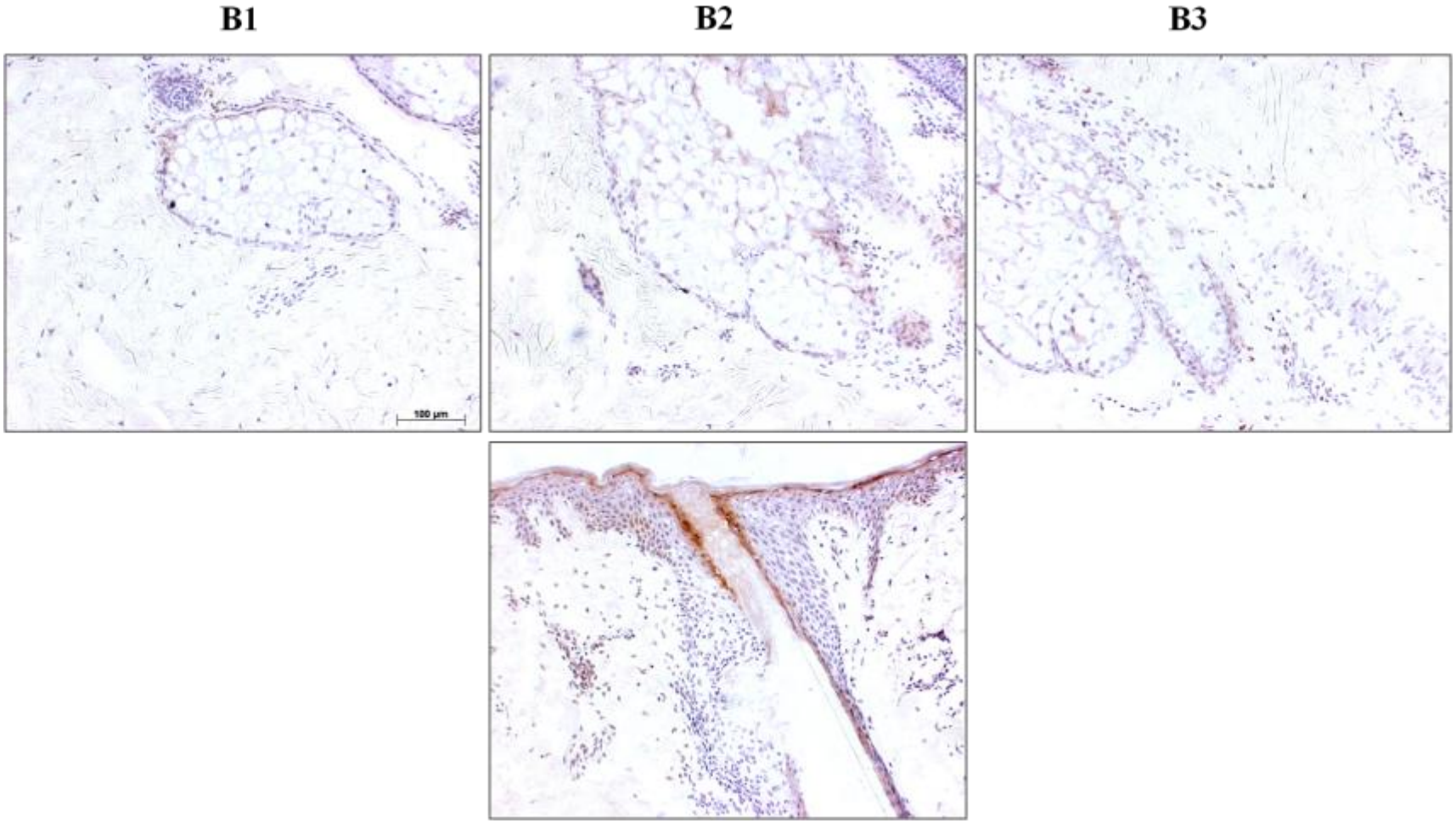

Figure 6.4 TGF-β1 immunoreactivity in the epidermal appendages. Representative photomicrographs from one case showing the immunoreactivity in the sebaceous glands (top panel). One photomicrograph (B2) from the same case demonstrated staining in the infundibular region of the hair follicle (bottom panel, no comparable sections from B1 or B3).

6.1.1.1 Image analysis

Quantification of the immunohistochemical staining was carried out using Image Pro Plus image analysis software, version 6.0 (Media Cybernetics, Bethesda, MD, USA). On quantification, it was demonstrated that the epidermal expression of TGF-β1 was upregulated in seventeen of the twenty cases and downregulated in remaining three cases 48 hours after the first treatment and the same trend was observed one week after the final treatment session (**Table 6.1**).

Table 6.1 Effect of IPL on the expression of TGF-β1 in the 20 cases

Isoform	48 hrs after 1st treatment vs. Baseline (B2 vs. B1)		1 wk after 4th treatment vs. Baseline (B3 vs. B1)	
	Upregulation	Downregulation	Upregulation	Downregulation
TGFβ1	17/20*	3/20	17/20*	3/20

*Statistically significant at $p < 0.05$

6.1.1.2 Statistical analysis

The data was analysed and compared using a one-way repeated measures analysis of variance (ANOVA) with time of measurement (B1 vs. B2 vs. B3) as a within-subjects factor. The sphericity assumption was met (Mauchly's test did not show a violation of sphericity). The ANOVA revealed that the mean difference in TGF-β1 expression was statistically significant between the different time points (B1, B2 and B3), $F(2, 38) = 10.789$, $p = 0.000195$. ANOVA indicates if there was an overall significant difference between the means at the different time points, but it does not indicate amongst which groups those differences occurred. However, post-hoc tests such as the Bonferroni correction does indicate which specific means differed. Post hoc comparisons were performed using the Bonferroni adjustment for multiple comparisons with the p value set at 0.05.

The Bonferroni correction revealed that IPL treatment elicited an increase (1.6-fold/63%) in TGF-β1 expression (**Figure 6.5**) from baseline to 48 hours after first treatment session (Mean ± Standard error of mean, SEM; 2.2 ± 0.4 *vs.* 3.58 ± 0.51, respectively) which was statistically significant (p = 0.004). Similarly, there was a statistically significant increase (1.6-fold/62%) in TGF-β1 expression from baseline to 1week after the last treatment session (increased to 3.55 ± 0.46; p = 0.007). However, there was no statistically significant difference between B2 (48 hours after the first IPL treatment) and B3 (1 week after the final treatment) ($p > 0.05$).

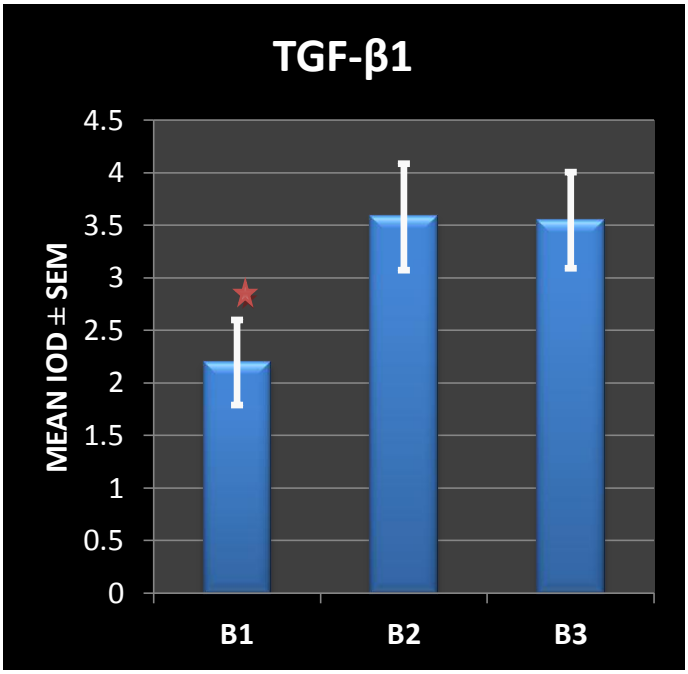

Figure 6.5 Effect of IPL on TGF-β1 expression. IOD: integrated optical density; Error bars denote standard error of mean, SEM; the red star denotes a statistically significant difference between baseline and post-IPL expression.

We can, therefore, conclude that IPL elicits a statistically significant increase in epidermal TGF-β1 expression 48 hours following the first treatment session and this increase was sustained until 1 week after the last treatment session.

6.1.2 TGF-β2

As for TGF-β1, the TGF-β2 antibody (sc-90) was selected according to the immunohistochemistry protocol employed in the study by Quan et al[229]. No specific trend in TGF-β2 staining was observed when comparing the baseline and the post-treatment sections. The localisation of the staining is as described below.

Epidermis and its appendages

Immunoreactivity to TGF-β2 was observed to be cytoplasmic. Staining was present in all the layers of the epidermis. However, it was most intense in the basal layer of the epidermis (**Figures 6.6** and **6.7**). In addition there was immunostaining of hair follicles, sweat glands and sebaceous glands (data not shown).

Dermis

TGF-β2 immunoreactivity was also observed in the dermis. Staining was moderate to intense in the dermal fibroblasts, inflammatory cells, and in the smooth muscle fibres of dermal vessels and arrector pili muscles.

Negative control and blocking peptide

The sections incubated in the absence of the TGF-β2 antibody (negative control) did not show any staining in any of the structures (**Figure 6.6**, top panel). In addition, preincubation of TGF-β2 antibody with the specific blocking peptide (Santa Cruz Biotechnology, catalogue no. sc-90 P) did not demonstrate any detectable staining (**Figure 6.6**, bottom panel).

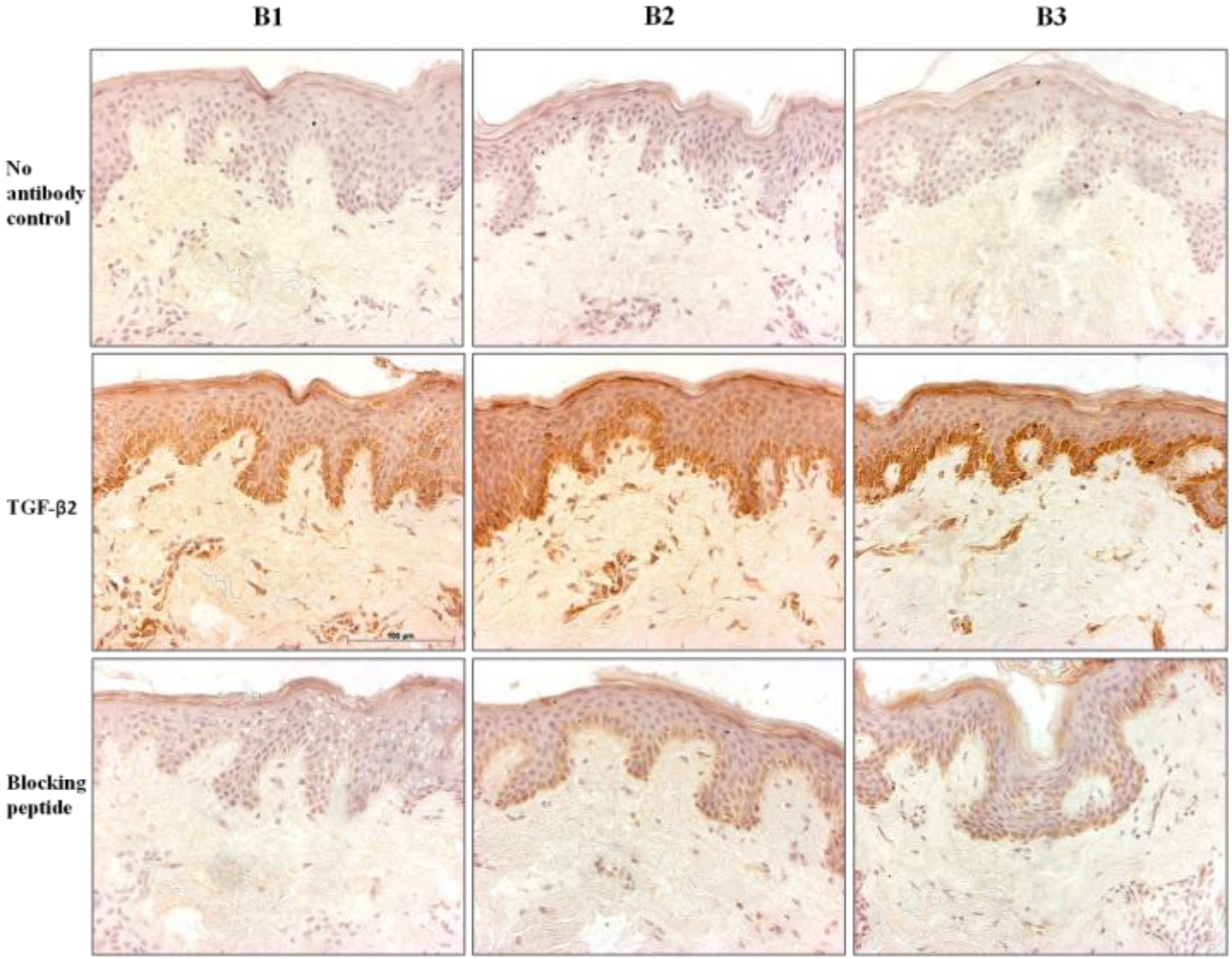

Figure 6.6 Immunohistochemical staining for TGF-β2 demonstrating increased intensity of staining in the post-IPL sections. Representative photomicrographs of TGF-β2 staining with antibody alone (middle panel), with blocking peptide (bottom panel) and without the primary antibody (top panel) at baseline (B1), 48 hours following 1st IPL session (B2), and 1 week after the 4th session of IPL treatment

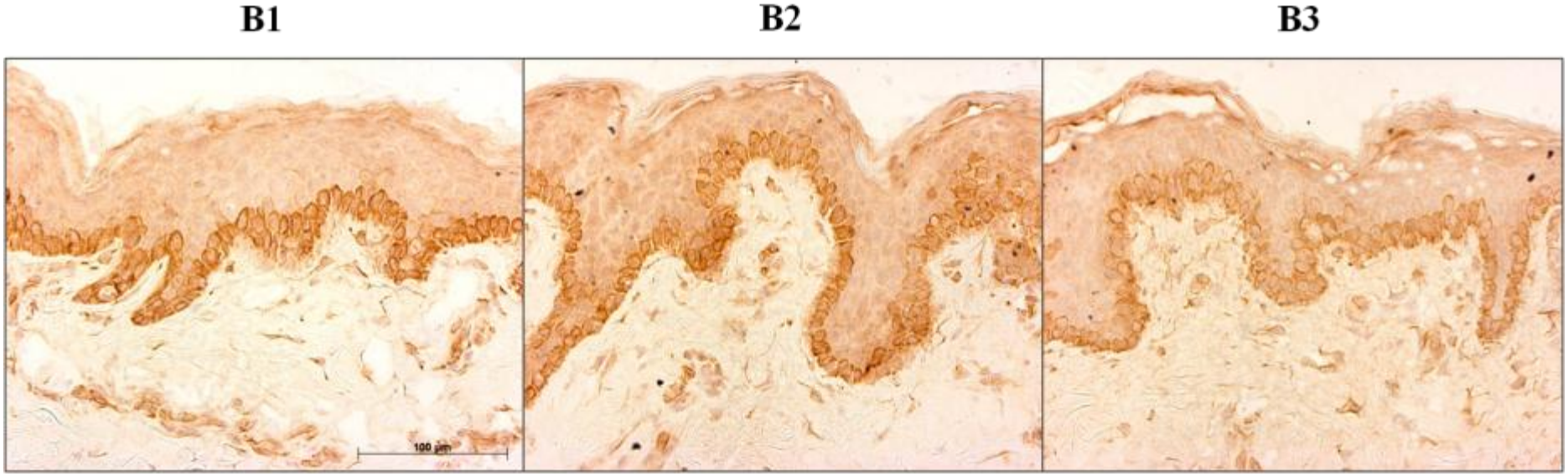

Figure 6.7 Immunohistochemical staining for TGF-β2 demonstrating a decrease in staining intensity from B1 to B3.

6.1.2.1 Image analysis

On quantification, it was demonstrated that the epidermal and dermal expression of TGF-β2 did not show any significant trends post-treatment at either of the two time points (**Table 6.2**).

Table 6.2 Effect of IPL on the expression of TGF-β2 in the 20 cases

TGFβ2 Isoform	48 hrs after 1st treatment vs. Baseline (B2 vs. B1)		1 wk after 4th treatment vs. Baseline (B3 vs. B1)	
	Upregulation	Downregulation	Upregulation	Downregulation
EPI	10/20	10/20	11/20	9/20
DERM	8/20	12/20	11/20	9/20

6.1.2.2 Statistical analysis

Repeated-measures ANOVA did not detect any statistical significance between the groups hence the post hoc Bonferroni test was not required in this scenario.

6.1.3 TGF-β3

The expression of TGF-β3 was studied by using a TGF-β3 antibody (sc-82) and similar to the other isoforms the immunohistochemistry protocol was based on the study by Quan et al[229].

As with TGF-β2 staining, no specific trend in TGF-β3 staining was observed when comparing the baseline and the post-treatment sections. The localisation of the staining is as described below (**Figure 6.8** and **6.9**).

Epidermis and its appendages

TGF-β3 immunoreactivity was predominantly cytoplasmic, however, in some cases speckled perinuclear staining was noted (**Figure 6.9**). Mild to moderate staining was present in all the layers of the epidermis. Staining was also present in the hair follicles, sebaceous glands and in sweat glands (not shown).

Dermis

TGF-β3 immunoreactivity was present in the dermis. There was mild to moderate staining of some dermal cells, probably inflammatory cells and fibroblasts.

Negative control and blocking peptide

The sections incubated in the absence of the primary antibody did not show any staining (**Figure 6.8**, top panel). In addition, preincubation of TGF-β3 antibody with the associated blocking peptide resulted in no detectable staining (**Figure 6.8**, bottom panel).

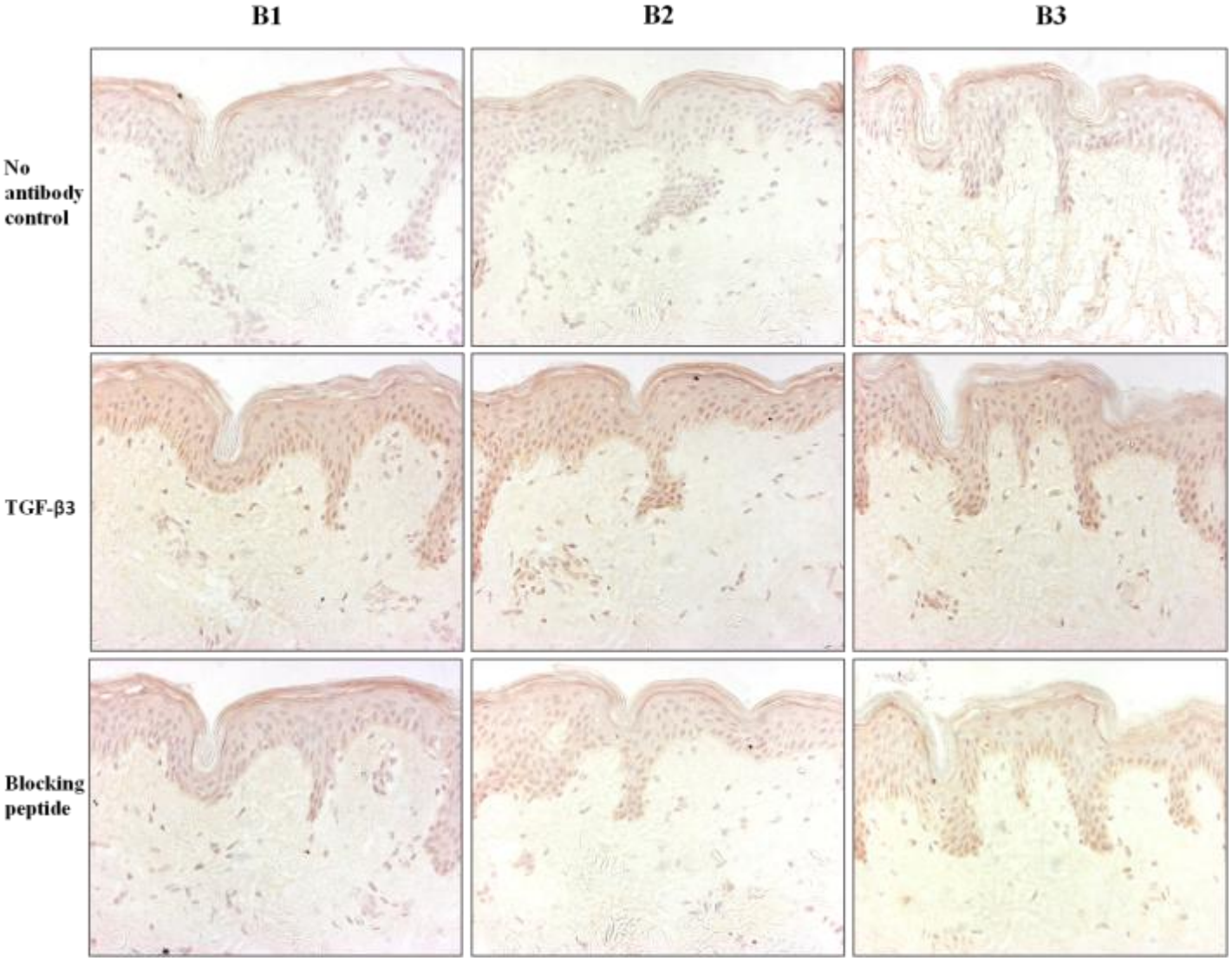

Figure 6.8 Immunohistochemical staining for TGF-β3. Representative photomicrographs of TGF-β3 immunohistochemitry are shown from one patient (middle panel). No detectable staining in the absence of the primary antibody (top panel) or with the corresponding blocking

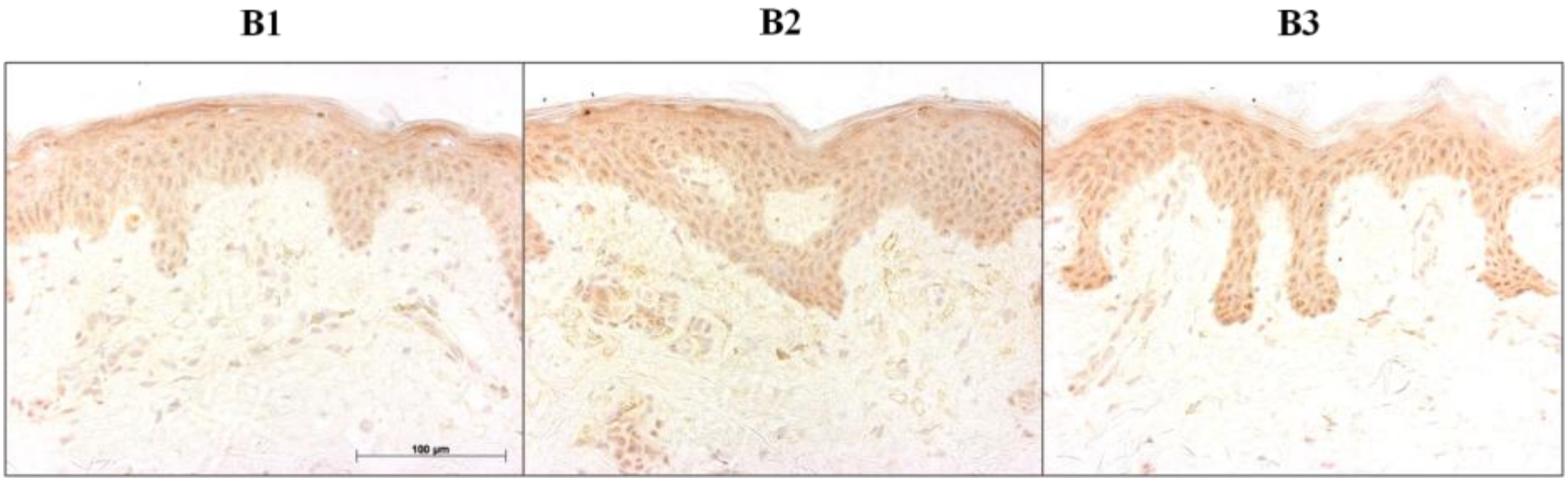

Figure 6.9 Representative photomicrographs of TGF-β3 immunoreactivity depicting a case wherein perinuclear speckling was present in addition to cytoplasmic staining

6.1.3.1 Image analysis

Akin to TGF-β2 protein, TGF-β3 did not show any specific trends post-treatment at either of the two time points (**Table 6.3**)

Table 6.3 Effect of IPL on the expression of TGF-β3 in the 20 cases

TGFβ3 Isoform	48 hrs after 1st treatment vs. Baseline (B2 vs. B1)		1 wk after 4th treatment vs. Baseline (B3 vs. B1)	
	Upregulation	Downregulation	Upregulation	Downregulation
EPI	11/20	9/20	11/20	9/20
DERM	9/20	11/20	8/20	12/20

6.1.3.2 Statistical analysis

Repeated-measures ANOVA did not detect any statistical significance between the groups hence the post hoc Bonferroni test was not required in this scenario.

6.1.4 Smad3

To determine whether TGF-β signalling had been activated the immunolocalisation of Smad3 to the nucleus was evaluated. An affinity-purified rabbit polyclonal antibody against a 20 amino acid synthetic peptide derived from the central portion of the linker domain of human Smad3 was utilized. The antibody was selected based on a staining procedure described in a publication by Kalinina et al[494].

Immunoreactivity

Pronounced nuclear staining for Smad3 was observed in the post-treatment sections (**Figure 6.10**, bottom panel and **Figure 6.11**). Immunolocalisation of Smad3 to the nucleus was observed in both epidermal and dermal cells. However, it was more predominant in the epidermis and the epidermal appendages (**Figure 6.12**). Smad3 immunoreactivity was only present in the nuclei of a few cells in the dermis, which were probably inflammatory cells and/or fibroblasts.

Negative control

Smad3 immunoreactivity was not detectable when sections were incubated in the absence of the primary Smad3 antibody (**Figure 6.10**, top panel). There was no blocking peptide available for this antibody.

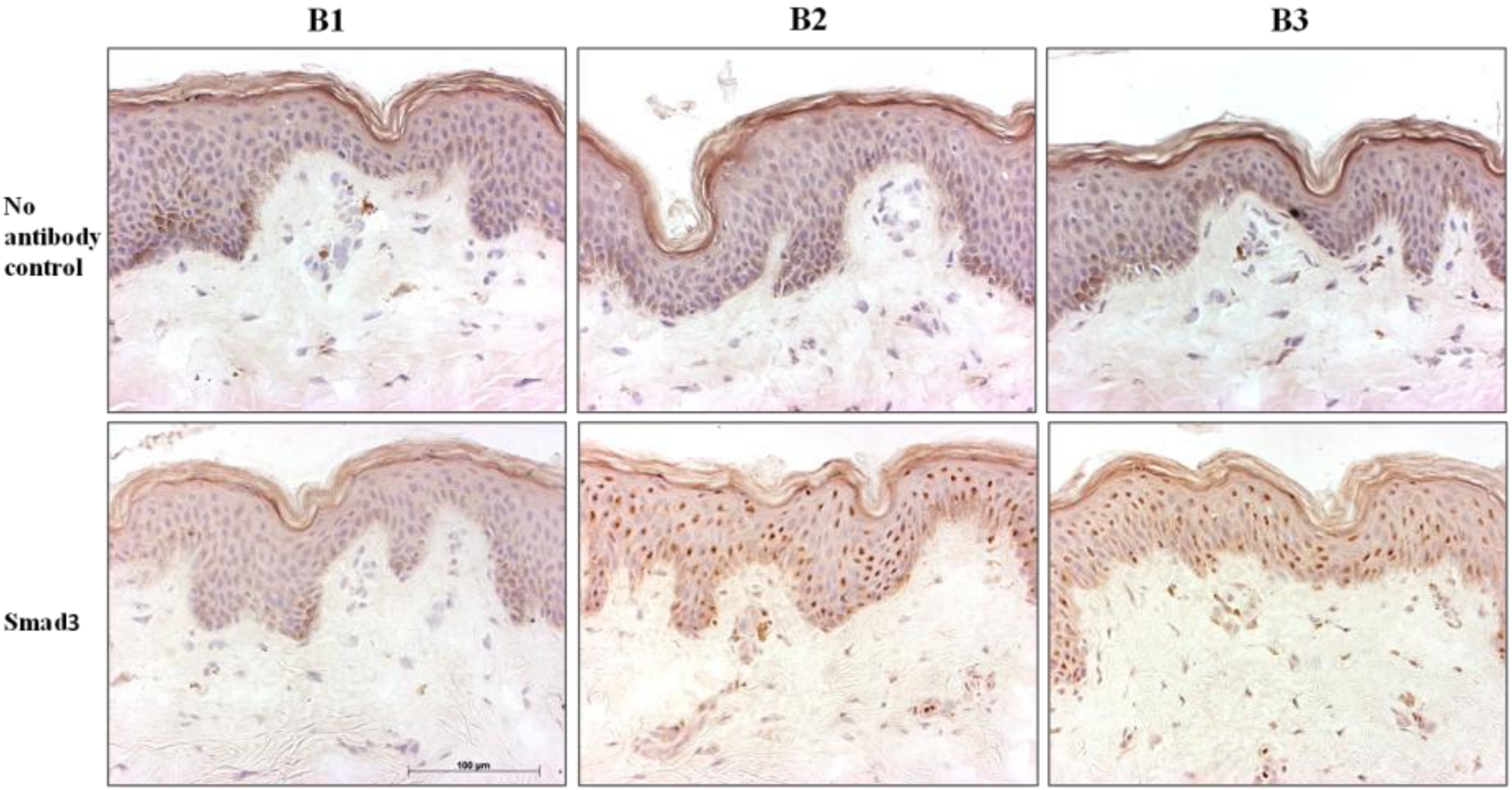

Figure 6.10 Immunolocalisation of Smad3 to the nucleus in the post-IPL sections (B2 and B3). Representative photomicrographs of smad3 staining are shown from one patient (bottom panel). No detectable nuclear staining in the absence of the primary antibody (top panel).

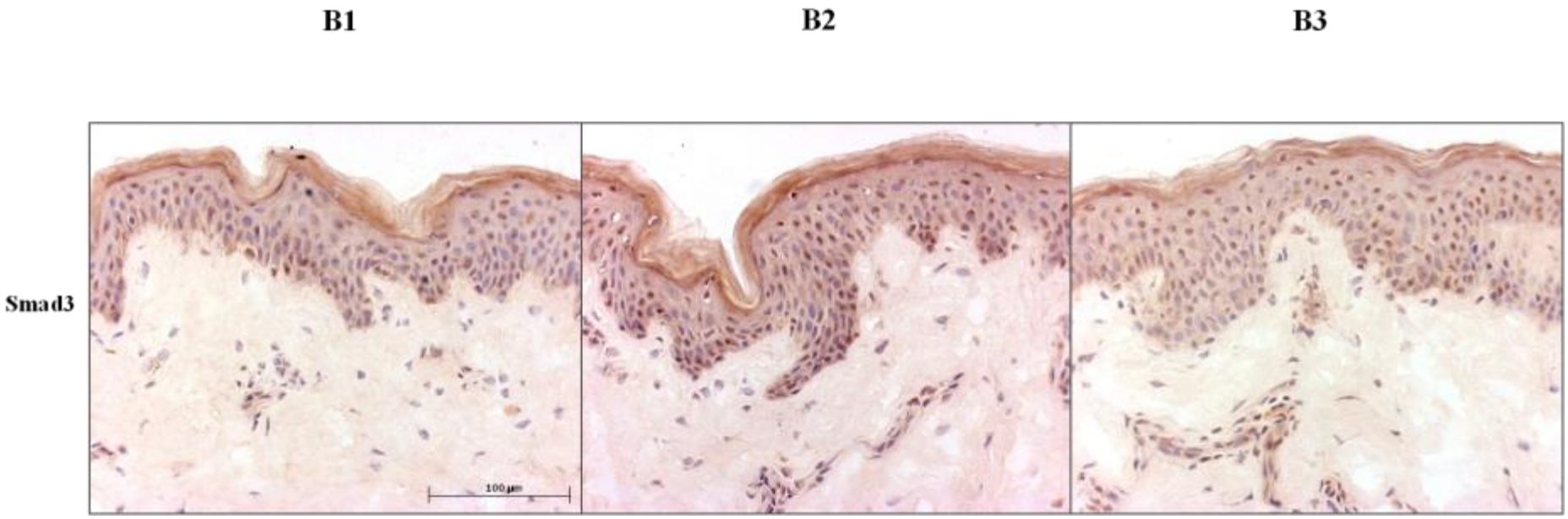

Figure 6.11 Representative photomicrographs of Smad3 staining from another case. Enhanced nuclear localisation of Smad3 in the post-IPL sections (B2 and B3).

B1 **B2**

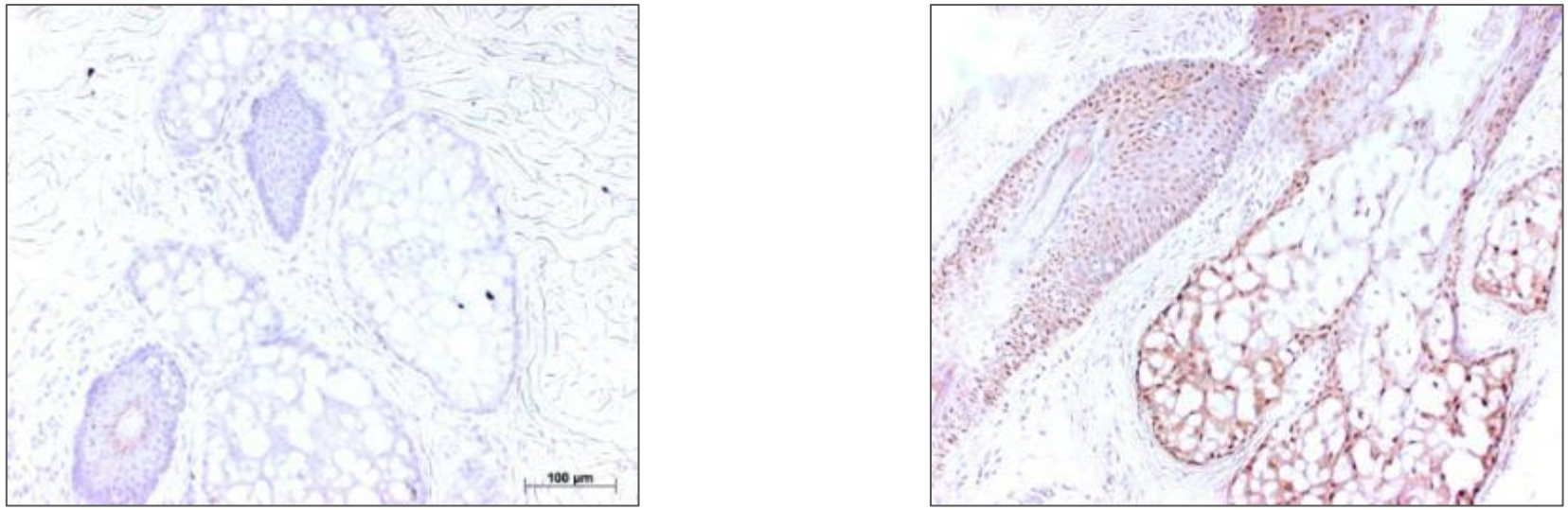

Figure 6.12 Smad3 immunoreactivity in the pilosebaceous unit. Representative photomicrographs (B1 and B2) from one case. Comparable photomicrograph for B3 was not available.

6.1.4.1 Image analysis

The immunoratio plugin of the ImageJ image-analysis software (available at: http://imtmicroscope.uta.fi/immunoratio/) was used to count the Smad3-stained nuclei and total nuclei, and the percentage of the Smad3 stained-nuclei was calculated.

In majority of the cases, there was an increase in the translocation of Smad3 into the nucleus post-IPL when compared to baseline (**Table 6.4**).

Table 6.4 Effect of IPL on the nuclear localisation of Smad3 in the 20 cases

Nuclear Smad3	48 hrs after 1st treatment vs. Baseline (B2 vs. B1)		1 wk after 4th treatment vs. Baseline (B3 vs. B1)	
	Upregulation	Downregulation	Upregulation	Downregulation
EPI	18/20*	2/20	19/20*	1/20
DERM	16/20*	4/20	15/20*	5/20

*Statistically significant at $p < 0.05$

6.1.4.2 Statistical analysis

The one-way repeated measures ANOVA revealed that the mean nuclear translocation of Smad3 differed significantly between the different time points, [epidermis: F (2, 38) = 23.884, p = 0.000000192 and dermis: F (2, 38) = 8.601, p = 0.001]. The Post hoc Bonferroni correction revealed that IPL treatment elicited a statistically significant increase (epidermis: 1.45-fold and dermis: 1.14-fold) in the nuclear translocation of Smad3 from baseline to 48 hours after first treatment session in both the epidermis (Mean $\pm$ SEM; 48.9 $\pm$ 3.6 *vs.* 71 $\pm$ 3.05, p = 0.000055, respectively) and dermis (Mean $\pm$ SEM; 57.18 $\pm$ 3.08 *vs.* 65.19 $\pm$ 2.65, p = 0.014, respectively). Similarly, there was a statistically significant increase (epidermis: 1.38-fold and dermis: 1.18-fold) in nuclear translocation of Smad3 from baseline to 1week after the last treatment session in the epidermis (increased to 67.35 $\pm$ 2.66; p = 0.00024) and dermis (increased to 67.4 $\pm$ 2.48; p = 0.008). However, there was no statistically significant difference between B2 (48 hours after the first IPL treatment) and B3 (1 week after the final treatment) ($p > 0.05$).

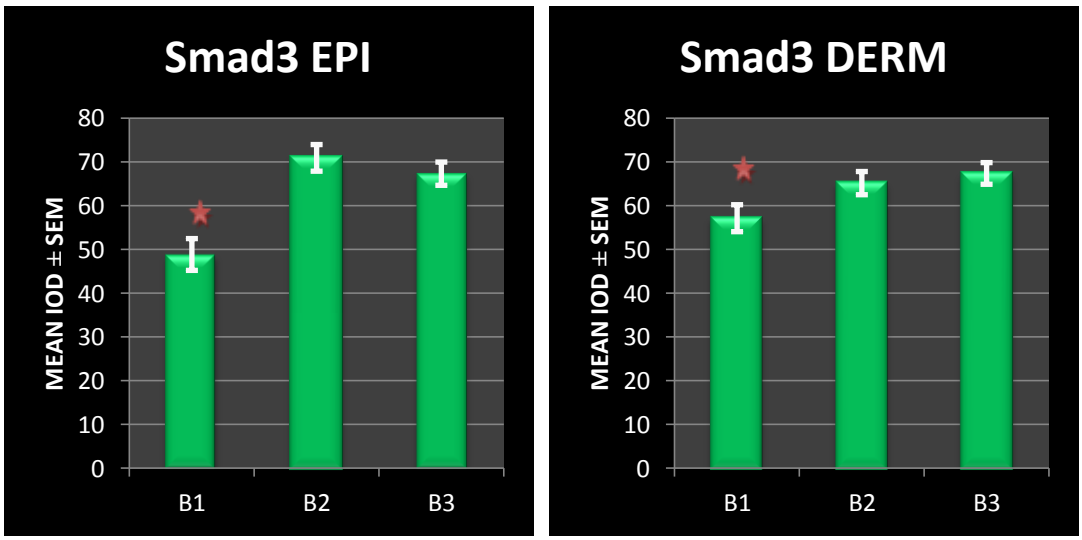

Figure 6.13 Effect of IPL on nuclear immunolocalisation of Smad3. IOD: integrated optical density; Error bars denote standard error of mean, SEM; the red star denotes a statistically significant difference between baseline and post-IPL expression.

We can, therefore, conclude that IPL irradiation results in the nuclear translocation of Smad3.

6.2 Effects on pro-inflammatory mediators IL-8 & MMP-1

6.2.1 Interleukin-8 (IL-8)

To determine whether IPL has an effect on the pro-inflammatory mediators implicated in acne pathogenesis, the expression of IL-8 was immunohistochemically evaluated using a mouse monoclonal antibody against human IL-8. The antibody selected and the staining protocol employed were based on a previous publication by Arici et al[495].

The trend of IL-8 expression at the various time-points was quite variable. A decrease in IL-8 staining was observed in the post-treatment sections in most of the cases (**Figure 6.14**). However, in a few cases there was an increase in staining post-IPL (**Figure 6.15**). The localisation of the staining is as described below.

Epidermis and its appendages

Immunoreactivity to IL-8 was observed to be both intracellular and intercellular. Moderate to intense staining was present in all the layers of the epidermis. However, it was more prominent in the upper layers of the epidermis (**Figures 6.14**). In addition there was staining of the hair follicles, sebaceous glands (**Figure 6.16**) and sweat glands.

Dermis

Immunoreactivity to IL-8 was moderate to intense in some cells in the dermis (probably inflammatory cells and fibroblasts). Prominent staining of the endothelial cells of the dermal vasculature was observed. In addition there was staining of the arrector pili muscle.

Negative control and blocking peptide

There was no detectable staining in the sections incubated in the absence of the primary IL-8 antibody (**Figure 6.14**, top panel). There was no blocking peptide available for this antibody.

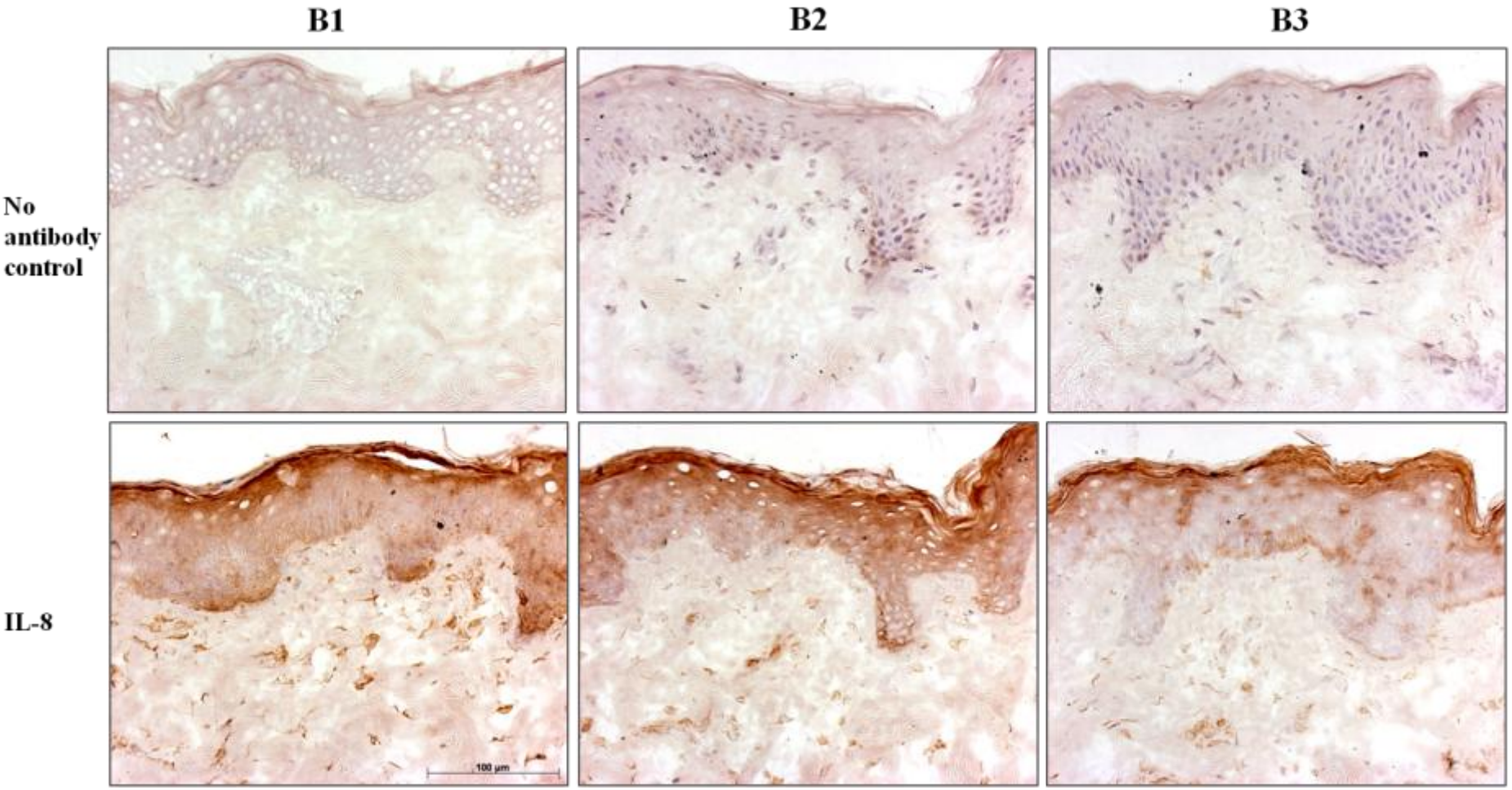

Figure 6.14 Immunohistochemical staining for IL-8 showing decreased staining in the post-IPL sections. Representative photomicrographs of IL-8 immunoreactivity in one case (bottom panel). No detectable staining in the absence of the primary antibody (top panel).

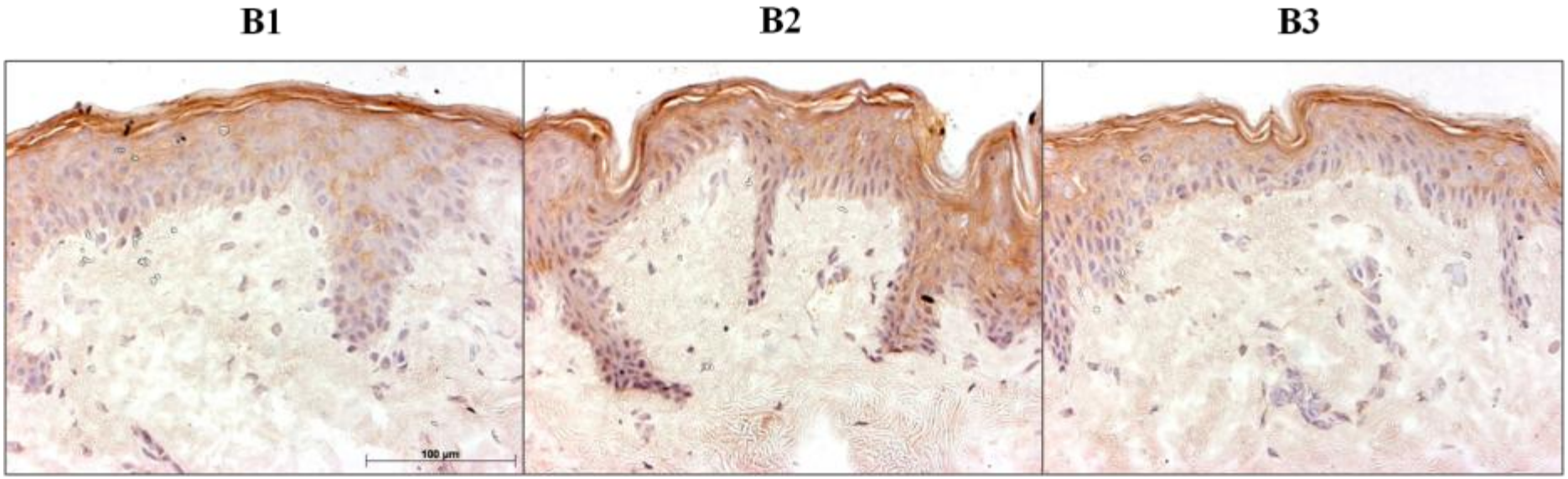

Figure 6.15 Immunohistochemical staining for IL-8 showing an increase in staining in B2 and a subsequent decrease in B3.

B1 **B3**

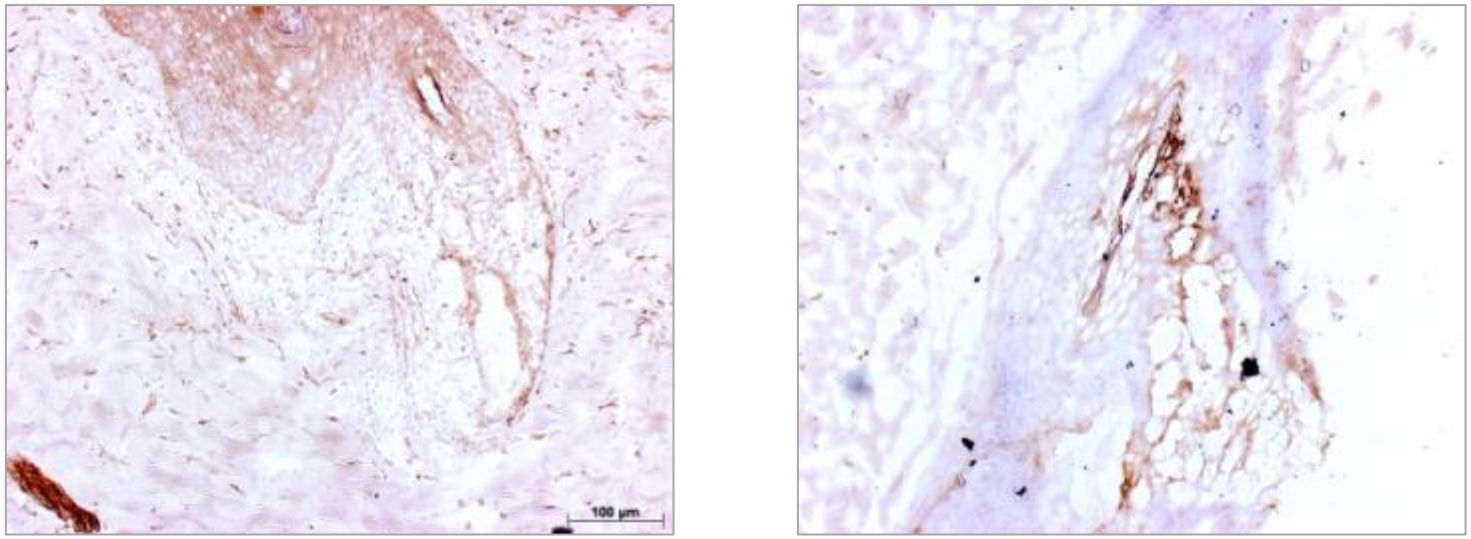

Figure 6.16 IL-8 immunoreactivity in the pilosebaceous unit. Representative photomicrographs (B1 and B3) from one case. Comparable photomicrograph for B2 was not available.

6.2.1.1 Image analysis

Quantification of the staining was carried out using Image Pro Plus 6.0 image analysis software. IL-8 expression showed a downward trend post-IPL in majority of the cases (Table 6.5).

Table 6.5 Effect of IPL on the expression of IL-8 in the 20 cases

IL-8	48 hrs after 1st treatment vs. Baseline (B2 vs. B1)		1 wk after 4th treatment vs. Baseline (B3 vs. B1)	
	Upregulation	Downregulation	Upregulation	Downregulation
EPI	6/20	14/20	5/20	15/20
DERM	4/20	16/20	7/20	13/20

6.2.1.2 Statistical analysis

Although a downward trend was observed (**Figure 6.17**), repeated-measures ANOVA did not detect any statistical significance between the groups hence the post hoc Bonferroni test was not required in this scenario.

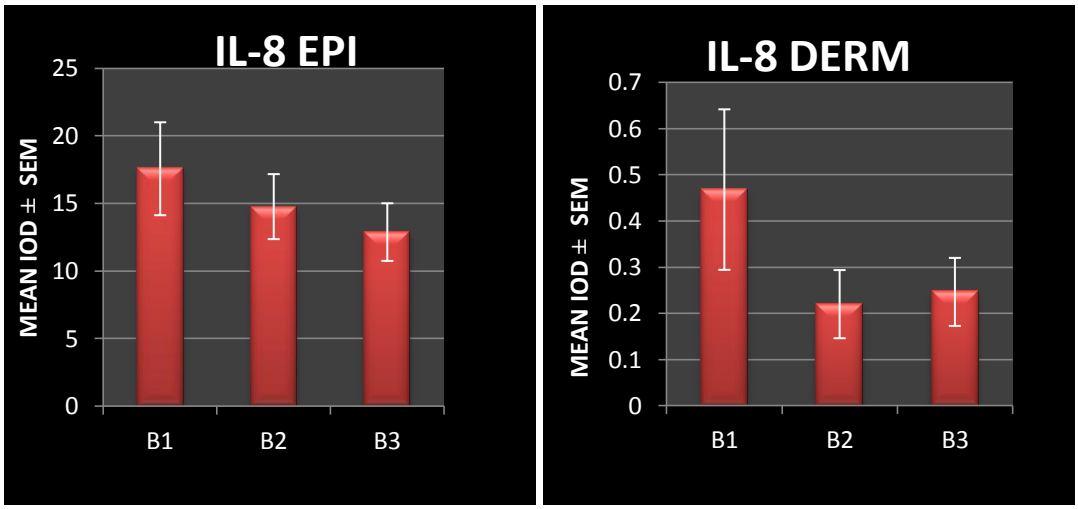

Figure 6.17 Effect of IPL on IL-8 expression. IOD: integrated optical density; Error bars denote standard error of mean, SEM.

6.2.2 Matrix Metalloproteinase-1 (MMP-1)

To determine whether IPL has an effect on matrix degrading enzymes that may play a role in acne inflammation and scarring the immunohistochemical evaluation of MMP-1 expression was carried out. A mouse monoclonal antibody against a synthetic peptide corresponding to amino acids 332-350 of human MMP-1 was utilised. We selected this particular MMP-1 antibody based on its previous use for immunohistochemical analysis in frozen sections of human skin as reported in a publication by Jalian et al[92].

Epidermis and its appendages

Moderate to intense immunoreactivity to MMP-1 was observed in all the layers of the epidermis (**Figure 6.18-6.20**). The staining was predominantly cytoplasmic but in some cases staining was observed both in the nuclei and in the cytoplasm (**Figure 6.19**) consistent with previous reports[503,504]. Moreover, Limb et al[504] reported that intracellular association of MMP-1 to the nuclei and mitochondria of cells confers resistance to apoptosis in those cells, which may contribute to cell survival. In addition there was staining of the hair follicles, sebaceous glands (**Figure 6.21**) and sweat glands.

Dermis

Immunoreactivity to MMP-1 was moderate to intense in some cells in the dermis. Prominent staining of dermal cells that appear morphologically similar to fibroblasts was observed.

Negative control and blocking peptide

Staining was not detectable in the sections incubated in the absence of the primary MMP-1 antibody (**Figure 6.18**, top panel). There was no blocking peptide available for this antibody.

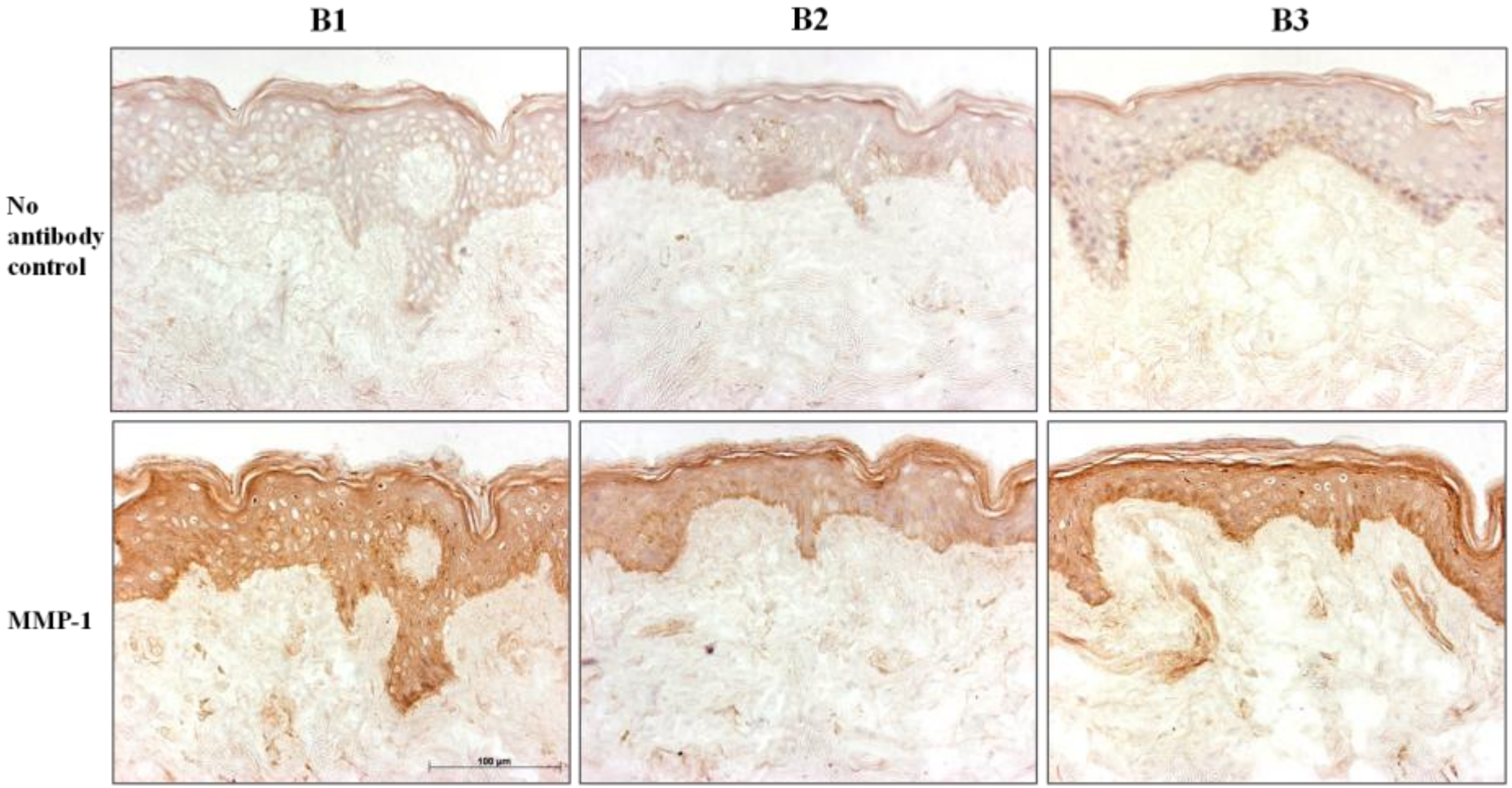

Figure 6.18 Immunohistochemical staining for MMP-1. There is an initial decrease in staining in B2 followed by a subsequent increase in B3 (bottom panel). No detectable staining in the absence of the primary antibody (top panel).

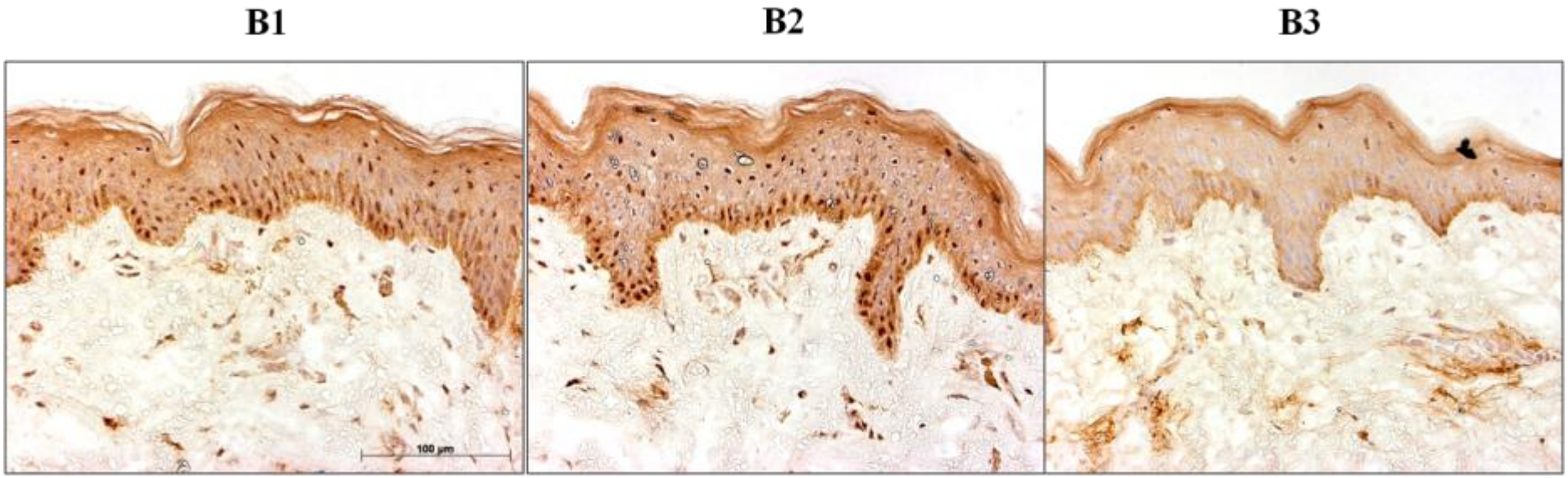

Figure 6.19 Immunohistochemical staining for MMP-1 demonstrating a case wherein staining was observed both in the nuclei and in the cytoplasm

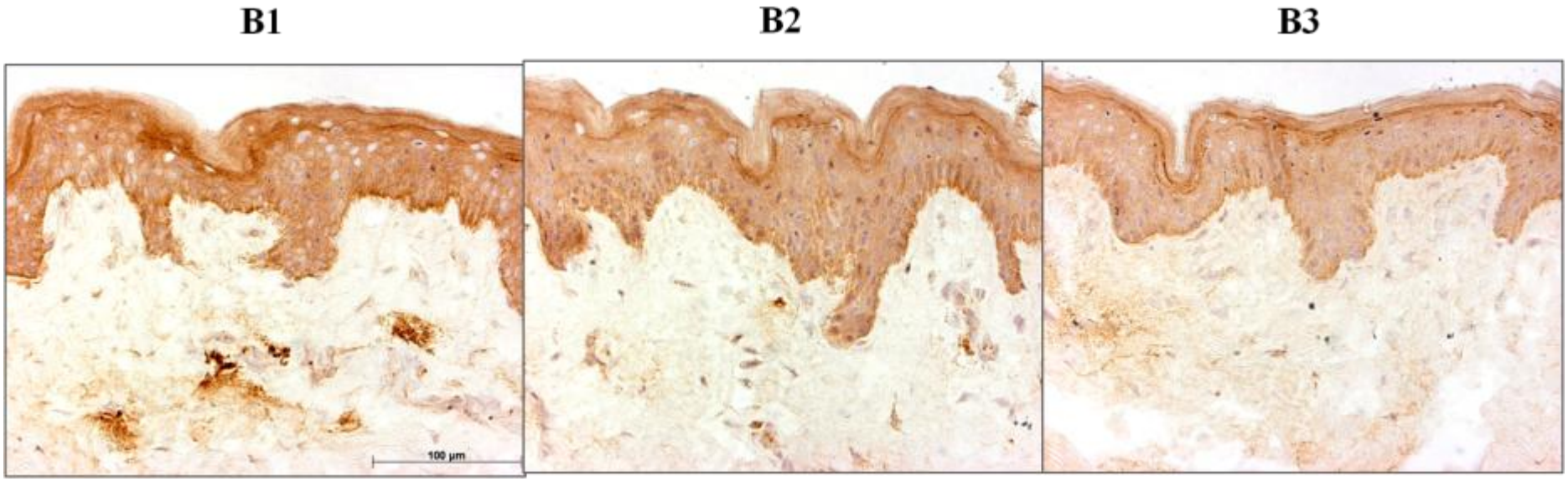

Figure 6.20 Immunohistochemical staining for MMP-1 demonstrating a progressive decrease in staining from B1 to B3.

B1 **B3**

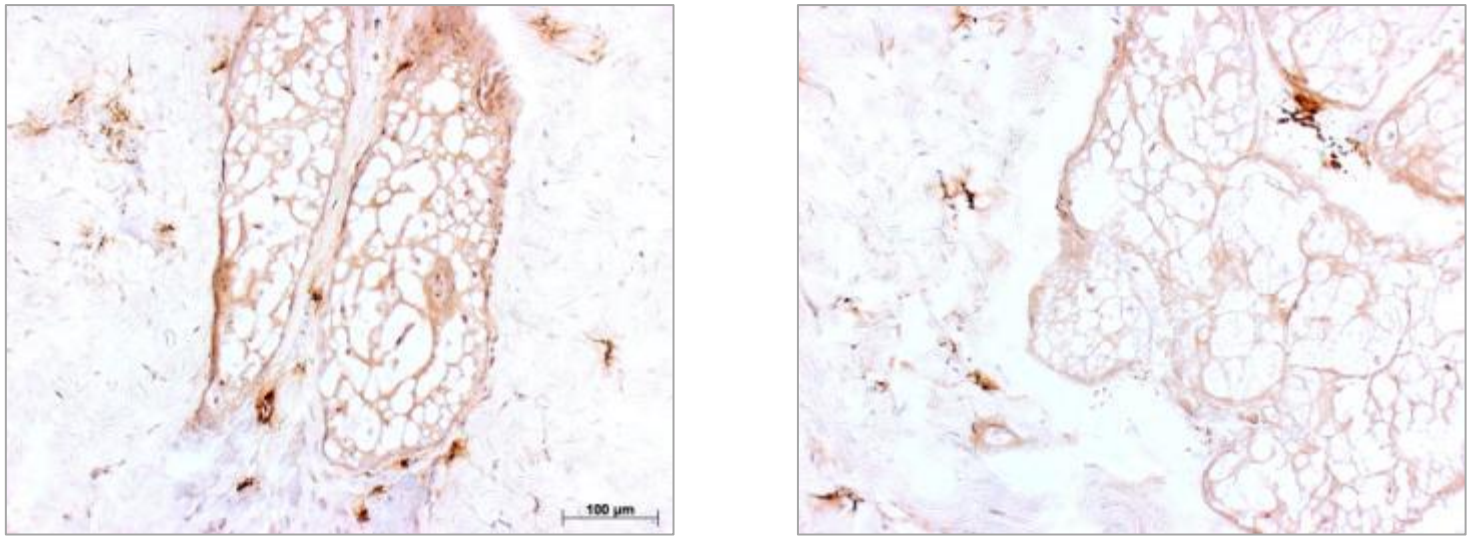

Figure 6.21 MMP-1 immunoreactivity in the sebaceous gland. Representative photomicrographs (B1 and B3) from one case. Comparable photomicrograph for B2 was not available.

6.2.2.1 Image analysis

Quantification of the staining was carried out using Image Pro Plus 6.0 image analysis software. MMP-1 expression showed a downward trend post-IPL in most of the cases (**Table 6.6**).

Table 6.6 Effect of IPL on the expression of MMP-1 in the 20 cases

IL-8	48 hrs after 1st treatment vs. Baseline (B2 vs. B1)		1 wk after 4th treatment vs. Baseline (B3 vs. B1)	
	Upregulation	Downregulation	Upregulation	Downregulation
EPI	6/20	14/20	5/20	15/20
DERM	6/20	14/20	4/20	16/20

6.2.2.2 Statistical analysis

On statistical analysis using the repeated-measures ANOVA, the mean MMP-1 expression in the epidermis was found to be significantly different between the different time points, $F(2, 38) = 3.322$, $p = 0.047$. However, on applying the post-hoc Bonferroni test no significance was found ($p > 0.05$ between all groups). This discordance could be attributed to a Type I error in the ANOVA or it could be due to the fact that the post hoc Bonferroni correction is quite a conservative test.

On the other hand, in the dermis repeated-measures ANOVA did not detect any statistical significance between the groups and hence the post hoc Bonferroni test was not required.

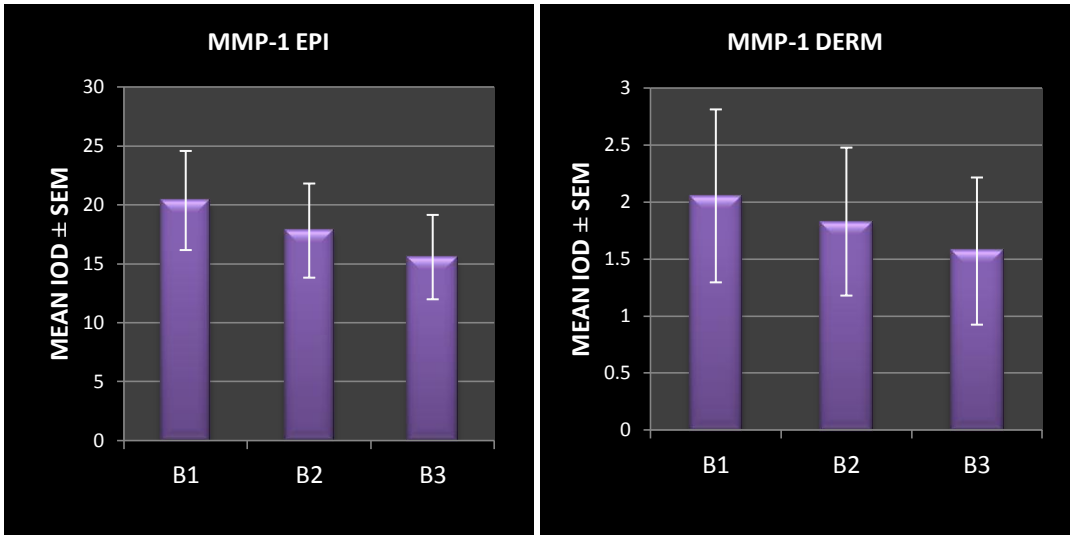

Figure 6.22 Effect of IPL on MMP-1 expression. IOD: integrated optical density; Error bars denote standard error of mean, SEM.

6.3 Western blotting

For the TGF-β isoforms, the corresponding blocking peptides were available to validate the specificity of their antibodies. However, blocking peptides were not available for the antibodies against Smad3, IL-8, and MMP-1. Therefore, to confirm the specificity of these antibodies western blotting was performed on cell lysates obtained from a breast cancer cell line and a HaCaT keratinocyte cell line.

6.3.1 Smad3

A band of approximately 52 kDa was detected (**Figure 6.23**) in the breast cancer cell line, consistent with the expected molecular weight of Smad3 (Molecular weight: 49-55 kDa).

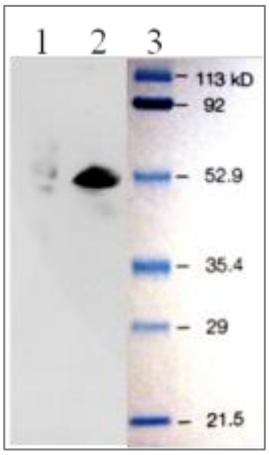

Figure 6.23 Western blot analysis of the antibody against Smad3. Lane 1: HaCaT keratinocyte cell line, Lane 2: Breast cancer cell line and Lane 3: low-range prestained SDS-PAGE molecular weight marker.

6.3.2 IL-8

There was a lot of cross-reactivity with this antibody and the bands did not correlate with the predicted molecular weight of IL-8 (Data not shown). Nevertheless, as per the company documentation this antibody is only suitable for performing immunohistochemistry.

6.3.3 MMP-1

No bands were detected for MMP-1 in any of the samples tested. This may be because MMP-1 was not expressed at detectable levels in these cells in culture or the epitope was destroyed during protein preparation for gel electrophoresis, thus affecting its ability to interact with the antibody.

Therefore, only the specificity of the Smad3 antibody could be validated from the western blotting analysis.

6.4 TaqMan low density array (TLDA) based quantitative real-time PCR

To evaluate the gene expression of TGF-β1, Smad3 and IL-8 a highly sensitive quantitative real-time-PCR assay based on TLDA technology was used. Based on the geNORM Visual Basic application, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was found to be the house-keeping gene with the most stable expression over the three time-points and was used as the endogenous control (active reference for normalisation). The threshold cycle (Ct) for each gene was measured in triplicate. The mean ΔCt values obtained by normalising the Ct values to the GAPDH levels were used to calculate the relative gene expression levels using the formula: $RQ = 2^{-\Delta\Delta Ct}$ (comparative Ct method). Gene expression is shown as fold change in average Ct value relative to expression levels at baseline. Due to a limited availability of samples there was only sufficient RNA from 7 cases.

There was a 1.33-fold increase in the expression of TGF-β1 at 48 hours following the first treatment, and one week after the final treatment session there was a 1.23-fold increase in expression (**Figure 6.24**)

6.4.1 Statistical analysis

Repeated-measures ANOVA did not detect any statistical significance between the groups hence the post hoc Bonferroni test was not required in this scenario. This may be attributed to the small sample size of seven cases, which violates the assumptions of parametric

testing. Hence, a non-parametric equivalent of the repeated-measures ANOVA, the Friedman's ANOVA test was applied to the data. However, the data was found not to be significant even with this test.

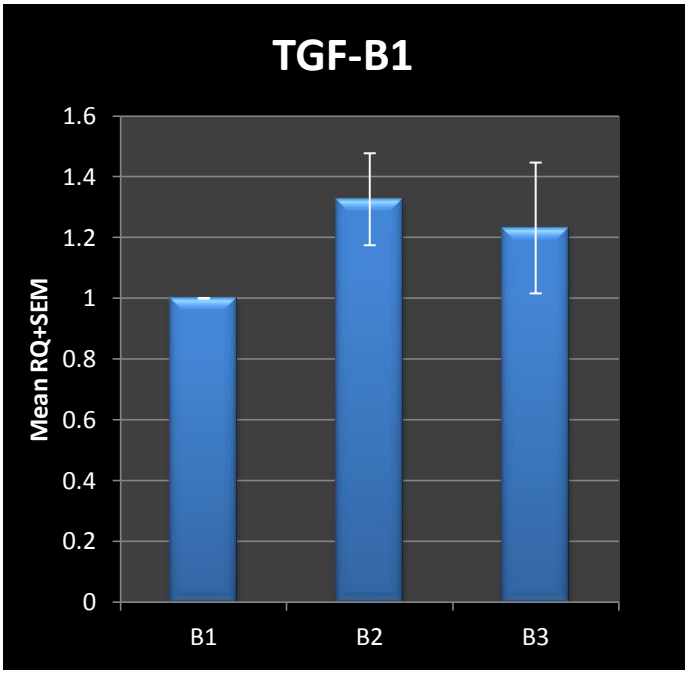

Figure 6.24 Mean fold-change in TGF-β1 gene expression at the different time-points. The data are represented as mean ± SEM relative to baseline and relative to the reference gene; RQ = relative quantity

Similarly, no statistically significant difference in gene expression were detected for Smad3 (**Figure 6.25**) or IL-8 (**Figure 6.26**). Nonetheless, for Smad3 our main focus was nuclear localisation rather than a change in gene expression. The data was included as Smad3 was present on the TLDA panel.

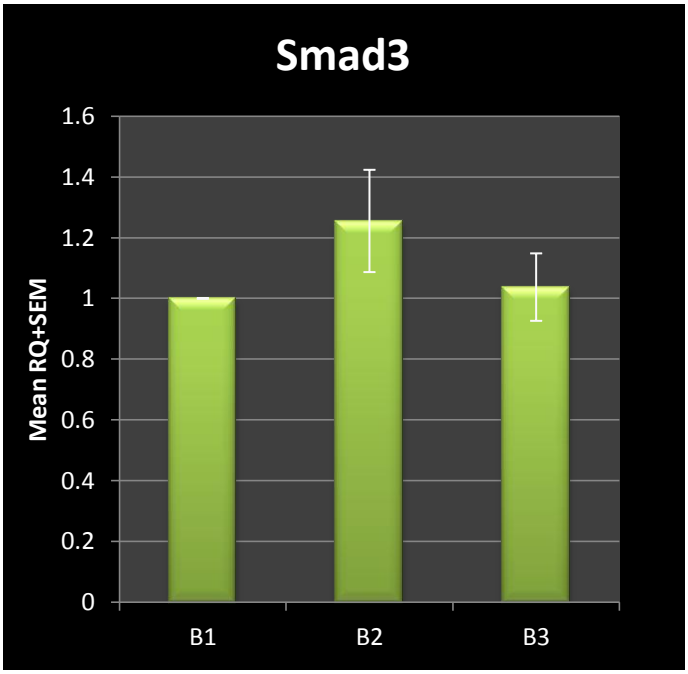

Figure 6.25 Mean fold-change in Smad3 gene expression at the different time-points. The data are represented as Mean ± SEM relative to baseline and relative to the reference gene; RQ = relative quantity

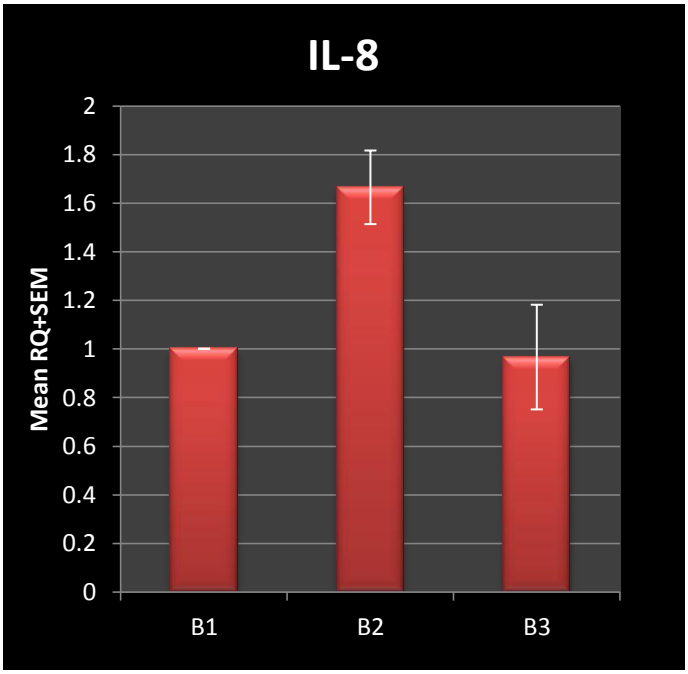

Figure 6.26 Mean fold-change in IL-8 gene expression at the different time-points. The data are represented as Mean ± SEM relative to baseline and relative to the reference gene; RQ = relative quantity

6.5 Correlation of results with clinical parameters

Correlation of the data on the expression of the various markers with the various clinical parameters such as inflammatory lesion count, non-inflammatory lesion count, sebum excretion rate and Leeds score (data kindly provided by Dr Marisa Taylor who conducted the clinical part of the study) were carried out using Pearson's product-moment correlation coefficient test. However, no significant correlation was found for any of the markers with any of the clinical parameters. One example is depicted in the graph below (**Figure 6.27**).

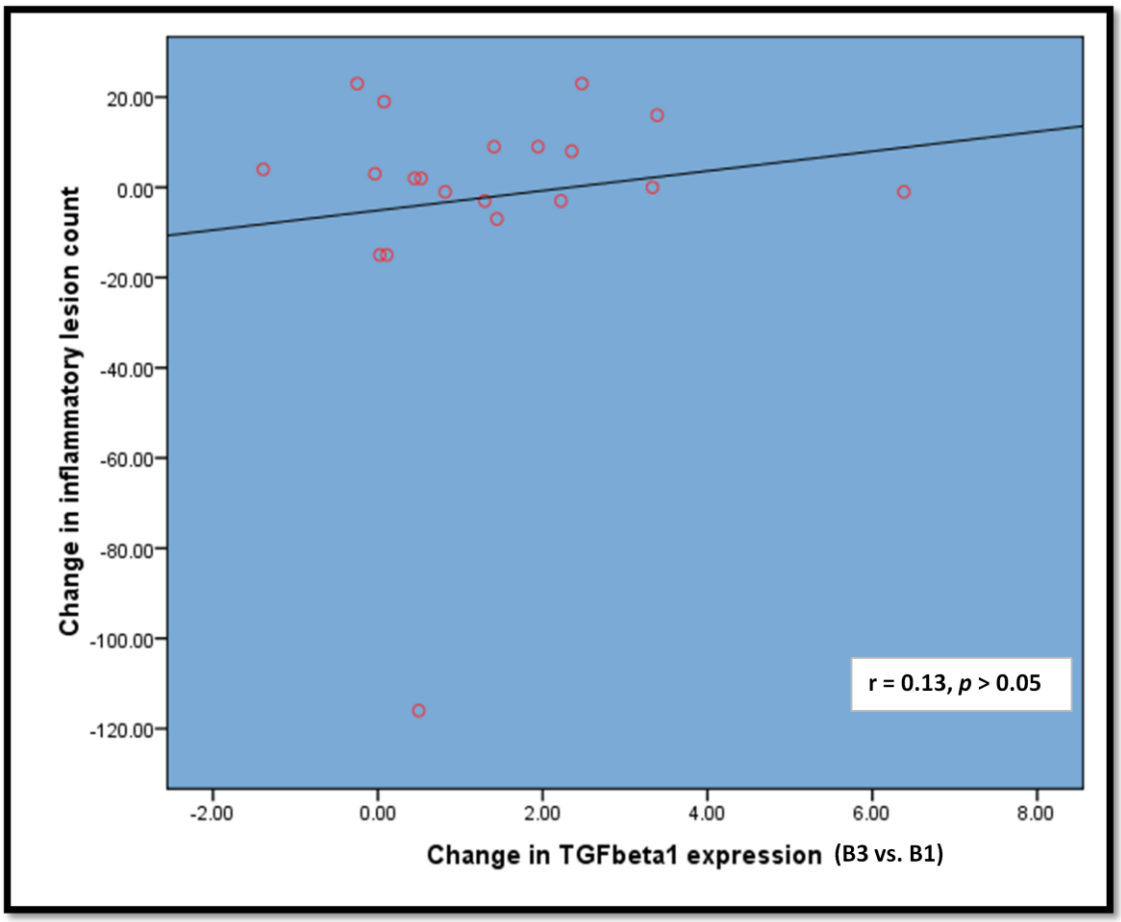

Figure 6.27 Representative graph of the correlation between change in inflammatory lesion count and TGF-β1 expression after 4 sessions of IPL treatment. There was no significant correlation between the two variables ($p > 0.05$); r represents the Pearson's product-moment correlation coefficient

6.6 Summary:

Immunohistochemical evaluation performed on the biopsies obtained from the 20 cases demonstrates that IPL upregulated the epidermal expression of TGF-β1 by 1.6 fold (p = 0.004) at 24hrs after the first treatment session and this increase was sustained at one week after the fourth and final treatment session (p = 0.007). The specificity of the staining was confirmed by using a blocking peptide to TGF-β1. However, the gene expression of TGF-β1 did not show a statistically significant change with IPL treatment (Table 6.7)

Table 6.7 Fold-change in TGF-β1 protein and mRNA expression after IPL treatment

TGF-β1	48 hrs after 1st treatment vs. Baseline (B2 vs.B1)	1 wk after 4th treatment vs. Baseline (B3 vs. B1)
Protein	1.6*	1.6*
mRNA	1.3	1.2

*Statistically significant at $p < 0.05$

IPL did not seem to affect the expression of the other TGF-β isoforms (TGF-β2 and TGF-β3).

Smad3 was demonstrated to be immunolocalised to the nucleus in the post-IPL samples when compared to baseline, suggesting activation of Smad3 and thus TGF-β signalling. The specificity of the Smad3 antibody used for immunohistochemistry was confirmed by western blotting.

Although not statistically significant, both IL-8 and MMP-1 expression showed a downward trend post-treatment in the majority of the cases. However, the specificity for either of these antibodies could not be confirmed by western blotting.

The next chapter discusses the implications of these results and also speculates on what future studies may be undertaken based on these findings.

Chapter 7: Discussion

7.1 Introduction

Light treatment is increasingly being considered as an alternative or complementary treatment option in the management of acne vulgaris. However, the research on this device is still in the infantile stages and the mechanisms responsible for clinical improvement are not clear. Most studies have laid emphasis on the clinical effect of intense pulsed light rather than elucidating its mechanism of action. There is a striking lack of studies in the literature that have investigated the molecular changes associated with intense pulsed light (IPL) treatment in acne. Only one other study could be detected within the search ranges used for this thesis[110]. The unregulated use of IPL by untrained medical staff and by non-medical operators in beauty establishments further warrants the need for understanding its underlying effect. This study attempted to contribute to the mechanistic explanation for use of IPL in acne-prone skin at the molecular level. This chapter discusses the general considerations drawn from the results and outlines the future studies that may be undertaken.

7.2 Interpretation of results

We hypothesized that IPL-induced TGF-β may play a potential role in the resolution of acne vulgaris, and therefore investigated the expression of the three mammalian TGF-β isoforms in skin biopsies obtained from twenty patients with mild to moderate inflammatory acne before (B1) and after IPL treatment (B2: 48 hours after the first IPL treatment and B3: 1 week after the fourth and final treatment). We have also examined the expression of one of the TGF-β signal transducers, Smad3 and two of the pro-inflammatory mediators implicated in acne pathogenesis, MMP-1 and IL-8. This was achieved by immunohistochemically evaluating and quantifying the expression of these markers by using image analysis software. In addition, quantitative PCR was employed to corroborate some of the data obtained from immunohistochemical analysis.

In this study the induction of TGF-β1 was epidermal and limited to the upper differentiated layers of the epidermis (the stratum granulosum and the lower layers of the stratum

corneum). This was consistent with the expression pattern reported by Levine et al[185] in normal skin.

Most of the currently published laser and IPL-photorejuvenation studies have usually focused on the expression of TGF-β1, and have not specified the effect on the individual isoforms. In this study, we have, for the first time, looked at the effect of IPL on the expression of the other two mammalian TGF-β isoforms as well.

The immunostaining for the TGF-β2 and TGF-β3 isoforms were much more intense and widely distributed than that for TGF-β1. The differential expression or localisation of the three TGF-β isoforms may be attributed to their differing receptor affinities and the distribution of receptors and may also be suggestive of their divergent functions in the skin. The specific role of each of the TGF-β isoforms in the human skin remains to be elucidated.

Based on quantification of the immunohistochemistry results by using image analysis software and statistical analysis, this study demonstrated that the epidermal TGF-β1 expression was significantly increased by 63% after the first treatment session and by 62% a week after the fourth session when compared to baseline, without any significant changes in the other isoforms. This may be due to the fact that the regulation of TGF-β2 and TGF-β3 expression is different from the regulation of TGF-β1 expression[267] . Further supporting the differences in the regulation of the TGF-β isoforms, a study conducted by Quan et al[229] reported that each TGF-β isoform was regulated differently by UV irradiation in human skin *in vivo*.

To determine whether the altered expression of TGF-β1 observed following IPL treatment was associated with changes in TGF-β1 gene expression, a TLDA based qRT-PCR was performed on the biopsies from seven cases. No significant differences in TGF-β1 mRNA levels were found in the post-IPL samples when compared to baseline. The modulation of TGF-β1 expression by IPL occurred in the absence of any significant changes at the mRNA level consistent with the TGF-β1 modulation by retinoic acid as reported by Fisher et al[445]. They demonstrated that treatment of human skin with Retin-A cream (0.1% retinoic acid) resulted in increased expression of epidermal TGF-β1 at the protein level but no changes were detected at the mRNA level[445].

Based on the data obtained by using keratinocyte-specific transgenic mouse models, Li et al[179] reported that the spatial pattern of TGF-β1 overexpression may determine its effects. They reported that suprabasal TGF-β1 overexpression was involved in inhibition of keratinocyte proliferation and suppression of cutaneous carcinogenesis at early stages, but promoted tumour invasion at later stages. Whereas, TGFβ1 overexpression in the basal layer of the epidermis and hair follicles resulted in a severe inflammatory and hyperproliferative skin disorder in the murine model[179]. Whether this is applicable to humans has not been determined and still needs to be investigated.

Enhanced TGF-β protein expression does not necessarily indicate increased TGF-β signalling. Therefore, to determine whether this increased TGF-β1 expression in the epidermis corresponds to increased activity, we examined the expression and immunolocalisation of one of its signalling transducers, Smad3 by immunohistochemistry in acne-prone skin. As discussed in Chapter 4, Smad3 signalling has been shown to play an important role in mediating the anti-proliferative effects of TGF-β on keratinocytes and also in mediating its stimulatory effects on ECM by dermal fibroblasts[478-480]. In addition, it has been demonstrated to be critical in mediating the immunomodulatory effects of TGF-β and also the TGF-β-induced negative regulation of MMP-1 is mediated through Smad3[241,242,266,336].

The presence of Smad3 in the nucleus is a direct indication of TGF-β signalling as nuclear translocation of Smad3 is an immediate response to TGF-β receptor activation [306]. This study has demonstrated for the first time an enhanced nuclear translocation of Smad3 following IPL treatment in the skin.

The increased percentage of nuclear staining of Smad3 in the post-IPL sections compared to baseline indicated that Smad3 was activated by stimulation of the TGF-β receptors by its ligand and was translocated to the nucleus. Thus, the data suggested that the IPL-irradiated acne-prone skin received signals of TGF-β in vivo. In addition, the immunolocalisation of Smad3 antibodies allowed direct visualisation of the cells that are responding to TGF-β signals. TGF-β1 was generally detected at the granular layer in the epidermis, whereas nuclear staining of Smad3 was seen in other epidermal layers, in the epidermal appendages and in a few dermal cells as well. Whether this observed

discrepancy was as a result of the contributions that were made from other isoforms remains to be determined. Nevertheless, the nuclear translocation of Smad3 following IPL irradiation suggests activation of the TGF-β signalling pathway.

Both IL-8 and MMP-1, which are implicated in acne pathogenesis showed a downward trend in their expression post-treatment in the majority of the cases. However, the changes were variable between different cases and therefore not statistically significant for either of these mediators. The failure to reach statistical significance may be attributed to the considerable variability in responses of the relatively small number of patients in the study. In addition, various extrinsic and intrinsic patient-related factors such as sun exposure, stress, hormones and characteristics of the stratum corneum may influence treatment effects and contribute to variability in responses.

Furthermore, the specificity for either of these antibodies could not be confirmed on western blotting. Due to the insufficient availability of frozen sections from the biopsies, alternative antibodies for these markers could not be tested. Therefore, these results need to be interpreted with caution.

TGF-β signalling has received much less attention in the context of acne vulgaris. Based on the understanding from the currently available literature, it has been suggested that TGF-β may play a role in resolution of inflammation in acne vulgaris by downregulating the inflammatory response via inhibition of pro-inflammatory cytokine production and through inhibition of the proliferation and differentiation of inflammatory cells[152,267,318,319]. Yuan and Varga[266] demonstrated that TGF-β/Smad3 repressed NFκB-specific gene transcription in dermal fibroblasts. TGF-β is also known to mediate the apoptosis and clearance of inflammatory cells[505]. In addition, TGF-β suppresses immune responses by promoting the function of regulatory T-cells by inducing the expression of Foxp3[324,325]. The suppressive role of regulatory T-cells and apoptotic cell clearance are described to be important in the resolution of innate and adaptive host immune responses[505]. In this respect it would be interesting to determine whether regulatory T-cells play a role in acne resolution.

TGF-β1 has also been demonstrated to suppress inflammatory cytokine-induced expression of MMP-1 and MMP-3[266,375,455,484-487]. Both of these matrix degrading enzymes

have been implicated in acne inflammation and scarring[5,81]. Hahm et al[283] demonstrated constitutive elevation of matrix degrading activity in mice with genetic disruption of TGF-β signalling. Thus, emphasizing the significance of TGF-β signalling in the repression of these enzymes. Moreover, TGF-β also induces the production of inhibitors of these metalloproteinases such as TIMP-1 (tissue inhibitor of metalloproteinases-1) and TIMP-3[279-282]. Therefore, TGF-β may also be involved in suppressing inflammation and extracellular matrix degradation mediated by MMPs in acne pathogenesis.

In addition, TGF-β1 has been demonstrated to be a potent inhibitor of keratinocyte proliferation in several studies[286,287]. Freedberg et al[506], suggested that TGF-β restores the IL-1 activated hyperproliferative keratinocytes to a healthy normal phenotype. TGF-β1 may therefore be involved in inhibiting the abnormal hyperproliferation of infrainfundibular keratinocytes seen in acne vulgaris and may interfere with microcomedo formation.

All of these effects of TGF-β have been demonstrated to be mediated through Smad3. Therefore, we hypothesised that IPL-induced TGF-β/Smad3 signalling may play a role in the resolution of acne vulgaris by inhibition of inflammation and keratinocyte proliferation, and by suppressing matrix degrading enzymes (**Figure 7.1**). Furthermore, increased FGFR2 signalling has recently been suggested to play a role in acne pathogenesis and TGF-β signalling has been reported to suppress downstream FGFR2 signalling by inducing the regulatory protein *Sprouty*, an important FGFR antagonist[451].

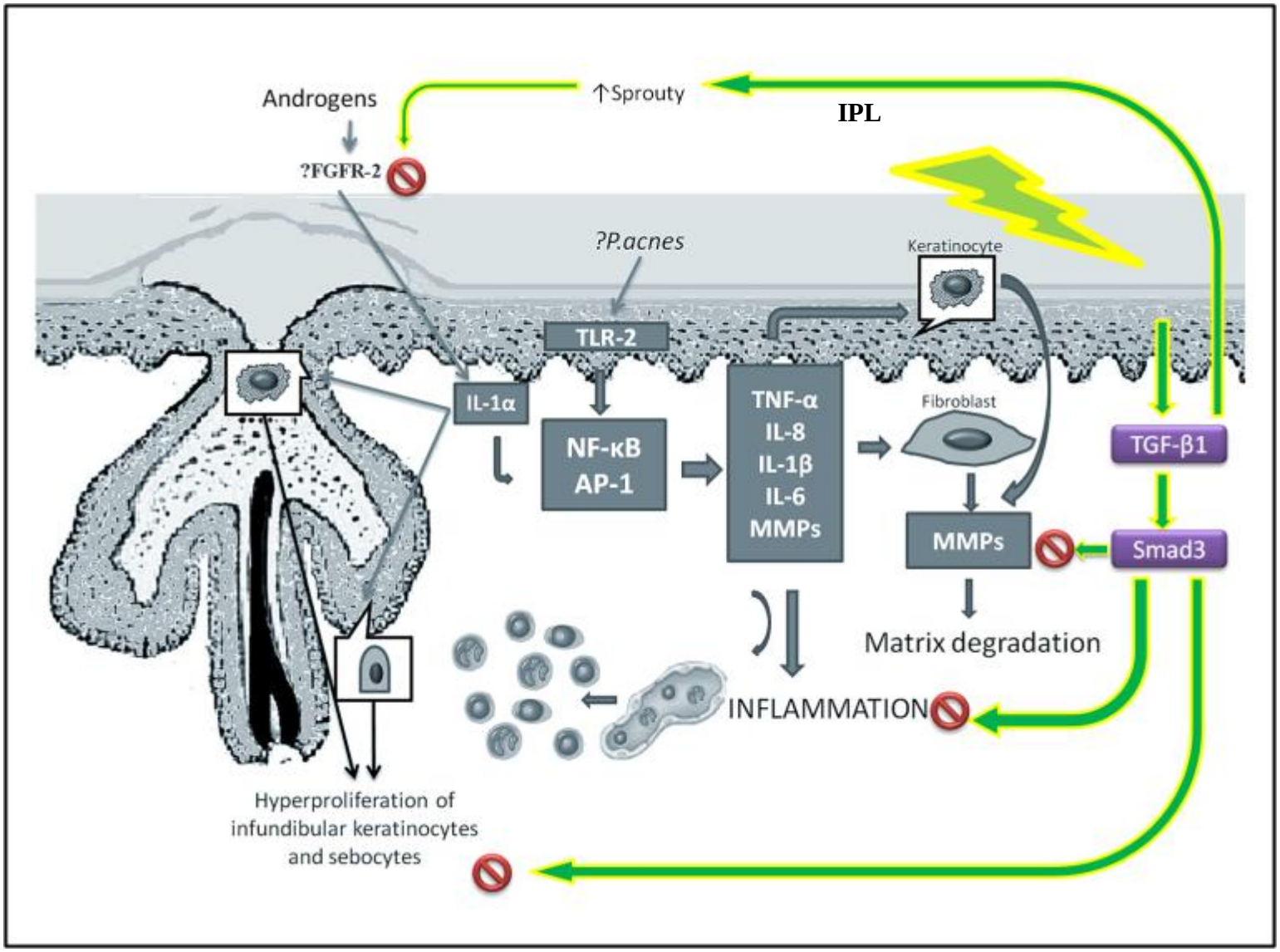

Figure 7.1 Schematic illustration of the proposed mechanism of action of intense pulsed light in acne vulgaris.

How IPL irradiation induces TGF-β1 production is not clear. It probably could be attributed to its photothermal action. In photorejuvenation, IPL is considered to induce heat-mediated cytokine activation[160]. Some hypothesise that it is induced as a wound healing response to photothermal injury[159,167]. Induction of TGF-β by heat shock proteins as a result of a heat-shock response has also been proposed[110]. Furthermore, TGF-β has been reported to be activated from its latent form by increases in temperature *in vitro*[218]. In addition, Fujimoto et al[507] demonstrated that keratinocytes cultured at a higher temperature (around 39°C) stimulate TGF-β1 production and suggested that under hyperthermal conditions, the epidermis can influence the functions of skin fibroblasts and matrix synthesis. This potential epidermal-dermal interaction may occur due to a paracrine influence of the TGF-β1 secreted from the keratinocytes. Future studies need to look into the activation mechanism responsible for the induction of TGF-β1 by IPL.

Modulation of TGF-β expression for attaining some of its therapeutic effects does not come without the possibility of triggering other undesirable effects such as the potential to induce fibrosis or to influence tumour initiation or progression[172,179]. The possibility of these effects underscores the need to understand the long-term effects of IPL treatment. It appears unlikely that the limited increase in IPL-induced TGF-β1 in the epidermis as demonstrated in this study would have the potential for mediating fibrosis. However, future studies need to address these issues.

The only study that evaluated the carcinogenic potential of IPL was a study conducted by Hedelund et al[508] in hairless, lightly pigmented female mice. This group reported that IPL treatment (Ellipse Flex system, DDD, Denmark; λ = 530-750 nm, fluence: 9.0 J/cm^2, spot size: 10 x 48 mm, single passes with double pulses, pulse delay: 10 ms and pulse duration: 2.5 ms) has no carcinogenic potential on its own and does not influence ultraviolet (UV)-induced carcinogenesis. Nevertheless, further long-term research is justified to assess whether IPL has a carcinogenic potential in human subjects.

Clinically, four sessions of IPL treatment (VPL Energist Ultra®; 530-950 nm Settings: 40 J/cm^2, 2 passes 15 pulses, 5 ms duration, 20 ms delay) at two week intervals resulted in an overall 28% reduction (p = 0.002) in the number of inflammatory lesions (data kindly

provided by Dr Marisa Taylor who conducted the clinical part of the study). However, the reduction in non-inflammatory lesions and sebum excretion rate were not statistically significant. An attempt was made to correlate the laboratory findings with the various clinical parameters. However, it did not show statistical significance, which may be attributed to the relatively small sample size and considerable variations between patients. In addition, the fact that the parameters were lesional and our findings were related to the non-lesional acne prone skin may also explain this discrepancy.

The increase in TGF-β1 expression demonstrated in this study although significant was not as profound as that reported in association with pulsed dye lasers and this may potentially be the explanation for the better clinical efficacy of pulse dye lasers when compared to IPL in treating acne vulgaris[110].

7.3 Limitations

This study is not without its limitations, some of which are mentioned in this section.
The quantification of protein expression was based on immunohistochemistry, which is semiquantitative. Although other methods such as western blotting and ELISA are more quantitative, they require pulverisation of the tissue resulting in the loss of morphological and spatial information. On the other hand, immunohistochemical analysis displays where the protein is expressed and in certain cases gives information about its bioavailability. However, if there were sufficient samples it might have been beneficial to do western blotting to obtain more reliable quantification.

The reproducibility and standardisation of immunohistochemistry has been questionable due to the diverse potential sources of variability including fixation conditions, specimen processing, reagents, detection methods, and interpretation of results[509]. To increase the accuracy of immunohistochemistry, this study has attempted to reduce the variability as far as possible by accurately following the staining protocol steps and timings, by using similar lot numbers of the antibodies and other reagents, by preparing a stock of the reagents that can be stored and by using standardised camera and computer settings for image acquisition. In addition, the system of grading specimens as either negative or positive or grading by the use of the pathologists 3- or 4-point scale is not considered

accurate or reproducible due to the high degree of intra-observer and inter-observer variability[509]. Moreover, these systems do not provide a truly continuous scale of measurement of protein expression[509]. The computer-assisted automated analysis programs eliminate some of these limitations to a considerable degree[509]. For these reasons, the Image Pro Plus image analysis software was utilised here. In addition, to minimising observer bias, the use of automated software for image analysis relinquishes the need to recruit personnel to analyse the results.

In view of the practical difficulties involved in timing the lesions, the biopsies that were available for analysis were taken from acne prone non-lesional skin in the vicinity of the lesion rather than lesional skin. Furthermore, for the purpose of accuracy in the comparative assessment of the sections, the epidermal appendages were excluded from the analysis because they were not present on all sections. Therefore, we were unable to visualise the effects of IPL on the expression of the various markers at the exact site of the lesion. Nevertheless, Jeremy et al[77] demonstrated that the pathology in acne vulgaris not only exists in the areas of the visible lesion, but also in the surrounding apparently normal looking skin.

There were only biopsies available from seven cases for studying the gene expression by PCR analysis. This may have introduced a type 2 error, which implies that the small sample size may have undermined the treatment effect by missing a true difference between the groups.

Overall, our study was relatively small in scale, and a larger sample would provide more robust analysis. Moreover, the study did not institute a control group, which would have allowed us to compare the specific molecular effects at the various time intervals between biopsies obtained from IPL-treated versus untreated skin.

Furthermore, the molecular effects were only examined up to 1 week after the last treatment session. Therefore, we do not have any idea about the long-term molecular effects of IPL treatment.

In retrospect, the western blotting employed for determining the antibody specificity should have been ideally carried out before performing the immunohistochemistry rather

than after. If the western blotting had been carried out prior to immunohistochemical analysis alternative antibodies could have been employed for IL-8 and MMP-1.

Interpreting dynamic processes by analysing biopsy material is challenging and the lack of a suitable animal model for acne hinders this process. Various attempts have been made by acne researchers to overcome this issue by using the syrian hamster flank organ and SZ95 sebocytes and by inducing *P. acnes* inflammation in mice[51,73,75]. However, these models are unable to reproduce the entire complexity of this disease.

7.4 Conclusion and Future Directions

The present study has provided some evidence on the potential molecular mechanisms that may be involved in the action of IPL on acne-prone skin. At this point we cannot make definitive conclusions, but can speculate that IPL-induced TGF-β1/Smad3 could assist in the resolution of inflammatory acne by inhibiting inflammation, matrix degradation and keratinocyte proliferation. This preliminary evidence although promising, needs further investigation as several questions still remain to be answered. Some specific areas that need be addressed in future studies are discussed below.

An important area of future research is to determine whether the TGF-β/Smad3 signalling pathway is directly involved in resolution of acne vulgaris. Clinical correlation and further experiments on the downstream effects of TGF-β1/Smad3 signalling need to be carried out to clarify whether the induction of TGF-β1 is a non-specific effect of IPL treatment or whether it actually mediates the therapeutic effects of IPL in acne-vulgaris. The full significance and the functional ramifications of TGF-β1/Smad3 signalling need to be further characterised. The effects of TGF-β1 on target mediators can potentially be investigated in organ cultures obtained from lesional and non-lesional skin from acne patients. Our data demonstrating nuclear translocation of Smad3 occurs due to TGF-β induced receptor-activation. Therefore, it would be interesting to look at the effect of IPL on the expression of the various TGF-β receptors. In addition, it would be intriguing to investigate the immunolocalisation of the inhibitory Smad, Smad7. At this point, we have not accounted for any non-canonical pathways due to limited knowledge on the effect of these pathways. As a result of practical constraints this study had a relatively small sample

size, did not have a control group and did not assess the long-term consequences of treatment. Therefore larger, controlled and blinded comparative trials with an extended follow-up are necessary to overcome these shortcomings and validate these initial findings.

It would also be particularly informative to have an idea about the timeline of the molecular effects by acquiring tissue samples at more frequent intervals during and also after the treatment period. Characterisation of the molecular events after treatment would also be needed to assess the long term consequences and the potential adverse effects. In addition, well-designed safety studies assessing the long-term effects would be prudent to explore whether IPL-induced TGF-β1 has a "dark" side such as any potential risks for fibrosis or carcinogenesis.

The molecular mechanisms involved in IPL treatment of acne vulgaris must be far more complex than the mere upregulation of TGF-β1 and may involve the interplay of several other cytokines, growth factors, and matrix degrading enzymes. Future studies should examine the effect of IPL on heat shock proteins and other inflammatory mediators of acne such as IL-1α, IL-1β and TNF-α. As more pieces of the puzzle are put together in context the picture will become a lot clearer.

Optical treatments have been reported to improve inflammatory acne on a short-term basis[510]. If this effect is confirmed to be mediated by TGF-β it could be explained by the fact that the active TGF-β induced by these treatments has a much shorter half life than its latent form and therefore its action is limited to the immediate therapeutic period. Future studies need to determine whether more sessions or more frequent sessions of treatment would be necessary to improve the treatment response.

The role of IPL in acne as a monotherapy is increasingly becoming questionable due to the temporary benefits and incomplete remission. The available literature suggests that combining it with other treatments would be more practical. In view of the fact that the inflammatory lesions showed a better response to IPL treatment compared to non-inflammatory lesions (as demonstrated in the clinical part of the study conducted by Dr Marisa Taylor) it would be advisable to combine the treatment with topical keratolytic agents to treat the non-inflammatory lesions.

Methodological constraints in both acne and IPL research have made evidence-based assessment of treatment challenging. Optimal settings for IPL in acne still remain to be established. To draw more meaningful and reliable comparisons from the data assimilated, future studies should have adequate sample size and need to address the issue of standardisation of the device, its parameters (fluence, number of passes, number of treatment sessions, interval between treatment sessions and technique employed) and the parameters for interpretation of outcomes. Moreover, our understanding of the contribution of various factors to acne development, exacerbation and remission is still incomplete. Knowledge on the light-based approaches will improve as the acne pathophysiology becomes better understood. Additional studies on the photoimmunomodulatory nature of light may enhance our understanding of optical treatments for acne as well as help improve the design and functions of future devices. In addition, well controlled studies that compare IPL with conventional acne therapies are needed.

This study may serve as a reference point for future studies and these findings could be extrapolated by performing similar studies with other devices and treatments used for acne. Understanding the mechanisms of the various treatments is necessary to overcome the current limitations of acne therapy. These findings could also be significant to the action of IPL in the treatment of other skin conditions.

In chapter 4 we have discussed the potential association between TGF-β signalling and some of the currently used treatments such as retinoids and other potential agents used in the treatment of acne vulgaris. It would be intriguing to explore this association in future studies. If it is conclusively proven to have a role in the resolution of acne vulgaris and found to be safe, it would be reasonable to develop potential strategies to modulate endogenous cutaneous TGF-β to control acne.

The application of IPL in the treatment of acne vulgaris is still a young and developing area. Much of the data from the currently available literature on IPL used for acne treatment are anecdotal or of suboptimal quality. Although, IPL seems to have a better safety profile and is well-tolerated by patients, its efficacy and sustainability appear to be inferior to the available conventional treatments. Moreover, the improvement of acne lesions with IPL is less convincing and less sustained when compared to that obtained with lasers and PDT. In one study, it was demonstrated that IPL effectively improved acne

sequelae such as red macules, irregular pigmentation and skin tone, but had no effect on the acne lesions[108]. This makes one contemplate whether the observed small improvement with IPL in acne is as a result of the improved appearance of the skin rather than from targeting the pathology.

Taking into account these facts, many obstacles still remain in the implementation of IPL therapy in acne management. Nevertheless, due to the limitations of the conventional acne treatments such as antibiotic resistance, patient compliance issues and teratogenicity, and the better tolerability of IPL it still has a potential role in acne management. At present its role is limited to the treatment of those who do not respond to conventional therapy, patients who are unable to tolerate the available conventional treatment options, patients who are non-compliant or in patients who specifically request this treatment. Further research and optimisation of the device parameters may broaden the horizons for IPL therapy in acne vulgaris. In view of the multifactorial nature of acne and the limited and short-lived efficacy of IPL, it would be beneficial to combine IPL with other conventional treatments or with ALA (ALA-IPL). We are still far from a thorough understanding of the molecular and signalling mechanisms involved in the action of this device due to considerable gaps in the understanding of acne pathogenesis, IPL treatment and TGF-β signalling. Therefore, the mechanisms underlying IPL action in acne vulgaris deserve further exploration as this may aid in the optimisation of this device and may assist in the clinical prerogative on its use in acne management. In addition, improved understanding of its mechanisms may provide clues on the pathogenesis of acne and may also provide novel insights into developing new therapeutic strategies for the management of this condition. Overall, it is clear that more research on a number of areas need to be carried out, to meet the increasing demand for a novel acne treatment that is effective, convenient to use, minimally invasive, well-sustained, has a rapid onset of action and has relatively few adverse effects.

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