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An Analysis of the Action of 530 – 950nm Intense Pulsed Light on the Global Severity & Inflammatory Markers in Mild to Moderate Acne Vulgaris

A thesis submitted for the award of the degree of

Doctor of Medicine

By

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Approval Sheet

AN ANALYSIS OF THE ACTION OF 530 – 950NM INTENSE PULSED LIGHT ON THE GLOBAL SEVERITY & INFLAMMATORY MARKERS IN MILD TO MODERATE ACNE VULGARIS

By

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Dedication

To my Father "Faithful & True", Who sustains all involved in this work and my parents, Crosswell & Norma Taylor, who sent me off on this venture with their blessings.

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Abbreviations

ANOVA	analysis of variance
AP-1	activator protein-1
APRT	adenine phosphoribosyl transferase
DAB	diaminobenzidine
DHT	dihydrotestosterone
ECM	extracellular matrix
ERK	extracellular signal regulated kinase
FGFR2	fibroblast growth factor receptor 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony stimulating factor
GNB	gram negative bacilli/ bacteria
GPB	gram positive bacteria
HKG	housekeeping gene
HSP	heat shock proteins
IL-1α	interleukin 1 alpha
IHC	immunohistochemistry
IKK	inhibitor of NF-κB kinase

IPL	intense pulsed light
JNK	*c*-Jun N-terminal kinase
MAL	methyl aminolaevulinate
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
ms	millisecond
NEMO	NF-κB essential modulator
NF-κB	nuclear factor kappa B
NOD	nucleotide-binding oligomerization domain
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphotidylinositol 3-kinase
PPARs	peroxisome proliferator–activated receptors
SER	sebum excretion rate
SCD	stearoyl coenzyme-A desaturase
SOCS3	supressor of cytokine signalling
SREBP	sterol regulatory element-binding proteins
STAT 3	signal transducer and activator of transcription 3

TGFβ	transforming growth factor beta
TIMP	tissue inhibitors of metalloproteinase
TLDA	Taqman® low density array
TLR 2	toll-like receptor 2
TNFα	tumour necrosis factor-alpha
TRAILR	TNF-related apoptosis inducing ligand

Abstract

Despite many studies on the action of yellow light in acne, it is still unclear whether it improves inflammatory acne or has photoimmunologic activity against pro-inflammatory pathways like toll-like receptor 2 (TLR2) and its down-stream cytokines e.g. TNF-α. This work sought to determine whether 530 nm IPL could cause a clinical improvement in acne and if its photo-mechanism of action involved modifications of the expressions of TLR2, TNF-α, IL-10 and IL-8.

Twenty-eight adult patients with mild to moderate acne vulgaris involving their backs received four 530-950 nm IPL treatments at two-week intervals. Assessments performed at baseline and one week after the final IPL session included inflamed and non-inflamed lesion counts, Leeds assessments and sebum excretion rate (SER). Biopsies within the treatment area were taken at these two time points and two days after the first irradiation. TLR2 expression was examined by immunohistochemistry and TaqMan® Low Density Arrays were used to measure changes in expression of TNF-α, TNFR, IL-8 and IL-10.

The data from 21 patients was included in the final clinical analyses. Inflamed lesions fell significantly by 28.0% ($p = 0.002$), but was not associated with significant changes in the Leeds score, SER or non-inflamed lesions. TNF-α expression fell by 17.6% ($p = 0.031$) at the end of therapy, and appeared to correlate with the percentage change in lesion counts in the subjects evaluated. TLR2 expression fell by 2.6% ($p < 0.001$) a week after the final irradiation, but bore no relationship to lesion counts. Neither IL-10 nor IL-8 was significantly affected.

Though 530nm IPL significantly reduces inflammatory lesions, treatment efficacy will have to be improved to make it a viable treatment option. Its mechanism seems to include an anti-TNF-α effect, independent of IL-10 up-regulation. This is a novel mechanism, not been previously described for 530nm IPL. Updated hypotheses are suggested in order to explore this phenomenon further.

Chapter 1

BACKGROUND 1

Acne Pathophysiology and its Therapeutic Targets

1. Acne Pathophysiology and its Therapeutic Targets

1.1 Introduction

As Western medicine evolves, alterations in the sub-cellular biological pathways have become the pivotal focus in the study of human ailments. Recent therapies are designed to manipulate and harness these pathways, coaxing a state of physiological imbalance back to health. The concept of 'targeted' therapeutics is not recent but rather, relatively crude tools have become more refined and continue to develop. In 2009, the US Food and Drug Administration approved the highest annual level of 'small-molecule' pharmaceuticals such as biologics [1]. Dermatology is now quite familiar with the use of biologics (e.g. anti-interleukin 12/23 and anti-tumour necrosis factor-α antibodies) [2] and more recently vemurafenib, a BRAF oncogene inhibitor said to modestly prolong survival in BRAF-V600E oncogene mutation-positive stage IV melanoma patients [3]. Molecular biology has discovered genetic targets, targeted diagnostics and targeted therapies. This has allowed us to re-think disorders and examine the probable reasons for a lack of success in treating them. Molecular techniques have also enabled closer analysis of therapeutic modalities with poorly characterised mechanisms of action.

Acne vulgaris management has also been studied using a reverse-engineering approach. The most heavily implicated factors in acne pathogenesis are comedogenesis, infundibular *Propionibacterium acnes* colonization, hormonally influenced seborrhoea, genetic susceptibility and inflammation [4-8]. Established management strategies tackle a combination of these aetiological factors with oral and topical antibiotics, anti-androgens,

anti-bacterials, anti-inflammatories, exfoliants and sebum secretion regulators. Many of these are quite useful but have a slow-onset of action fraught with undesirable side effects such as dryness, bleaching of hair and clothing, birth defects and the development of antibiotic resistance [9-11]. The result is poor patient compliance and left untreated in predisposed individuals, acne results in disfiguring scars and keloids, which are difficult to manage once established.

These difficulties have caused us to seek therapeutic alternatives such as laser and light devices [12, 13]. Intense pulsed light (IPL) is one such device. It is a polychromatic, adjustable, incoherent light source that has been successfully used to treat acne either on its own or in combination with a photosensitizer which augments its action [10, 14-16]. *In vitro* studies suggest that red light (620-750 nm) is anti-inflammatory by inhibiting cytokine release from macrophages [17] while blue light (450-495 nm) photo-inactivates *P. acnes*. However, specific published references regarding the mechanism of action of intense pulsed light are few.

1.2 Acne pathogenesis: Classical and Neo-classical Concepts

The classical concept of acne pathogenesis is largely based on a vast body of evidence from eminent dermatologists and scientists such as WJ Cunliffe [18], AM Kligman [8], JS Strauss [19] and G Plewig [20]. In this model, microcomedone formation is the initiating event [8]. These non-inflamed lesions are caused by retention hyperkeratosis of the epithelium within the sebaceous follicle. The resultant comedone produces a mechanical obstruction to sebum outflow and provides an environment suitable for *Propionibacterium acnes* proliferation. *P. acnes* metabolises these lipids, producing free fatty acids which are comedogenic and irritant to the surrounding dermis [8]. On-going hyperproliferation

disrupts the comedonal wall, releasing epidermal and fatty acid debris into the dermis. This incites an inflammatory response producing inflamed, polymorphic, acne lesions [8]. Then, the prevailing view was that diet had an obscure role to play in acne pathogenesis [8], but a single study on the effect of chocolate consumption on acne [21], now hotly refuted [22], suggested otherwise.

In the 'neo-classical concept', a specific T-cell mediated inflammatory response is the initiating event in acne pathogenesis [23], followed by comedone formation. Previously, a lymphocytic infiltrate in the vicinity of early lesions (< 24 hours) had been noted but was deemed to be an early post-comedonal event [24, 25]. The mechanism of inflammation is hypothesized to be a Type IV hypersensitivity reaction where *P. acnes* moieties are the probable antigenic compounds [26, 27]. This model is still inadequate as mere suppression of inflammation does not suppress acne lesion formation, signalling the need for further study.

Seborrhoea is still a necessary co-factor within the pathogenetic pathway and comedogenesis is triggered by the initial inflammatory response. Genetic factors have been given greater emphasis now that diagnostic tools are widely available to discover them. The following section is a re-construction of the neo-classical concept from initiation to resolution of the acne lesion. Thereafter each pathogenetic factor: *P. acnes*, comedogenesis, seborrhoea and genetic susceptibility will be expanded and pro-inflammatory therapeutic targets identified.

1.2.1 Neo-classical View: Evolution and Dissolution of the Acne Papule

1.2.1.1 Initiation

In genetically susceptible individuals, pilosebaceous follicles are inherently more sensitive to circulating androgens. At the onset of puberty, the rise in circulating and local androgens ‘switches on’ sebaceous glands within predisposed follicles encouraging sebocyte differentiation, proliferation and lipid production [4, 28]. Hence, larger sebaceous glands that secrete more sebum develop. Add to this a diet rich in dairy, simple carbohydrates and monounsaturated fats, the ensuing hyperinsulinaemia increases peripheral production of androgenic metabolites, worsening the seborrhoea. In acne-prone skin, interleukin 1 receptor (IL-1R) is up-regulated on keratinocytes within the epidermis, enabling minute concentrations of IL-1, which would have otherwise been overlooked by normal cells, to trigger an immune response [23, 29] and stimulate hyperkeratinisation of the infra-infundibulum and sebaceous ducts [8]. This follicular ‘hypervigilance’ represents the baseline state of the acne prone pilosebaceous unit.

The skin’s microflora would have been processed and presented by dendritic cells to lymph nodes in early life, producing a bank of memory T-cells. Once an individual-specific titre of microbial stimulant e.g. *P. acnes*, is bound by toll-like receptors (TLRs) and defensins, then cytokines and chemokines are released by the recruited lymphocytes, neutrophils and mononuclear cells. The cytokines then attach to their receptors within the epidermis, infundibulum and sebaceous glands to initiate an inflammatory cascade.

1.2.1.2 Progression

TLRs on keratinocytes, sebocytes and dendritic cells activate signalling cascades that enlist transcription factors and signal transducing phosphokinases such as nuclear factor kappa B (NFκB) and mitogen-activated phosphokinase (MAPK) [30]. In response to these messages, a host of chemokines are then transcribed including tumour necrosis factor α (TNFα), IL-8 and IL-6 that in turn, recruit leucocytes. These lymphocytes, monocytes, neutrophils and macrophages then release more cytokines. Keratinocytes within the epidermis, infundibulum and sebaceous duct are stimulated to proliferate and resist desquamation by interleukin-1 alpha (IL-1α) and IL-1β, forming a cornified blockade to the outflow of sebum. This marks the beginning of the comedone.

If the patient is also experiencing stress such as a family crisis, the neuropeptide substance P is released. Substance P augments the response of sebaceous glands to cytokines such as IL-1α, IL-6 and TNFα and prepares the surrounding vasculature for the chemotactic phase of an inflammatory response by up-regulating cellular adhesion molecules such as intercellular adhesion molecule (ICAM) and vascular adhesion molecule (VCAM) [31, 32]. Stress also causes an increase in corticotropin releasing hormone (CRH) which is pro-inflammatory [33, 34], sebo-stimulatory [35] and pro-comedogenic [36].

As a consequence, escalating ductal blockage by sticky keratinocytes leads to the eventual disruption of sebaceous and infundibular ducts producing dermal irritation by released fatty acids. Damaged keratinocytes and TLR2 provocation contributes to dissolution of the extracellular matrix through the release of matrix metalloproteinases (MMPs) from monocytes [37], sebocytes and keratinocytes [38]. The resultant lesions present as papules, pustules and nodules. The severity of these acne lesions is dictated by the size of the

initial cellular infiltrate, the volume and volatility of the sebaceous lipids [39] and the underlying magnitude of androgenic stimulation.

1.2.1.3 Resolution

Cutaneous surveillance mechanisms in the form of immune-modulatory cytokines such as IL-10 are released to modulate the response. However, these measures are rendered ineffective in those affected by acne vulgaris who have either lower circulating levels of IL-10 [40] or a slow release compared to normal controls [41]. Incorporating the *'keratinocyte activation cycle'* as described by Freedberg et al. [42] in the context of wound healing, the activated epithelial cells that release IL-1α causes the release of chemotactic cytokines such as IL-6, TGFα, IL-8 and GCSF, that in turn send multiple signals for cellular proliferation, re-epithelialisation and endothelial selectin expression. Eventually, as extracellular matrix is synthesised, the basement membrane is restored and TGFβ levels increase. These autocrine and paracrine signals form a negative feedback loop resulting in deactivation of keratinocytes [42]. The wound then heals and the papule resolves.

Persistent lesions may occur because the deactivating signals are not all present. A chronically disrupted basement membrane or high levels of the activating cytokines may continue to stimulate this process. Holland et al. [26, 43] demonstrated that patients with scarring acne have a greater proportion of memory T-cells at the beginning of the inflammatory process. They also have more CD68+ macrophages lingering in their acne papules many hours after a similar infiltrate in individuals with non-scarring acne would have waned. This chronicity leads to delayed healing of dermal wounds and hence scarring [26, 43].

Table 1.1: Therapeutic Targets for Visible Light within Acne Inflammation. A list of major targets that can affect inflammation.

Pathogenetic Group	Targets
Pro- inflammatory Cytokines	IL-8, IL-6, TNFα, ICAM-1, VCAM
Anti-inflammatory Cytokines	IL-10, TGFβ
T-cells	CD4, CD8
Growth Factors	GCSF

1.3 Propionibacterium acnes (P. acnes)

1.3.1 Is P. acnes a Primary or Secondary Therapeutic Target?

Anti-bacterial and antibiotic therapies have been dutifully prescribed based on observational studies which show that *P. acnes* undoubtedly plays a role in acne pathogenesis:

(i) There is a progressive increase in *P. acnes* counts from pre-adolescence to early adulthood [44]

(ii) Persons with active acne have higher densities of *P. acnes* compared to normal controls [45]

(iii) The face and upper trunk have significantly higher *P. acnes* and sebum levels compared with the lower trunk and extremities, which is in keeping with the common distribution of acne vulgaris [46]

(iv) *P. acnes* can stimulate hamster sebaceous lipogenesis via prostaglandin J [47]

(v) The density of *P. acnes* positively correlates with the sebum excretion rate (SER) [45] as well as the total mass of several sebum components [46]

(vi) *P. acnes* is able to incite a robust inflammatory response and a specific T-cell response [48]

(vii) Antibiotic resistance can be associated with difficult-to-treat acne [9, 49]

However, whether *P. acnes* colonisation is a primary or secondary aetiological event in acne lesion initiation is still debatable [44]. The presence and quantity of *P. acnes* cultured from skin of individuals with and without acne do not always correlate with the existence or severity of acne [50, 51] and after therapy, improvement does not always coincide with a reduction in *P. acnes* colony counts [52]. It is noteworthy that tetracyclines are said to be effective because of their anti-inflammatory action and not their direct anti-bacterial properties [53]. The dual effect of tetracyclines reduces both the antigenic load and the inflammation caused by *P. acnes*.

1.3.2 *P. acnes* and Innate Immune Response Targets

Micro-organisms like *P. acnes* can prompt the secretion of a variety of cutaneous anti-microbial peptides e.g. defensins and cathelicidins. These mediate bactericidal or bacteriostatic actions through cell membrane disruption [54]. There are two main defensin subfamilies: α and β, where β-defensins predominate in human skin [55]. There are many types of β-defensins including human β-defensin 1 (hBD1), hBD2, hBD3 and hBD4 [56]. Defensins are housed in neutrophilic granules [55] and released as pro-peptides, which are then cleaved to release an active cationic moiety that interacts with the negatively charged bacterial surface proteins [57]. The hydrophobic portion of the peptide integrates with the bacterial membrane leading to cytoplasmic leakage, inhibition of nucleic acid synthesis [55] and complete cellular disruption [57]. Defensins are regulated by a complex relationship between skin flora, toll-like receptors (TLRs), nucleotide-binding

oligomerisation domain (NOD) receptors, cytokines, chemokines and members of the specific and adaptive immune responses [57]. hBD1 and hBD2 can bind to chemokine receptors such as CCR6 attracting dendritic cells, monocytes and T-lymphocytes [55]. Stimulators of hBD2 expression include IL-1α, IL-1β, TNFα, protease inhibitor 3 and lipocalin (anti-microbial compound found in tears) [54, 58, 59]. The addition of IL-1R antagonist to cultures containing bacterial lipopolysaccharides suppressed the increase in hBD2 mRNA expression [58]. These findings suggest that the innate immune response mediated by keratinocytes is through IL-1 production.

The inflammatory effects of *P. acnes* do not require the organism to be alive or present in its entirety [60, 61]. Keratinocyte expression of TLR2 and TLR4 *in vitro* was increased in cultured cells in the presence of supernatants containing membranous peptidoglycans and lipopolysaccharides, cytosolic contents or membrane proteins [62]. The peptidoglycan-lipopolysaccharide moiety had a more stimulatory effect compared to the other cellular components.

1.3.3 *P. acnes* as a Photodynamic Target

Both *P. acnes* and *P. granulosum* synthesise coproporphyrin III (CPIII) as their major porphyrin [63, 64]. Both the *P. acnes* bacterium and CPIII molecule can stimulate IL-8 release from keratinocytes *in vitro*, inciting inflammatory acne [65]. Irradiation of *P. acnes* with a suitable wavelength of light leads to photoactivation of CPIII, followed by destruction of the organism, resulting in an improvement in acne [66]. The maximal excitation peak of CPIII is at 403 nm (Soret band) [64] with smaller peaks at 500 and 530 nm [67]. Regarding photodynamic efficacy, CPIII is 1.1 and 20 times more efficient at

producing singlet oxygen than other endogenous porphyrins such as haematoporphyrin or eosin [68].

Table 1.2: Therapeutic Targets induced by *P. acnes*

Pathogenetic Group	**Targets**
P. acnes moieties	TLR2, TLR4, IL-8, IL-6, IL-1α, hBD2, Prostaglandin J
Porphyrins	Coproporphyrin III, metalloporphyrins

1.4 Comedogenesis

In 2000, Cunliffe et al. [7] confidently stated “Hypercornification is an early feature of acne and precedes inflammation”. Jeremy et al. [23] cast doubt on this dogma as they reported a significant population of memory and effector T-cells in clinically normal pilosebaceous follicles prior to comedo formation. In their study, timed observations of papules less than 6 hours old revealed augmentation of the pre-existing adaptive immune response without disruption of the basement membrane and no obvious hyperproliferation. Earlier literature on the genesis of the acne papule is partially supportive of an inciting inflammatory event [25, 32, 69], but one can hardly dispute the fact that comedones are a feature of acne and require treatment. An understanding of comedogenesis will help to identify potential therapeutic targets for IPL.

Comedogenesis is a result of abnormal hyperproliferation and shedding of corneocytes into the pilosebaceous duct [7]. Over the past 40 years, abnormal shedding has been suggested to be secondary to alterations in corneocyte tight junctions and desmosomes [70, 71], intra-ductal linoleic acid deficiency [72] and recently, an accumulation of lipid peroxides, break down products of sebum [73]. Guy, Green and Kealy [74] observed that the infundibulum of pilosebaceous units in facial skin hyperproliferated under the influence of interleukin 1 alpha (IL-1α) and could be blocked by the use of IL-1 receptor antagonist (IL-1ra). Further exposure of these infundibula to a clinically therapeutic concentration of 13-cis retinoic acid reduced the rate of cell division [74], and hence ductal obstruction, in keeping with the known topical effects of retinoids in acne [75]. Furthermore, earlier studies which showed that biologically active IL1α-like material has been isolated from open comedones [76]. Cutaneous keratinocytes express IL-1α and IL-1β receptors where IL-1α receptors predominate in the epidermis and IL-1β receptors are more prevalent in sebaceous acinar walls [77, 78]. Renne et al. [79] found that IL-1β released by cultured keratinocytes promoted inflammation by increasing the release of interferon γ (IFNγ) by T-cells. Hence keratinocytes through IL-1α and IL-1β is a key promoter of both comedogenesis and inflammation.

Cunliffe and colleagues [7] hypothesised that comedones, like hair follicles, naturally go through cyclical periods of exacerbation and resolution based on observed changes in the expression of Ki67 and K16. Downie et al. [80] modelled a resolving acne lesion and showed that isolated sebaceous glands, harvested from post-surgical chest and breast skin, de-differentiated to keratinocyte-like morphology after exposure to IL-1α, TNFα and IFNγ. Therefore pro-comedogenic cytokines also orchestrate the resolution of a comedone. This process of de-differentiation was previously described by Strauss & Kligman [81] in

1958, where it was noted that sebaceous glands traumatised with phenol, long-term occlusion or repeated needling, developed a keratinocyte-like morphology.

1.4.1 Pro-comedogenic Targets and P. acnes

Ingham et al. [82] used normal human epidermal keratinocytes (NHEK) harvested from neonatal foreskin showed that *P. acnes* was able to significantly provoke IL-1α production by keratinocytes. *P. acnes* is also able to increase keratinocyte proliferation, as indicated by increased Ki67 expression in the epidermis of skin explants [83]. Ki67 is a nuclear protein present within cycling cells [84] and in non-involved skin of acne patients, Ki67 is up-regulated in the acroinfundibulum [85]. Thus, it might be inferred that *P. acnes*, which colonises the infra-infundibulum, contributes to comedogenesis. However, rather than observing increased keratinocyte proliferation, Akaza et al. [86] noted strain specific reductions in Keratin 1 (K1), K10, K6 and K16 mRNA levels after exposure to different *P. acnes* isolates *in vitro* despite a statistically significant increase in pro-inflammatory cytokine release. It is possible that the inconsistencies amongst these studies could be attributable to the use of cultured keratinocytes [82, 86] versus more biologically representative models such as skin explants [83].

Table 1.3: Therapeutic Targets in Comedogenesis

Pathogenetic targets	Targets
Ductal hyperproliferation	IL-1α , IL-1β
	P. acnes
	S. epidermidis
Pro-comedogenic	TNFα, IFNγ, IL-1α

1.5 Androgenic Hormones, Sebaceous Glands and Seborrhoea

1.5.1 The Skin as an Endocrine Organ

The skin produces hormones *de novo* from cholesterol and can also metabolise them [87, 88], making it an obvious target for medical intervention. A significant proportion of these hormones and many of the resident enzymes are dedicated to androgen-metabolism. Androgen receptors have been localised to many sites within the skin: keratinocytes, fibroblasts, endothelial cells and both peripheral and central portions of the sebaceous gland [89]. Sebaceous glands have a particular role to play as they express a variety of receptors, implicated in acne pathogenesis, for androgens [90], oestrogens [91], thyroid stimulating hormone (TSH) [90], histamine [92], prostaglandins [93], corticotrophin releasing hormone (CRH) [33, 94], melanocortin-1 [95-97] and peroxisome proliferator-activated receptors (PPAR) [98-100]. Androgen receptors on keratinocytes and sebocytes present a possible mechanism by which androgens stimulate hyperkeratinisation and seborrhoea [101]. In these sites, testosterone is converted to its potent metabolite DHT via two isoforms of 5α- reductase, type I and type II [102], where type I predominates [103, 104]. Thiboutot and colleagues [87] studied hair follicles in breast skin from 82 donors and found that 5α reductase activity was highest in the sebaceous gland and duct followed by the follicular infra-infundibulum and lastly the epidermis. 5α-reductase activity is greater in acne-prone skin (face) when compared to other sites [103] and was slightly higher in individuals with acne than those without [105].

Three beta– and 17β-hydroxy steroid dehydrogenase (17β-HSD) are enzymes required for androgen metabolism within the skin (**Figure 1.1**). Like 5α reductase, they also have greater activity in sebaceous glands compared to the dermis and epidermis [106, 107].

However, 5α reductase has 2-3 fold higher activity than 17β-HSD in sebaceous glands [108], but there is little difference in their activities in cultured epidermal and infra-infundibular keratinocytes [105]. Both enzymes have greater activity in men [105, 109] and are not associated with the presence of acne in women [105]. Furthermore, despite their increased activity in acne prone areas [106], their up-regulation in acne subjects is not statistically significant [105]. Referring to **Figure 1.1**, it is clear that antagonists of any of these enzymes, or hormone receptors (DHT, testosterone or DHEAS) are potential therapeutic targets and the ultimate aim would be to deliver these antagonists directly to the skin and /or sebaceous glands.

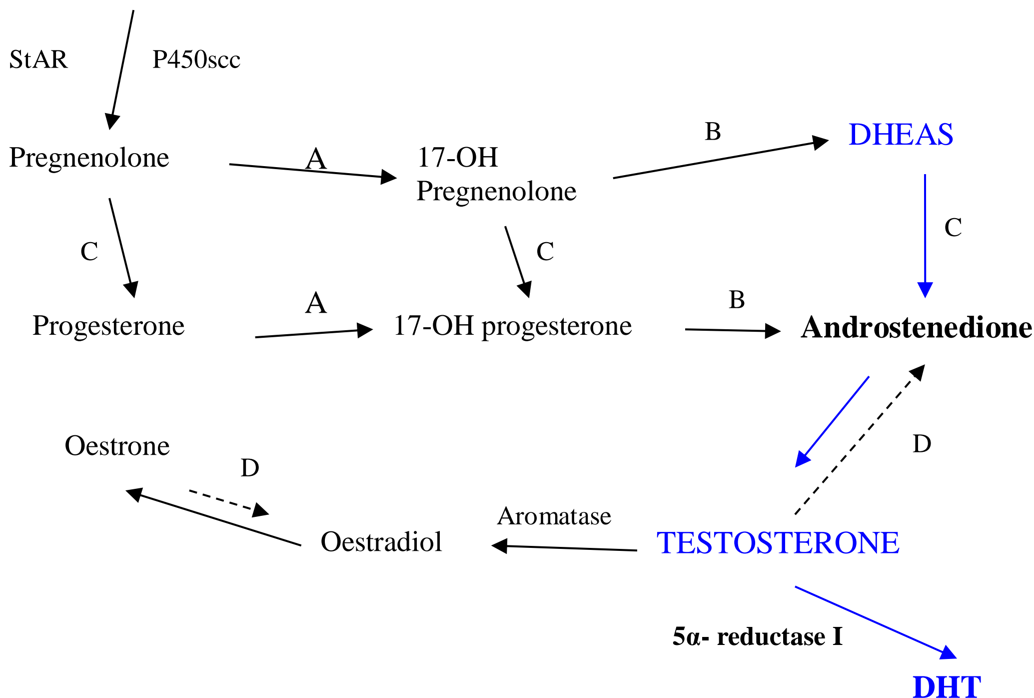


Figure 1.1: Androgen and Sex Hormone Metabolism in the Skin

KEY: *3β-HSD* – 3β-hydroxysteroid dehydrogenase; *17β-HSD* – 17β-hydroxysteroid dehydrogenase; *DHEAS* – dehydroepiandrosterone ; *DHT*– dihydrotestosterone; *P450c17* – cytochrome P450 17-hydroxylase; *P450scc* - P450 side-chain cleavage enzyme; *StAR* – steroid acute regulatory protein facilitates the transfer of cholesterol from the outer to the inner mitochondrial membrane. 17β HSD II predominates in the sebaceous glands. *Adapted from Thiboutot et al. [87, 105, 108, 110] and Chen et al. [88].*

1.5.2 Androgens and Seborrhoea

Seborrhoea has long been associated with the presence of acne [111, 112] which is very much under androgenic control:

i. Adults with complete androgen insensitivity, a condition in which mutations in the androgen receptor gene renders them ‘insensitive’ to circulating androgens [113] producing a phenotypic female but genetic male, do not excrete appreciable levels of sebum [114]

ii. Males castrated prior to the onset of puberty do not develop acne [115] and 60% of pre-pubertal boys treated with topical testosterone exhibit increased sebum output [116]

iii. Post-adolescent castration or oophorectomy in acne patients results in the gradual resolution of their acne [115] and their sebum excretion rates fall below that of normal subjects [117, 118]

1.5.3 Androgens, Acne and Gender

It should follow that males have a higher incidence and greater severity of acne when compared to females. Some epidemiological studies confirm this [119-121] but, there is not a strict positive relationship between androgen levels, the presence of acne and its severity in men [108].

Females with severe acne have significantly higher levels of a circulating androgen precursor, dehydroepiandrosterone (DHEAS), than their compatriots with mild to moderate acne or normal controls [122-124]. Carmina et al. [122] and Lucky et al. [123] report a very specific association between acne severity and DHEAS serum

concentrations. A similar association was not found with other androgenic hormones (i.e. testosterone and progesterone) amongst women with varying severities of acne. These findings have been confirmed *in vitro* where SZ95 sebocytes of female-origin were incubated with various substances including progesterone, pregnenolone, DHEA and IGF-1 [125]. Of these, DHEA induced maximal testosterone production [125]. A useful extension of this study would be to compare the response of male-derived and female-derived SZ95 sebocytes. Some smaller studies involving women with post-adolescent acne disagree. They identified significantly higher levels of free testosterone and DHT in acne patients than normal controls [108, 126]. Hence, it is clear that both DHT and DHEAS are implicated alone or separately in the development of acne vulgaris highlighting the ability of a number of androgenic compounds to cause acne although they may not exhibit the same potency.

Regarding inflammation, cultured human sebocytes express significantly higher levels of IL-6 and TNFα (mRNA & protein) when cultured with DHT [127]. In a murine system, Weinstein et al. [128] showed that androgens were not immunostimulatory. Rather, they caused an inhibition of antigen presentation in male mice and androgen-supplemented female mice. In humans, this observation is partially supported by Holland et al. [119] as females with mild acne had a measurable difference in their peripheral blood immunocyte counts when compared to normal controls but the male cohort did not exhibit similar increases unless their acne was moderately severe or worse [119]. They postulated that this phenomenon meant that males did not mount a protective response until it was too late – when their acne was already quite severe, accounting for their greater representation in groups with moderate and severe acne.

1.5.4 Sebum

Sebum changes in quality and quantity with age, such that the volume of sebum excreted increases in adulthood and declines with advancing age (41+ years) [129-131]. Few studies have examined the composition of sebum directly from the sebaceous glands [132, 133]. Nasr [132] reported that skin surface lipids closely approximate to sebum but the cadaveric sebaceous glands that he examined (from 53 people of various ages) did not contain free cholesterol. He surmised that cholesterol may be synthesised by the ductal keratinocytes and secreted onto the skin surface. A large percentage of pre-pubertal skin surface lipid is made up of cholesterol and cholesterol esters but adult sebum contains shorter-chain length fatty acids (C16), squalene, triglycerides and fatty alcohols [98, 134-136]. This difference may reflect a surge in the cutaneous consumption of cholesterol at the onset of puberty for the production of androgens and other hormones.

Amongst the fatty acids, high levels of Δ6 C16 fatty acids are present, including sapienic acid and its derivative sebaleic acid, a polyunsaturated substrate of palmitic acid [137]. These two fatty acids are unique to humans and are converted to a potent chemoattractant, 5-oxo-octodecadienoic acid, in neutrophils and keratinocytes via lipoxygenase enzyme activity [137]. The increased sebum excretion rate (SER) and change in its composition in pre-pubertal children was accompanied by greater propionibacterial densities [129, 138]. Notably, in an investigation by Mourelatos et al., children who maintained a low SER throughout the 2½ year study did not develop acne [138]. Triggers for this localised process are currently uncharacterised but hypothetically, due to the observed synchrony between puberty and acne, is likely to be also under the control of the hypothalamo-pituitary-adrenal axis. People without acne or with mild acne may secrete more protective FFA components, such as lauric acid, a 12-carbon fatty-acid, which accounts for only 1-

2% of the FFA moiety in sebum but is 4–60 times more potent than benzoyl peroxide against *P. acnes*, *S. epidermidis* and *S. aureus in vitro* [139]. An experimental ointment containing lauric acid was able to reverse intradermal inflammation caused by *P. acnes* in murine epidermis [139].

An individual's propensity for inflammatory acne vulgaris is heavily modulated by environmental and dietary factors. As IPL is unable to directly influence a person's diet, the roles of dietary factors in acne pathogenesis [140-142], androgen expression [143, 144], lipogenesis [145] and sebum composition [142, 146] will not be discussed here but are explained more fully in our review article [147] (**Appendix 4**).

Table 1.4: Therapeutic Targets within Sebogenesis Key: *CRH*, corticotrophin releasing hormone; *FFA*, free fatty acids; *MC-1*, melanocortin 1.

Pathogenetic Group	Targets
Androgens	DHEAS, DHT, Testosterone, Androstenedione
	Androgen receptors
Sebaceous glands	Receptors, enzymes, prostaglandins
Sebum	CRH, MC-1, cholesterol, FFA, triglycerides

1.6 Genetic Susceptibility

A number of studies carried out in adolescents and young adults from Iran, China, France and the UK [120, 148-150] report a strong familial tendency for acne, where the index cases tend to present at a younger age [120]. A cohort of British patients with '*adult persistent acne'* (26 – 78 years) also had increased rates of similarly affected first degree relatives when compared to normal controls [151]. The increased risk of developing acne in the presence of a positive family history ranges from 2.3 [149] to 4.69 [120]. A retrospective study of 1,557 pairs of monozygotic and dizygotic twins living in the UK showed significant familial clustering [152]. Forty-one per cent of those with acne had children similarly affected, 47% had siblings and 25% had parents also affected [152]. In the unaffected (86% of the cohort), the percentages of children, siblings and parents with acne were 17%, 15%, and 4% respectively. To date there have been only a few published genetic candidates to account for this heredity.

1.6.1 CAG Repeats

The androgen receptor gene (Xq11-12) has 8-35 CAG trinucleotide repeats in the transactivation domain [153]. The length of this repeat sequence inversely correlates with the sensitivity of the androgen receptors. Therefore, persons with longer CAG repeats have lower incidences of androgen-mediated disease such as prostate cancer, polycystic ovaries and acne [121, 153, 154]. Yang et al. [121] and Pang et al. [153] found male acne patients tended to have 23 or less CAG repeats and normal controls had more. A similar association in females was seen only in Pang's study despite having a similar ethnicity and sample size to Yang's study, therefore the strength of this association remains unclear.

1.6.2 Other Polymorphisms

Polymorphisms in genes for TNFα [155, 156], TNFR2 [157], TLR2 [157], IL-1α [155], cytochrome P450 1A1 (which codes for retinoic acid metabolising enzymes) [158, 159], CYP17 – 34T/C (another P450 gene that codes for 17α-hydroxylase) [110, 160], CYP21 (gene coding for 21-hydoxylase) [161] and fibroblast growth factor receptor 2 (FGFR2) [162] are possible contenders for the modulation of acne severity.

FGFR2 mosaicism may be associated with epithelial dysregulation resulting in cancers of the breast, uterus and skin [163] and has been identified in a localised acneiform naevus in a single individual [162]. FGFR mosaicism is also responsible for the nodulocystic acne seen in Apert's syndrome, a dominantly inherited condition characterised by craniofacial abnormalities and syndactyly [164]. Melnik, Schmitz & Zouboulis [164] have made an elegant case for FGFR2's role in acne pathogenesis and how its manipulation is crucial to the mechanism of action of antibiotics, anti-androgens and retinoids. Here, fibroblasts synthesise FGF7 and FGF10 in response to DHT [164]. FGF7 and FGF10 produced by fibroblasts are ligands for FGFR2b found on suprabasal keratinocytes and sebocytes and DHT augments FGF7 and FGF10 transcription [28]. FGFR2b binding leads to transcription of IL-1α, which encourages hyperkeratinisation of epithelia as well as proliferation and fatty-acid synthesis in sebocytes [164]. Whilst this hypothesis is a possibility, as yet, there are no *in vitro* or *in vivo* studies to substantiate it.

Polymorphisms are normal variants of a gene and therefore the polymorphisms mentioned above may not represent a disease state. In 1975, an overview of acne by Kligman [8], stated that acne was *"...a polygenic disorder in which the phenotypic expressions represent the sum of many genes each of which is not strongly active by itself... (does) not follow simple Mendelian patterns (and) can be strongly influenced by external agencies*

and have variable expression." In spite of swathes of new information, there have been no real revelations about the precise genetic factors associated with acne in almost 40 years. Hence, the all-encompassing term 'genetic susceptibility' is often used when discussing acne pathogenesis.

Since acne is so common amongst humans, is it possible that it is an evolutionary double-edged sword? Is it a distant and ultimately more complex relative of the sickle cell trait which offers some protection against certain types of malaria in endemic countries, but can be deadly in homozygous carriers [165]? Bloom [166] theorises that the unsavoury appearance of typical adolescent acne serves to avert early mating and improves at a time which coincides with psychosocial maturity and greater readiness for parenthood. Kligman [8] also cites papers which take a more mechanistic view. They propose that by adulthood, the follicular epithelium becomes less permeable to irritant fatty acids and 'hardened' to external irritants leading to the resolution of acne.

Table 1.5: Potential Genetic Therapeutic Targets

Pathogenetic Group	Targets
Androgen receptor polymorphisms	CAG repeats
Androgen metabolism	CYP17 , CYP 21, P450 1A1
Fibroblast regulation	FGFR2
Immunological disruption	TNF-α, TNFR, TLR2, IL-1α
Evolutionary modulation	?

1.7 Conclusion

A number of the therapeutic targets identified are implicated in innate and adaptive immune mechanisms, comedogenesis and sebogenesis. Later, molecules from these lists will be identified as having the potential to be IPL-modifiable. The following chapters also discuss the principles of IPL devices and the available evidence for the use visible light in acne therapy thus identifying some gaps in our knowledge with respect to IPL as a therapeutic alternative.

Chapter 2

BACKGROUND 2

The Basis for Visible Light in Acne

2. The Basis for Visible Light Use in Acne

2.1 Introduction

Light as a therapeutic alternative in acne management is not a new concept. Sunlight has long been considered a natural remedy for acne and physician-administered UVA and UVB were once used as alternative treatments for acne [167]. UVB is known to promote cellular apoptosis and disrupt Langerhans cells' antigen presenting function by reducing their dendricity and antigen presenting molecules (e.g. MHC II) [168-171]. UV radiation has now fallen out of favour due to the wide availability of more effective treatments without the additional photo-carcinogenic effect associated with it. Visible light (400 -700 nm) has a greater depth of penetration than UV light and hence is able to reach the deeper sites of acnegenesis such as the sebaceous duct and gland [172-174].

Commonly cited justifications for the use of light-based therapies to treat acne are that they widen the therapeutic options available, offer treatment to those unable to take or have failed standard therapy, may improve compliance, does not add to the problem of antibiotic resistance and reduces the need for long-term antibiotics [175-178]. An assortment of laser and light sources has been used to treat acne with variable success [179, 180]. Comparing lasers and broad-spectrum light sources, the latter is advantageous in that they are cheaper to run, versatile and portable [181]. Intense pulsed light (IPL) is a broadband incoherent light source which utilizes xenon lamps to emit photons at various energies along a continuous spectrum (500 -1300 nm) via a computer-controlled capacitor bank [182, 183]. Many IPL systems allow a range of treatment parameters through the manipulation of cut-off filters [182]. This filtered light irradiates target tissues to produce thermal, mechanical or chemical damage of target chromophores such as melanin within

the epidermis and haemoglobin in the vasculature, whilst sparing surrounding tissues [184, 185]. Thus, IPL systems are used for a number of indications including hair removal, photorejuvenation, dyschromia, rosacea, telangiectasia, vascular malformations, actinic keratoses and acne [181, 186-190]. The rest of this chapter describes light-skin interactions, IPL parameters and their effect on clinical performance and finally, a brief summary of clinical trials examining incoherent visible light use in acne.

2.2 Light-Skin Interactions

The observed clinical outcome in irradiated tissue is equally dependent upon the properties of the tissue being irradiated and the radiant source [191, 192]. The desired result arises from a combination of chemical, mechanical, thermal and immunological interactions, the extent of the insult and the tissue's healing response.

2.2.1 The Effect of Light in Light-Skin Interactions

2.2.1.1 Photothermal Reactions

In photothermal reactions, absorbed light is converted to heat. The temperature attained, the sustained duration at that temperature, and time between subsequent episodes of heating induces local changes and pathological processes in the tissue [191, 193]. These changes are summarised in **Table 2.1**.

2.2.1.2 Photomechanical Reactions

Short pulses which deliver high energies can cause rapid heating and expansion of the target tissue leading to rupture. Clumping of nuclear chromatin, epidermal and dermal vacuolisation can occur. Elastic recoil of the tissue can cause additional secondary damage. These mechanical/acoustic effects are exploited in the treatment of renal calculi via shock wave lithotripsy [193].

2.2.1.3 Photochemical Reactions

This requires the presence of an endogenous or exogenous chromophore. Photons are absorbed by the chromophore, converting it to an excited state. In this state, cross-linking, bond-breaking, free-radical formation or destruction of the chromophore may occur. In photodynamic reactions, a photon of light reacts with a photosensitive dye e.g. coproporphyrin, in the presence of oxygen to produce oxygen free radicals [191]. The free radicals injure cells and subcellular organelles such as mitochondria.

2.2.1.4 Photoimmunological Reactions

This term refers to the immunomodulatory effects of non-ionising electromagnetic radiation (light) on cytokines and immuno-competent cells [194]. The use of UVA and UVB as immunosuppressants in inflammatory skin diseases is well-documented as is their ability to induce skin cancer. Visible light can also induce useful and pathological immune reactions e.g. solar urticaria [195].

Table 2. 1: Photothermal Effects of Laser-Tissue Interactions. *Adapted from Thomsen [193]*

Photo-thermal Interaction	Temperature at Onset (ºC)	Histopathologic Result
Low-temperature damage	40 +	Reversible cell injury, enzyme & protein denaturation, cell death, cell shrinkage, membrane rupture
Higher-temperature damage	58+	Hyalinization of collagen, elastin denaturation
Water-dominated Processes	100 +	Water vaporisation, vacuolar rupture (popcorn effect), tissue ablation through fragmentation
High Temperature Ablation	300 – 1000+	Tissue ablation & carbon vaporisation

2.2.2 Influence of the Skin in Light-Skin Interactions

The optical properties (scatter and absorption), thermal capacity, mechanical properties (elasticity, tensile strength), composition (water, endogenous and exogenous chromophores), anatomy (physical arrangement of organelles within the tissue) and physiology (metabolic status) of the skin affect the attainment of the desired end point [193].

At the stratum corneum-air interface, 5% of incident photons are reflected (Fresnel's reflection) whilst the remainder traverse the epidermis into the turbid dermis [172, 196]. Depending upon the component wavelengths of the incident light, select structures within the epidermis and dermis absorb, scatter and transmit the remaining photons (**Figure 2.1)** [197]. Optical properties of dermal collagen fibres and the subcutaneous fat below determine how much scatter occurs and hence the final depth of penetration of incident

light [172, 198, 199]. Temperature changes within human skin can change the lipid's fluidity, orientation and stacking order, altering the skin's optical pathways [198]. These complex and varied optical properties of skin contribute to the constant flux noted in estimations of the dose of delivered light [174, 191].

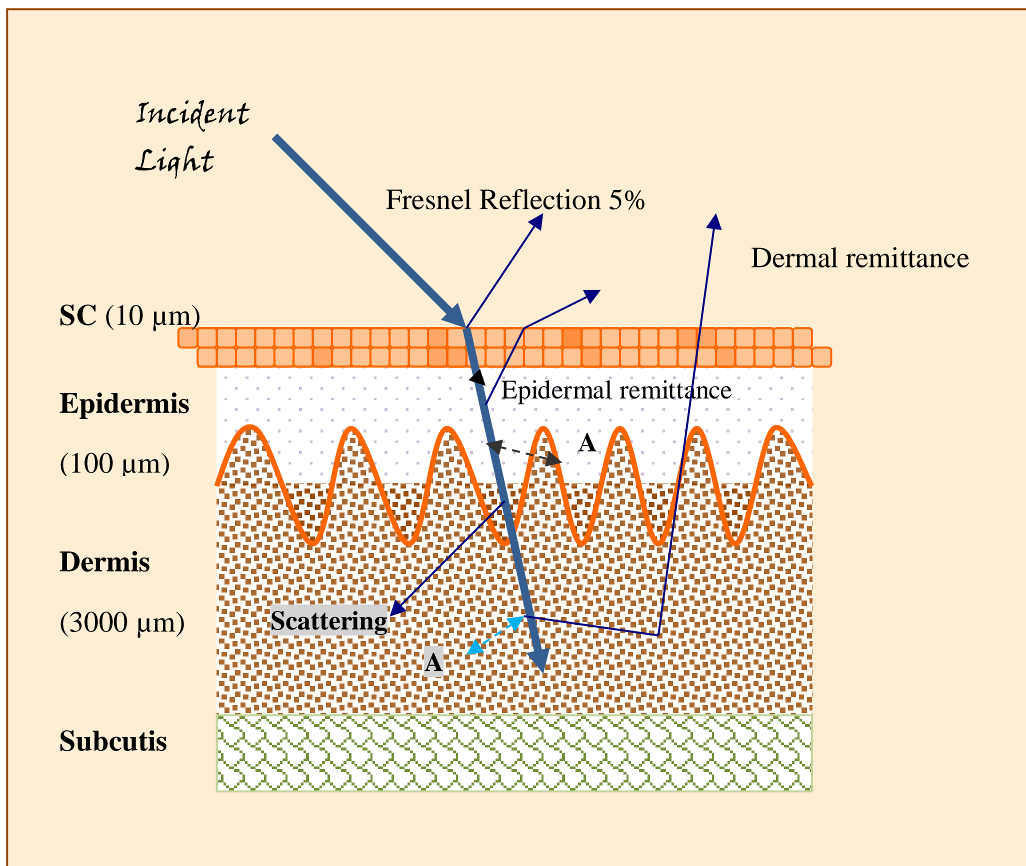


Figure 2. 1: Schematic Representation of the Optical Pathways in the Skin [172, 196, 197]. KEY: *SC* – stratum corneum*; A* - absorbed radiation. *Adapted from Anderson & Parrish [172] and Krishnaswamy & Baranosky [197].*

2.2.3 Chromophores

"Chromophores" are the bonded, unsaturated atoms in a molecule that dictate its absorption properties. The epidermis contains many chromophores including melanin, urocanic acid, nucleic acids, water and amino acids. The dermis consists of a proteinaceous matrix of collagen, elastin and fibroblasts in which capillary plexi and appendages sit [199]. Collagen surrounds key chromophores such as haemoglobin, bilirubin and water [172, 198].

2.2.3.1 Melanin

Absorption peaks for melanin and its metabolites lie within the 250 – 1200 nm range with the largest absorption peak at 335 nm followed by a sharp reduction at longer wavelengths [200]. The method of induction of melanin and the individual's native skin phototype dictate its absorption properties [191, 201]. Melanin particles released as a result of irradiation (410 - 610 nm light) produce 'immediate pigment darkening' and are less absorbent than melanin released in response to radiation between 610 and 720 nm [200]. Between 650 and 850 nm, darkly pigmented skin (Fitzpatrick skin type VI) scatters less and absorbs more radiation than lightly pigmented skin (Fitzpatrick skin types I – IV) [199]. At 980 nm, each skin type exhibits similar absorption coefficients indicating that absorption by melanin at or beyond this wavelength is minimal [172].

The absorption spectra within the visible light and near infra-red ranges show that melanin is a competing chromophore for oxyhaemoglobin and its absorptive properties supersede oxyhaemoglobin at wavelengths longer than 630 nm (**Figure 2.2**). Thus, the use of visible light (400 – 700 nm) in darkly pigmented skin to target dermal structures is likely to be

associated with an unacceptably high risk of excessive thermal injury to the epidermis, the main reservoir of melanosomes.

2.2.3.2 Haemoglobin

Absorption curves for haemoglobin (Hb) vary widely with small deviations in its structure [202]. OxyHb, deoxyHb, metHb and foetal Hb all have very different absorption coefficients for a given wavelength. For example, Kimel et al. [202] quoted an 8% difference between adult oxyHb and foetal oxyHb at 596 nm whereas adult deoxyHb has a 4% lower coefficient than foetal deoxyHb at the same wavelength. To complicate matters further, intravascular temperatures after laser irradiation were dependent upon the vessel diameter and wall thickness [202]. Therefore, we can surmise that autonomic stimulation e.g. drugs, mood and physical exercise, can cause vasodilatation or constriction, will affect how light interacts with intravascular haemoglobin. This should be taken into account when assessing the treatment efficacy of IPL and other light-based therapies.

2.2.3.3 Water

The skin may contain as much as 80% water. Thus water acts as a major chromophore and determines many of the skin's thermal properties [193]. Water's main absorption peak is at 3,000 nm [203], but strongly absorbs any wavelength over 1300 nm [193]. Tissues approaching water's boiling point (100ºC) are prone to intracellular vacuolar formation, desiccation and disruption from rapidly expanding steam [193]. Lasers emitting within the infra-red spectrum e.g. Erbium YAG (2,940 nm) and CO_2 lasers (10, 600 nm) exploit these properties and are used for tissue ablation, coagulation and cutting [203, 204].

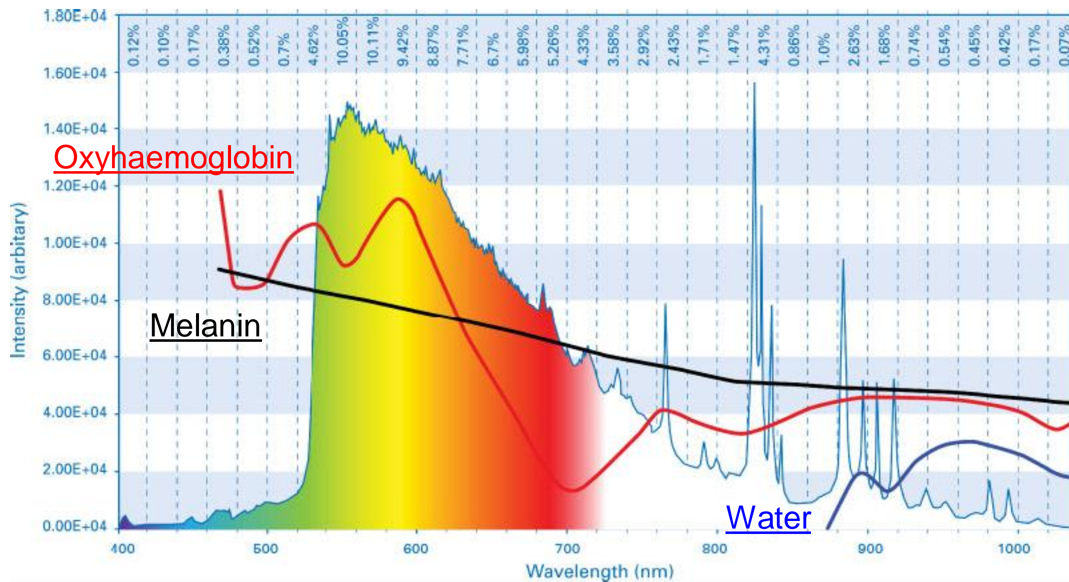


Figure 2. 2: Standardised Spectral Output of a Typical Xenon Lamp (*iPulse*, Cyden Ltd). This was measured in 20 nm bandwidths as a percentage of the total energy beneath the curve. Cut-off filters have excluded ultraviolet light. Ash et al. [182] have overlaid the absorption curves for melanin (black), oxyhaemoglobin (red) and water (blue) to illustrate chromophore absorption characteristics. Note oxyhaemoglobin is the favoured chromophore between 510 and 620 nm, thereafter melanin supersedes both oxyhaemoglobin and water until approximately 1100 nm (not shown).

2.3 The IPL Apparatus

2.3.1 IPL Parameter Definitions

In addition to light-skin interactions, intrinsic parameters of the IPL device also alter dosimetry and ultimately, clinical performance. The medical applications of any IPL device are dependent upon the theory of 'selective photothermolysis' [173, 193, 205]. If photothermolysis is to be selective, three criteria need to be fulfilled: (1) the wavelength chosen must be sufficiently and almost exclusively absorbed by the target chromophore; (2) the pulse duration must be less than the thermal relaxation time (approximately 200 -

600 ms for skin) to reduce collateral damage and; (3) the energy delivered must be sufficient to cause destruction of the target chromophore [203]. Wavelength, spectral output, time-resolved spectral output, fluence, pulse duration pulse profile and spot/footprint size are defined and explained with respect to their relevance in light therapy below.

1. **Wavelength:** is the distance from peak to peak or trough to trough in a sinusoidal waveform. It is usually expressed in metres (m). To tailor a sufficient clinical effect by confining the greatest thermal damage to specific targets, the wavelength should match the highest absorption peak of the target chromophore (e.g. 595 nm for oxyhaemoglobin) [202].

2. **Average Spectral Output:** describes the component wavelengths emitted by the lamp. Those below 500 nm are associated with increased risk of retinal damage. Cut-off filters have a key role to play in the accuracy and effectiveness of the light distributed. Spectral output should match the target chromophore whilst avoiding other chromophores in the skin to prevent collateral damage. The spectral output of an IPL device is necessary to perform comparisons between equipment [185].

3. **Time-resolved Spectral Output:** describes the portion of the entire pulse during which effective wavelengths are being emitted at their most advantageous intensities [184, 202].

4. **Fluence**: a measure of energy delivered per unit area in joules per m^2 (J/m^2) [206]. An appropriate fluence would be one which thermally destroys the target

chromophore without causing damage to the surrounding tissues. The change in fluence over time in seconds is the fluence rate or energy density, expressed as Watts per m^2 (W/m^2) [206].

5. **Pulse Duration**: is the time, in milliseconds (ms) required for a waveform to reach its full magnitude and may also refer to the sum of sub-pulse durations in a pulse train [184]. The pulse duration is critical especially where it has to be matched with the thermal relaxation time of the target to get maximum clearance. The stated pulse duration should, but may not consist of thermally effective radiation [182].

6. **Electrical Discharge Pulse Shape**: describes the energy entering the xenon lamp. This energy may be constant (square pulse) or variable ('free discharge') [182].

7. **Footprint**: describes the spot size or beam emitted from the lamp. Narrow beams become depleted with depth while larger beams achieve greater depths of penetration and hence have an increased treatment efficacy [207].

2.3.2 Nature of an IPL Device

In an IPL device, an electrical charge is applied to a large capacitor or capacitors arranged in series or parallel [182]. Under the control of a computer, this energy is released to the gas-filled (xenon or argon) flashlamp envelope. The electrical energy is converted to electromagnetic radiation (light) as excited gas particles return to their resting state releasing photons of light. The wavelengths emitted by the hand piece are controlled by

interposing filters that allow only the desired wavelengths to be transmitted. A typical circuit diagram of a flashlamp's power supply is illustrated in **Figure 2.3**.

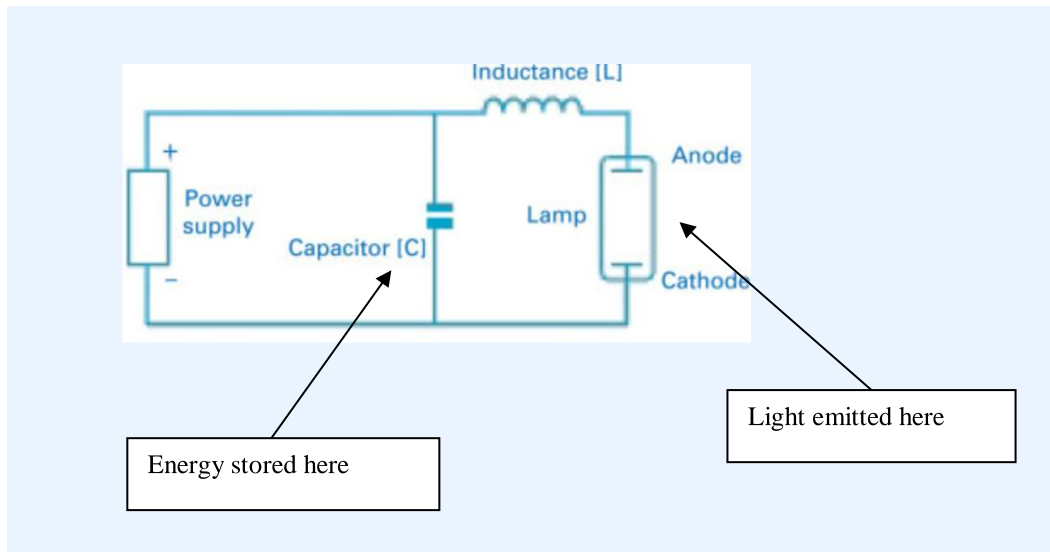


Figure 2. 3: Typical Circuit Diagram of Flashlamp Power Supply. *Adapted from Ash et al. [182]*

2.3.3 IPL Device Classification

IPL devices can be classified by their electrical discharge pulse shape [182] as a free discharge, constant current or grouped pulsed system. The features of each type of system and potential weaknesses in their design are discussed below.

2.3.3.1 Free Discharge Systems

Many free discharge systems e.g. *Chromolite* (Chromogenex Technologies Ltd., Swansea, UK), do not produce long pulse durations hence their optical output varies with time (**Figure 2.4a**). Spectrophotometry measurements by Ash et al. [182] revealed that

Chromolite claimed to have a 15 – 17 ms pulse duration, however, only 3 – 4 ms of this consisted of useful energy, the rest being largely low-intensity near infra-red radiation [182]. Hence, the thermal relaxation times (TRTs) of the pulse do not match that of blood vessels or hair follicles (25 – 55 ms).

To combat this effect, manufacturers of free discharge systems have designed these IPLs to deliver a train of high energy, short pulses to match the TRTs of these structures. This strategy however may lead to prolonged treatment regimens and far more adverse events as the shortened energy discharge time does not allow for thermal relaxation of the target. Other chromophores may collect a high amount of this energy leading to excessive erythema, burning and possibly scarring [182]. 'Flooding' of the flashlamp may also cause an undue proportion of emitted light as shorter wavelengths and the production of heat. Such systems tend to compensate with skin cooling. Operator dependent skin cooling introduces additional variability and affects the shot-to-shot energy delivery.

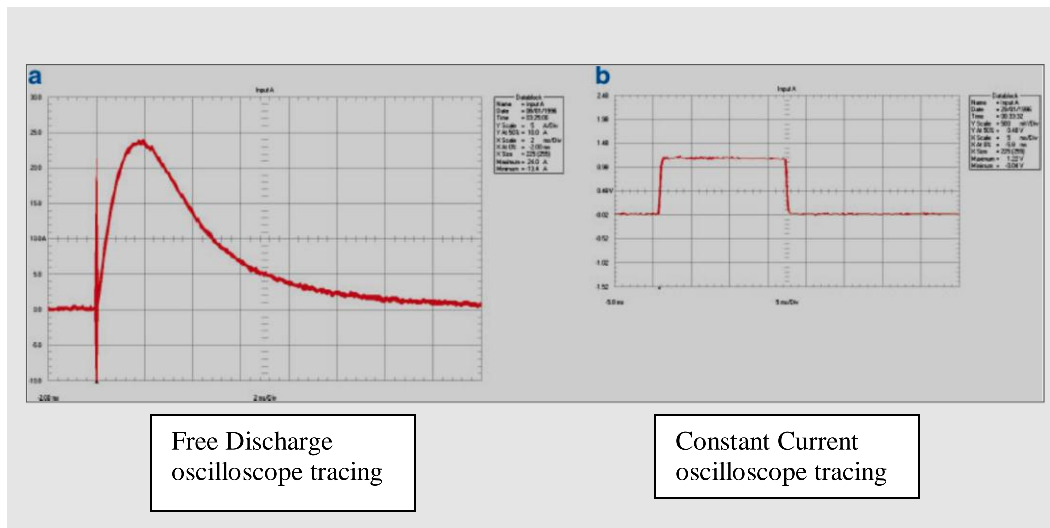


Figure 2.4: Typical Oscilloscope Tracings for a Free Discharge and a Constant Current IPL Device (a) A free discharge IPL tracing (*Chromolite*, Chromogenex Ltd.) (b) The 'square pulse' discharge profile of a constant current IPL (iPulse, Cyden Ltd.). Each tracing has been plotted on a graph against time. *Illustration adapted from Ash et al. [182].*

2.3.3.2 Constant Current Systems

The constant current or 'square pulse' systems generate energy the same way as free-discharge IPLs do. However, the energy is delivered at an optimal, constant level throughout the pulse via a partial discharge capacitor, forming a 'square' rather than the skewed bell curve seen with free discharge systems [184]. The light energy is released steadily across the pulse that lasts for 10 – 50 ms, compared with 3 – 4 ms constant phase of the free discharge system (**Figure 2.4b**). Consequently, these square pulse systems can be quite efficacious at lower fluences. Additionally, the relatively low but constant light energy reduces the risk of epidermal damage and in theory, almost eliminates the need for skin cooling [182]. But, even in constant current systems, much of the energy may be wasted if discharged at unhelpful wavelengths from the xenon flashlamp.

2.3.3.3 Grouped-pulse Systems

Grouped-pulse systems utilise short-pulse stacking to produce longer pulse durations. This however does not avoid the exponential decay of the energy at the end of the pulses, leaving the more clinically useful discharge at the beginning of the pulse (**Figure 2.5**).

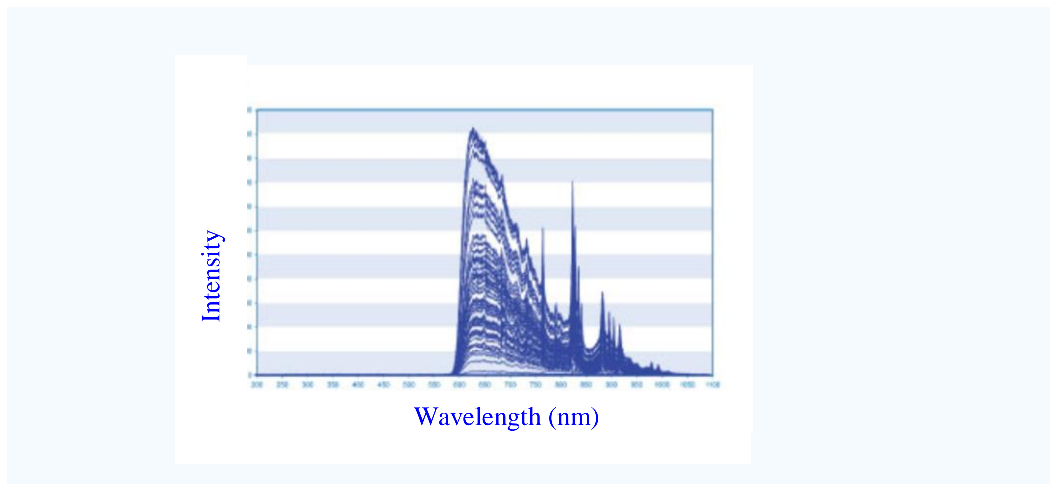


Figure 2.5: Spectral Distribution Graph of a Grouped Pulse Free Discharge IPL (*Ellipse*, DDD, Denmark). Each trace was taken at time intervals of 1 millisecond, producing groups of sub-pulses discharging even spectral outputs over the entire train of sub-pulses. *Illustration and caption adapted from Ash et al. [182]*

2.3.4 IPL Device Variability

Even where IPL devices have similar average spectral outputs, they cannot be viewed as being equivalent in efficacy. Hence, correlating inter-study outcomes can be difficult where identical devices have not been used. There is also some doubt surrounding the accuracy of the claims of IPL manufacturers.

Town et al. [184] inspected 18 IPL machines (manufactured in Europe with the exception of 1) and discovered that 2 manufacturers exaggerated the size of the applicators; only 8 devices had a medical CE mark; 11 of 30 IPL heads discharged fluences more than 20% below the displayed level and 8 were more than 10% above that displayed. Admittedly, the manufacturers' methods of measuring energy density may have differed from those used by authors of this study. Marked differences were also noted between the claimed and/or displayed pulse durations and the measured values for non-medical CE marked devices [184]. IPLs with medical CEs were consistent. Only 2 of 18 IPLs had a true square pulse tracing and 3 utilised pulse-stacking to achieve a near constant current system waveform. One IPL system claimed to have a triple pulsing system, but the machine tested produced only 2 pulses, therefore only delivered approximately 67% of the advertised energies. Seven of 30 IPL applicators emitted greater than 1% UV light, despite the cut off filter wavelengths being significantly higher. Only 34.5% of filters were within 20 nm of their stated cut-off filter wavelength.

2.3.5 Adverse Effects of IPL

Unwanted extension of IPL's photothermal effects may occur. Blue light is heavily absorbed by epidermal melanin leading to post-inflammatory hyperpigmentation and pain [208]. Wavelengths of 632 – 940 nm have very little effect on keratinocytes [209], but are a source of free radicals and heat [210].

UV radiation induces DNA mutations through thymine dimerization [182, 211]. Even with the removal of UV light by filtering, visible light may potentially encourage lipid peroxidation and micronuclei formation, markers of oxidative stress and DNA damage [211, 212], which is directly proportional to the light intensity [210]. Botta et al. [213]

compared micronuclei formation induced by visible light (400 – 800 nm) with a UVA/ visible light combination (300 – 800 nm) in cultured keratinocytes. Though UVA caused greater DNA damage, visible light also caused statistically significant genotoxicity which increased with the fluence. Similarly, an IPL device (520 – 750 nm, 2.5 ms single pulse, 9 J/cm^2, 3600W/cm^2) was compared with UVA (90mW/cm^2) and UVB (130mW/cm^2) [211]. Though IPL did not cause DNA damage as UVB did, there was a 6-fold increase in lipid peroxides 15 minutes post-irradiation, which was double that for UVA alone [211]. Conversely, Pflaum et al. [214] conducted similar experiments and noticed DNA containing single strand breaks was very rare in keratinocytes and completely prevented using a 400nm cut-off filter. Also, the magnitude of the damage was partially dependent upon the amount of endogenous porphyrins within the cells. In the context of acne vulgaris, it is conceivable that patients with higher concentrations of porphyrin-producing *P. acnes* within the treatment area may experience more side-effects but potentially greater improvement in their inflamed lesions.

Cells repair this damage via excision, daughter strand repair [215] and thymine-dimer repair through photolyase restriction enzymes in the presence of 300 – 600 nm light (termed photoreactivation) [216]. In young patients, this repair process is complete within 24 hours of irradiation [215].

2.3.6 An Ideal IPL Device

Based on the preceding discussion, an ideal IPL device in the clinical setting is a constant current system delivering the desired filtered wavelengths to the target chromophore, coagulating it with no or minimal collateral damage. This effect should be predictable and equivalent to that stated in the manufacturer's specifications for the chosen device. It

should have the versatility to be manipulated by a trained operator where the desired spectral output, pulse duration and fluences are consistently reproduced throughout the life of the hand piece.

2.4 Clinical Efficacy of Incoherent Light in Acne

The current literature surrounding incoherent visible light therapy in acne must be examined considering the dynamic nature of three components: light source, subjects and treatment schedules. Many studies were open and uncontrolled therefore several authors mentioned the need for more randomised controlled studies with an extended follow-up period [217-219]. Whether or not prolonged follow-up would have been truly possible in a typically young population, where some subjects would have invariably experienced steadily worsening acne, is debatable. Competing regimes are discussed according to their predominant wavelengths i.e. blue, red, yellow or broad-spectrum light (Outlined in Tables 1–3, **Appendix 2**).

Blue light induced a 26 – 70% improvement in inflammatory lesion counts but had a minimal effect on non-inflammatory lesions [52, 220-224]. On average 40 – 80% of patients can expect to appreciate a clinically observable improvement in their acne [220, 221, 225-228]. Therefore, blue light is a relatively successful method of treating inflammatory acne in suitable patients. Direct comparisons with conventional therapies are few. Gold et al. [224] found that the efficacy of blue light therapy (*Blu-U*™ *Blue Light Photodynamic Therapy Illuminator Model 4170*, DUSA Pharmaceuticals, MA, USA; λ not stated) was comparable to 1% clindamycin solution when evaluated after a month. The consensus on the prolongation of the response to blue light is mixed. If assessed, improvement does not appear to extend beyond 2 months after cessation of therapy [220-

224]. The combination of blue and red light is able to reduce inflammatory lesion counts by 50 – 80% [229-231] which is similar to the rates quoted for blue light alone. However, the addition of red light appears to add endurance to the duration of the improvement [230-232].

Where red light was used as monotherapy, there are opposing reports on the duration of its efficacy [217, 233]. Two months post-therapy, Na et al.'s [233] cohort experienced a nearly universal relapse 2 months after therapy whereas Zane et al. [217] (*PDT 1200,* Waldmann Medical Division, Villingen-Schwenningen, Germany; λ = 580-740nm) described continued improvement of their global acne grade when measured at the same time point. It must be noted that Na et al. used a home-use hand held device (*SoftLaser SL30*, Beurer GmbH, Germany; 630 – 670 nm) that delivered a much lower total fluence than Zane et al.'s device. This may explain why Sadick et al.'s [232] cohort, who also used hand held devices (*Omnilux Clear-U*™*,* Phototherapeutics Inc., CA, USA; λ= 415 and 633 nm) managed to maintain a reduction of their mean inflammatory count of 70% 8 weeks after cessation of therapy as their devices delivered much higher irradiances. A possible interpretation of these findings is that red light tends to produce a prolonged remission of inflammatory acne once the intensity/strength of the exposure exceeds a currently undefined threshold level.

Sigurdsson et al. [234] treated 23 patients with mild to moderate acne vulgaris on the body with broad spectrum lamps filtered (*Robax*, Schott, Germany) to emit three wavelength profiles. The lamp housing (*Philips HP3136*, Netherlands) was fitted with different high-pressure arc lamps: full-spectrum light + UVA (*Philips HPA* 400W), violet light (*Philips HPM-10* 400W + *UVILEX* 390-filter, Desag, Germany) and green light (Philips thallium

lamp + KV-470 filter) in an attempt to see which was most effective. Full-spectrum and violet light both produced similar, statistically significant reductions in the number of inflammatory lesions. Of note, in this experiment, the fluences of violet light for both full-spectrum and violet light were similar, suggesting that the photoactive portion lies in the violet range. They also performed split-chest or split-back experiments treating with combinations of their light sources. There is no mention of the use of shielding to carry out these split-field experiments and predictably, intra-patient comparisons found no difference between any of the wavelength profiles. At the end, they concluded that though violet light showed the most promise, visible light was not effective as monotherapy.

Yellow light may be able to elicit similar treatment efficacies as those reported for blue light; however there is much less data supporting incoherent yellow-spectrum light sources in acne. Sami et al. [218] was able to elicit a 41.7% decline in inflammatory counts one month after weekly IPL (*Epi-C Plus®,* Funo, Italy; 550 – 1200 nm, 22 J/cm^2, 30 ms pulse) but Chang et al. [14] (*Ellipse Flex IPL*, DDD, Hørsholm, Denmark; 530 – 750nm, 7.5 – 8.0 J/cm^2) did not demonstrate a similar improvement. This outcome is not surprising as all subjects were inexplicably given full-face benzoyl peroxide and unilateral IPL irradiation [14]. Such a regime obscures the true contribution of the light source as monotherapy. Also, in Sami et al.'s study, the total energy and number of treatments were delivered were double that stated in Chang's paper i.e. once weekly treatments for 6 weeks vs. 3 weeks. Choi et al. [181] described a single-blind split-face trial comparing the effects of 585-PDL with a 530 – 750 nm Ellipse Flex IPL system (*Ellipse Flex IPL*, DDD, Hørsholm, Denmark) on mild acne. Initially, IPL caused a greater improvement but at 4 and 8 weeks after the 4th and final treatment, PDL had slightly better reductions in inflammatory and non-inflammatory lesions. Using the Leeds Revised Acne Grading

scale, their mean acne severity fell from 2.5 to 1.0 unit for PDL and 1.3 units for IPL 8 weeks after the last treatment [181]. This illustrates that yellow light may have an effect on mild acne with prolonged improvement in the medium term.

2.4.1 Summary of Incoherent Light in Acne

Blue light is efficacious in acne vulgaris however, the effects are confined to inflammatory acne, are moderate at best, and the duration of the improvement is restricted to about 4 weeks. Of course, it should be noted that many studies did not extend their follow-up beyond this period, or those that did lost a significant number of their cohort to follow-up. Blue and red light act synergistically producing larger improvements in mean lesion counts, including comedones, when compared to blue light alone. The studies using red light modalities universally achieved a minimum reduction of 50% in inflamed lesions or the acne severity score. The inclusion of red light appears to prolong the efficacy of blue light, extending it to 8 weeks. The literature on yellow incoherent light is scarce and less conclusive. Two of three studies attained a reduction in inflamed lesions of at least 66% when measured 1 month after completion of therapy. Only one of these three studies reported non-inflamed as well as inflammatory lesion counts.

2.5 Conclusions

As several publications demonstrate, light is a pharmacological agent which can induce specific changes in the skin. These photothermal, photomechanical, photochemical and photoimmunological effects are dependent upon the composition and content of the skin through which it passes and the colour, width, strength and duration of the irradiant beam. These latter light-dependent properties are dictated by the physics of the apparatus that

produces them, where a constant, controlled beam consisting of the desired wavelengths can produce clearance of the acne lesions without unwanted photo-induced side-effects. Together, these form the basis for the use of visible broad-spectrum light in acne.

Chapter 3

BACKGROUND 3

Potential Mechanisms of IPL's Action in Acne

3. Potential Mechanisms of IPL's Action in Acne

3.1 Introduction

Acne vulgaris is an inflammatory disease modified by endogenous and exogenous factors. However, prior to 2012, published observations on the mechanism of specific wavelengths of IPL in acne, in *vivo* or *in vitro*, were lacking. A single study by Fan et al. (2012) investigated the mechanism of 420 nm IPL in a Sprague-Dawley rat acne model [235]. They based their study on the ability of 420 nm light to photoactivate endogenous CPIII in *P. acnes* causing photodynamic destruction of the bacteria and resultant improvement in inflammatory acne. In their model, inoculation of *P. acnes* isolates into the ear of the treatment group resulted in an increase in the expression of TNFα (4 fold) and MMP-2 (2 fold) while control groups did not exhibit a significant increase in either cytokine. In the intervention group, mRNA expression of TNFα and MMP-2 returned to baseline after 6 irradiations with 420 nm IPL. The untreated group did not experience a similar reduction and hence the authors concluded that 420 nm IPL's anti-inflammatory effect was secondary to *P. acnes* destruction. Thus, IPL is able to directly cause a photochemical or photodynamic reaction and secondarily induce an immunological effect. Relevant studies investigating the use of incoherent visible light in acne will be discussed from the perspective of light-skin interactions. This is followed by greater focus on the cytokines and receptors with the greatest potential of being modulated by 530 nm IPL.

3.2 *Visible Light – Acne Interactions*

3.2.1 Photochemical/ Photodynamic Interactions

Blue light has a well-known antimicrobial effect [68, 236] that is dependent on photoinactivation of *P. acnes* through a coproporphyrin-mediated photodynamic reaction. This leads to intracellular membrane disruption, leakage and cell separation [236]. In fact, blue light may be such an effective photoactivator of coproporphyrin that the addition of an exogenous photosensitiser may not improve its efficacy [208, 237]. In an *in vitro* study, Ashkenazi et al. [236] quantified the inhibition of bacterial growth after 3 illuminations with narrow band blue light (NBBL; *Clear Light™,* Lumenis Ltd., Yokneam, Israel; 407 – 420 nm; 20 mW/cm^2 or 75 J/cm^2) with a 1 day interval between each treatment. Colony counts were reduced by 5 orders of magnitude. Conversely, *in vivo* studies that assessed *P. acnes* inhibition by colony counts, cultures and PCR assays pre- and post- light treatment (blue and red) failed to show a quantitative reduction in *P. acnes* despite clinical improvement [52, 222, 225]. Pollock et al. [238] treated selected areas on the backs of 10 acne patients with diode laser PDT (CeramOptec GmbH, Bonn, Germany, λ= 635 nm, 25 mW/cm^2, 15 J/cm^2). This caused a significant reduction (68.97%, $p< 0.05$) of inflammatory lesions but *P. acnes* colony counts were not significantly reduced at the end of the 3-week treatment period. *P. acnes* can recolonize as early as 10 days post-treatment [239] thus the absence of a change may be due to timing of the assessments.

3.2.2 Photothermal Interactions

Elman & Lask [240] treated 19 volunteers with broad spectrum light and heat (*Clear Touch*™, Radiancy Inc., NY, USA; λ= 430 – 1100 nm, 3.5 J/cm^2). The peak emission

wavelengths were not stated, but were said to be predominantly green/yellow light. The investigators observed a steady reduction in inflamed and non-inflamed lesions up to 2 months post therapy with mean lesion count reductions of 85%. Basing their hypothesis on the Arrhenius equation (temperature increases of 10°C double the rate of a chemical reaction), they surmised that the addition of heat accentuated any improvements made using visible light alone. Elman and Lask [240] did not venture any views about the underlying mechanism. As heat-killed *P. acnes* is still immunogenic [241], direct bacterial heating is unlikely to play a role in resolving acne lesions. However, vascular coagulation and the induction of heat shock proteins are two potential mechanisms of action.

3.2.2.1 Photocoagulation

Vascular coagulation destroys the conduit for inflammatory cell migration and thus may indirectly contribute to IPL's anti-inflammatory mechanism of action. Treatment of cutaneous vascular malformations, telangiectatic vessels and diffuse redness is dependent upon a light source's ability to heat intravascular haemoglobin to at least 70˚C leading to coagulation of aberrant vessels [185]. Pulse width is important as larger vessels (150 – 300 μm) may not be coagulated at their bases even in the presence of high fluences [185]. This is especially relevant regarding the choice of apparatus as free-discharge IPLs are less efficient at sustaining useful pulse widths to completely coagulate larger targets [182]. In the absence of clinical data, predictions of tissue temperatures, and hence an adjustment of treatment parameters, can be made using mathematical models [173, 185, 242].

3.2.2.1.1 Photocoagulation and Sebaceous Glands

IPL and coherent light sources have been successfully used to treat sebaceous gland hyperplasia with and without an exogenous photosensitizer [243-246]. *In vivo* confocal microscopy performed immediately after treatment of a hyperplastic sebaceous gland with 585 nm PDL revealed that the damage was confined to the blood vessels and improvement in the hyperplastic epithelium became discernible 2 weeks after [244]. This study [244] suggests that sebaceous gland shrinkage occurs after photocoagulation of its vascular supply. In the context of acne vulgaris, both Chang et al. [14] and Glaich et al. [247] mention self-reported reductions in oiliness after yellow-spectrum light therapy but sebum measurements were not done to substantiate these claims. Unfortunately, objective sebutape measurements by Orringer et al. [219], who used PDL (*NLite* Laser, ICN Pharmaceuticals, Calif., USA; 585nm), did not reveal any changes in sebum output.

3.2.2.2 IPL and Heat Shock Proteins

IPL, through heat shock protein 70 upregulation, may induce a wound healing response in human and murine skin [248-250], leading to acne resolution (see Chapter 1, Section 1.2.1.3).

Heat shock protein (HSP) release is an evolutionarily conserved ability of organisms to respond rapidly to stressors such as sudden increases in temperature (heat or cold), oxidative stress and toxins [251, 252]. HSPs protect the cell by forming complexes with unfolded or denatured peptides and proteins produced as a result of the stressor [252]. HSPs ensure correct re-folding or re-assembly of these proteins and increase the tolerance of exposed cells to further injury [252]. The function of HSPs is not confined to cell

protection but also has a role in the regulation of cell growth and proliferation. HSPs are commonly classified according to their molecular weights in kDa e.g. HSP 60, 70 and 90 [253].

The evidence of IPL's action on heat shock protein expression is limited. Small animal and human studies suggest that IPL can induce heat shock proteins. A study evaluating the immunohistochemical expression of HSP70 in 9 women after a course of 560 nm IPL (make and model not stated; 560 nm cut-off, 28–35 J/cm^2) over 5 months showed a 'slight' increase in the number of dendritic cells expressing HSP70 [249]. Helbig et al. [254] obtained skin explants from 35 volunteers and exposed groups of five to three different light sources: 585 nm light emitting diode (LED), 633 nm LED (*LEDA SCR*, Quantel-Derma, Erlangen, Germany; 20 – 120 Jcm^{-2}) and 1540 nm Er: glass laser (*Aramis,* Quantel-Derma, Erlangen, Germany; 2 mm spot, 30 Jcm^{-2}). The LED output was delivered at various fluences in pulsed and non-pulsed modes. After irradiation with an Er: glass laser, HSP70 was uniformly up-regulated in the epidermis of explants, which was maximal between days 1 and 3. The 585 nm and 633 nm pulsed LED did not elicit a similar response. This study [254] suggests that high-intensity pulsed LED may not necessarily induce HSP70. However, Wang et al. [250] reported that irradiation of murine skin with IPL (action spectrum not stated) up-regulated HSP70 expression. Taking these studies together, IPL more than likely increases HSP70 expression in human skin.

3.2.3 Photoimmunological Interactions

Light has a variety of immunological actions that vary with the predominant wavelength of light. An *in vitro* study using 530 nm IPL showed up-regulation of IL-10 in cultured keratinocytes [255] and pulsed dye laser is known to increase TGFβ1 expression in treated

skin [13]. Both of these cytokines have anti-inflammatory effects. Shnitkind and colleagues [256] incubated two immortalized keratinocyte cell lines (HaCaT and hTERT) with TNFα and IFNγ and then exposed them to narrow-band blue-light (NBBL) and/or UVB. The expression of IFNγ's downstream cytokines, IL-1α and ICAM-1 were measured. NBBL alone reduced IL-1α and ICAM-1 concentrations by at least 70% in both cell lines. Its effect was synergistically augmented by UVB.

Boros-Gyevi et al. [257] presented an interesting abstract at the 2009 Annual Meeting of the American Academy of Dermatology. They sought to determine whether broad-spectrum IPL (wavelengths not stated) had any effect on delayed type hypersensitivity reactions (DTH) in photo-damaged and normal skin. The DTH was induced by Mantoux testing and measured by the number of CD1a+ Langerhans cells in skin biopsies taken before and after IPL irradiation. After a course of IPL (3 treatments at 3 week intervals), the number of CD1a+ Langerhans cells was higher in IPL-treated photodamaged skin than untreated chronically photodamaged skin. Cell numbers were also high in normal, photo-protected skin [257]. Based on these findings they concluded that IPL was able to induce an immunological reaction. Though a full publication of this abstract is still outstanding, their findings suggest that IPL may have a direct immuno-stimulatory or 'immuno-redemptive' role in skin physiology. As previously mentioned (Chapter 1), it has been theorised that the cellular infiltrate in acne pathophysiology is reminiscent of a DTH reaction [26], suggesting the possibility that IPL exacerbates rather than suppresses the inflammatory reaction. Further study, such as that presented in this thesis, will be required to clarify this issue.

3.2.4 Summary

It is evident that different wavelengths of light have divergent biological effects. **Table 3.1** revisits the therapeutic targets identified in Chapter 1 and lists the most likely light-skin interactions responsible for light's efficacy in acne.

Table 3. 1: The Potential Mechanisms of Action of IPL against Acne Pathophysiology's Therapeutic Targets

Pathogenetic Factors	Therapeutic Targets	Visible Light-Acne Interactions
P. acnes	CPIII, TLR2/TLR4	Photodynamic Photoimmunological
Pro-inflammatory Cytokines	IL-8, IL-6, TNFα, IL-1β ICAM, VCAM	Photocoagulation Photothermal Photoimmunological
Anti-inflammatory Cytokines	IL-10, TGF-β	Photoimmunological
T-cells	CD4 (Th), CD8 (Tc), CD3+ CD4- CD8- (Ts)	Photoimmunological Photocoagulation
Ductal Hyperkeratosis	IL-1α, IL-1β **GCSF, *EGF*	Photothermal Photoimmunological **EGF, GCSF - unknown*
Growth Factors	GCSF	Unknown
Sebaceous Gland	Sebocytes	Photothermal
Sebogenesis	Prostaglandins, CRH, PPARs, steroidogenic enzymes	Unknown

Pathogenetic Factors	Therapeutic Targets	Visible Light-Acne Interactions
Genetic Susceptibility	CAG repeats, AR, FGFR, CYP17, CYP21, TNFR2, TLR2, FGFR2	? Photothermal DNA damage

3.3 Photo–therapeutic Targets for Yellow IPL

The trial in this thesis focuses on the actions of a broad-spectrum yellow IPL source. Light-skin interactions pertinent to yellow-spectrum light are: (1) Photodynamic inactivation of *P. acnes* (2) Photocoagulation of blood vessels surrounding the pilosebaceous gland and (3) Direct and indirect photo-immunomodulatory effects. As previously discussed, clinical improvement does not always coincide with a reduction in *P. acnes* colony counts [52, 222, 225], therefore, like Fan et al. [235], indirect assessment by measuring inflammatory cytokine expression is a reasonable way of assessing IPL's immunomodulatory effects.

Five of the nine pathogenetic factors listed in Table 3.1 are potentially addressed by the photo–immunological mechanisms attributed to visible radiation. TLR2, TNFα, IL-8 and IL-10 contribute to each of the three phases in acne pathogenesis: initiation, evolution and resolution. They are discussed in more detail below followed by a unifying hypothesis of yellow IPL's mechanism of action in acne.

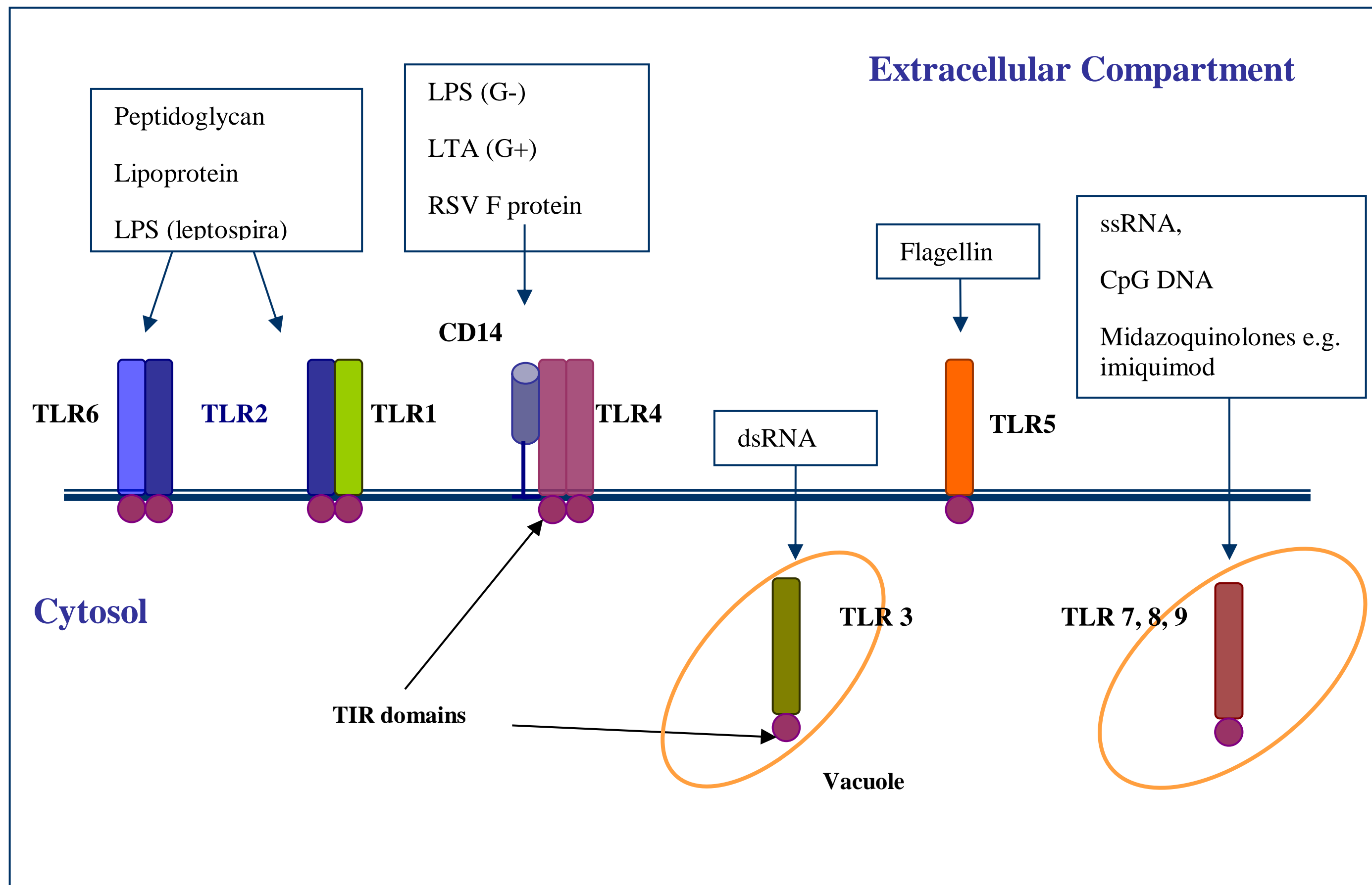
3.3.1 Toll-like Receptors

Toll-like receptors (TLRs) detect highly conserved molecules or patterns on the cell surface of microbes. These patterns are called pathogen-associated molecular patterns (PAMPs) [258]. Pattern recognition receptors (PRRs) bind PAMPs resulting in either activation of a signalling cascade which drives a specific immune response or non-specifically renders the bound microbe more susceptible to phagocytosis and destruction through complement activation [259]. Toll-like receptors (TLRs) are signalling PRRs [258, 259] consisting of two domains.

Outer TLR domains are structurally diverse and consist of leucine-rich repeats producing at least ten different classes of receptor (TLR1–10) in humans [62, 259-262]. They are generally extracellular but some TLRs (3, 7 and 9) are intra-vacuolar [263]. Diversity within the extracellular domain of TLRs dictates the ligands to which they bind. See **Figure 3.1.**

TLR4, one of the first mammalian TLRs described, binds lipopolysaccharides (LPS) in gram-negative bacteria (e.g. *E. coli*), lipoteichoic acid and heat-shock proteins (HSP60 and HSP70) released from necrosed or virally lysed cells [258, 261, 263, 264]. The ligand for TLR10 is still unknown. TLR2 recognises an unusually broad range of cellular components including gram-positive cell wall peptidoglycans (PGNs), atypical lipopolysaccharides [265], lipoteichoic acid, lipoarabinomannans from mycobacterial cell walls [62, 260, 266] and HSP 70 [264]. This versatility and specificity is due to its cooperation with TLR1 and TLR6 [258].

TLR's have an intracellular domain, the Toll/IL-1 receptor (TIR) that is highly conserved and similar across all TLR family members. Stimulation of signalling pathways via TLRs mobilises gene transcription of immunomodulatory chemicals (IL-6 and TNFα), costimulatory molecules on antigen presenting cells (iNOS) and anti-microbial peptides such as defensins [258, 259, 267]. These signalling pathways may be either unique to each TLR or 'communal' and often involve IL-1R as well. Despite the commonality amongst the beginnings of TLR pathways, activation of each results in diverse and differential cytokine expression [268]. For example, monocytic and dendritic (CD11+) TLR2 stimulation induces IL-6, TNFα and IL-12 secretion [266]. TLR7 and TLR9 stimulation primarily stimulates type I interferon (IFN) production, which is appropriate for their viral ligands [269]. However, the gene products which control this specificity are still unknown [268].



Note: TLR2 heterodimerises with both TLR1 and TLR6 resulting in a diverse number of ligands. TLRs 3, 7, 8, & 9 are intra-vacuolar receptors whilst the rest are transmembranous. CD 14 is a co-recognition molecule for TLR2 and TLR4. **KEY**: *G+,* gram positive; *G –,* gram negative; *LTA* – lipotechoic acid; *LPS* – lipopolysaccharide; *TIR* – Toll/ IL-1 receptor; *RSV* - respiratory syncytial virus.

Figure 3.1: Diagrammatic Representation of Ligand Specificities of Selected TLRs [258, 262, 263, 270, 271]**.**

3.3.1.1 Toll-like Receptor Localisation

Epidermal keratinocytes and corneocytes are the first anatomical barrier and therefore play a major role in innate immunity. TLR1–6 and TLR 9 are expressed by all keratinocyte cell lines [263, 272]. However, it is stimulation of TLR2 – 5 and TLR9 that cause cytokine and chemokine release [273] supporting the finding that TLR1 and TLR6 require TLR2 for any meaningful function. In human skin, TLRs are found on a variety of other cell types including SZ95 sebocytes (TLR2, 4 and 6) [274, 275], monocytes (TLR2) [30] and dendritic cells (DCs) (TLR1–5, 7–9) [266]. As would be expected for a highly specialised immunological system, different dendritic subsets have different TLRs and therefore respond to different ligands [266]. Regarding the sites involved in acne, sebocytes express TLR2, 4 and 6, and TLR2 has also been found on macrophages surrounding the pilosebaceous unit.

3.3.1.2 P. acnes Binds Preferentially to TLR2

Live and heat-killed strains of *P. acnes* can induce keratinocytes and monocytes to release pro-inflammatory cytokines [30, 86, 276] and anti-microbial peptides [54, 58] through toll-like receptor (TLR) activation. An atypical gram positive bacterium like *P. acnes* could be recognised by either TLR2 or TLR4 on epidermal and dendritic cells [30]. But, a number of studies suggest that *P. acnes* preferentially binds TLR2.

P. acnes-induced cytokine production by monocytes is largely TLR2-dependent [30]. Kim et al. [30] were unable to detect IL-6 production after intra-peritoneal seeding of TLR2 knockout mice with *P. acnes* but wild-type, TLR1$^{-/-}$ and TLR6$^{-/-}$ mice responded appropriately [260]. Also, pre-incubation of human monocytes with anti-TLR2 reduced

IL-12 production by 65% [260] but this reduction was not reproduced when an anti-TLR4 antibody was used [260]. Thus, these experiments show that TLR2 is the main receptor that recognises *P. acnes* products in these models.

Stimulation of the inflammatory TLR2 pathway by *P. acnes* is more robust in comparison to non-pathogenic bacteria. The induction of TNFα and IL-12 release from peritoneal macrophages after *P. acnes* stimulation is double that of *S. epidermidis*, a non-pathogenic commensal [277, 278]. However, the anti-inflammatory activity of *S. epidermidis* is significantly greater as IL-10 induction was 2.5 times the amount elicited by *P. acnes* [277]. This may represent the mechanism by which skin commensals exist without eliciting an inflammatory response.

3.3.1.3 TLR2 Suppression is Beneficial in Acne

TLR2 is more strongly expressed than TLR4 in acne-involved epidermis [62] and peripheral blood monocytes [279] when compared to normal controls. Pre-incubation of keratinocytes with anti-TLR2 and anti-TLR4 antibodies cause partial abolition of hBD2 and IL-8 production [60]. The authors explained this phenomenon as being due to the presence of TLR2 co-receptors (TLR1 and TLR6), which were still able to mediate certain signalling pathways. Dispenza and colleagues [279] hypothesised that the long-term efficacy of isotretinoin in acne may be due to TLR2 suppression. Using flow cytometry, they measured TLR2 expression on PBMCs 6 months after completion of a 20 week course of isotretinoin (n= 8). TLR2 expression was reduced by approximately 50% ($p < 0.01$) compared to baseline levels [279]. They suggested that correction of the 'dysregulated' innate immune response contributes to long-term remission of the disease after isotretinoin therapy.

3.3.1.4 Toll-like Receptor 2 Signalling

Now that TLR2 has been established as the key TLR in the disordered immune response in acne patients, a short description of TLR2 signalling will establish that its stimulation is likely to be the initiating event in acne pathogenesis.

After lipopeptide or peptidoglycan portions of bacterial cell walls bind to the extracellular portion of TLR2/6 or TLR2/1, MyD88 (Myeloid Differentiation primary response protein 88*)* is recruited to the TIR domain of the receptor along with MAL, the bridging adaptor [259, 280-283] (**Figure 3.2**). This interaction facilitates the binding of the death-domains of MyD88 and IRAK 4 (IL-1 Receptor Associated Kinase 4) which then binds and phosphorylates IRAK1. The MyD88/IRAK4/IRAK1 complex then activates TRAF6 (TNF Receptor-Associated Factor 6) and IRAK1/TRAF6 dissociate from the toll receptor.

TRAF6 recruits TAK1 (TGFβ Activated Kinase 1) and TAB 1 & 2 (TAK1 binding proteins 1 & 2) [282]. ECSIT *(*Evolutionarily Conserved Signalling Intermediate in Toll pathways) also binds to TRAF6 and indirectly modulates the function of TAK1 [284], playing a role in the generation of reactive oxygen species [285]. The TRAF6/ TAK1/ TAB1/ TAB2 complex enters the cytosol, leaving IRAK1 behind on the cell membrane for degradation.

TRAF6 is then degraded by ubiquitination enzymes, e.g. Ubc13 and Uev1A, and the TAK1/TAB1/TAB2 complex can induce signal transduction via two separate pathways, NF-κB (Nuclear Factor kappa B) or MAPK (Mitogen-Activated Protein Kinase) [286, 287]. In the NF-κB pathway, TAK1 phosphorylates the IKK (IκB Kinase) complex that in turn phospho-degrades IκB (inhibitor of κB). The nuclear localisation sequence of NF-κB

becomes exposed and it is now free to enter the nucleus to regulate transcription. In the MAPK pathway, its members are sequentially phosphorylated, activating ELK-1 (Ets-like protein 1) and AP-1 (Activator Protein 1) [288]. These transcription factors enter the nucleus to regulate gene transcription.

3.3.1.4.1 NF-κB and MAPK Pathways in Acne

NF-κB and AP-1 activation has been demonstrated in acne lesions. Kang et al. [289] observed that AP-1 was significantly up-regulated in acne involved skin compared to normal skin and found clear nuclear localisation of NF-κB within perifollicular and epidermal keratinocytes over and above that seen in adjacent biopsies of uninvolved skin and normal control skin. Elevated NF-κB expression was accompanied by significant increases in mRNA levels for IL-8 (3,015 fold), IL-1β (16 fold), IL-10 (46 fold) and TNFα (2.6 fold) compared with non-lesional skin. *In vitro*, Grange et al. [290] incubated HaCaT and primary keratinocyte cultures with *P. acnes* (strain ATCC 6919) and found that both AP-1 and NF-κB promoter regions of the IL-8 gene were activated. Demonstration of the activation of both these pathways provides additional evidence for the central role of TLR2 signalling secondary to *P. acnes* binding in acne pathogenesis.

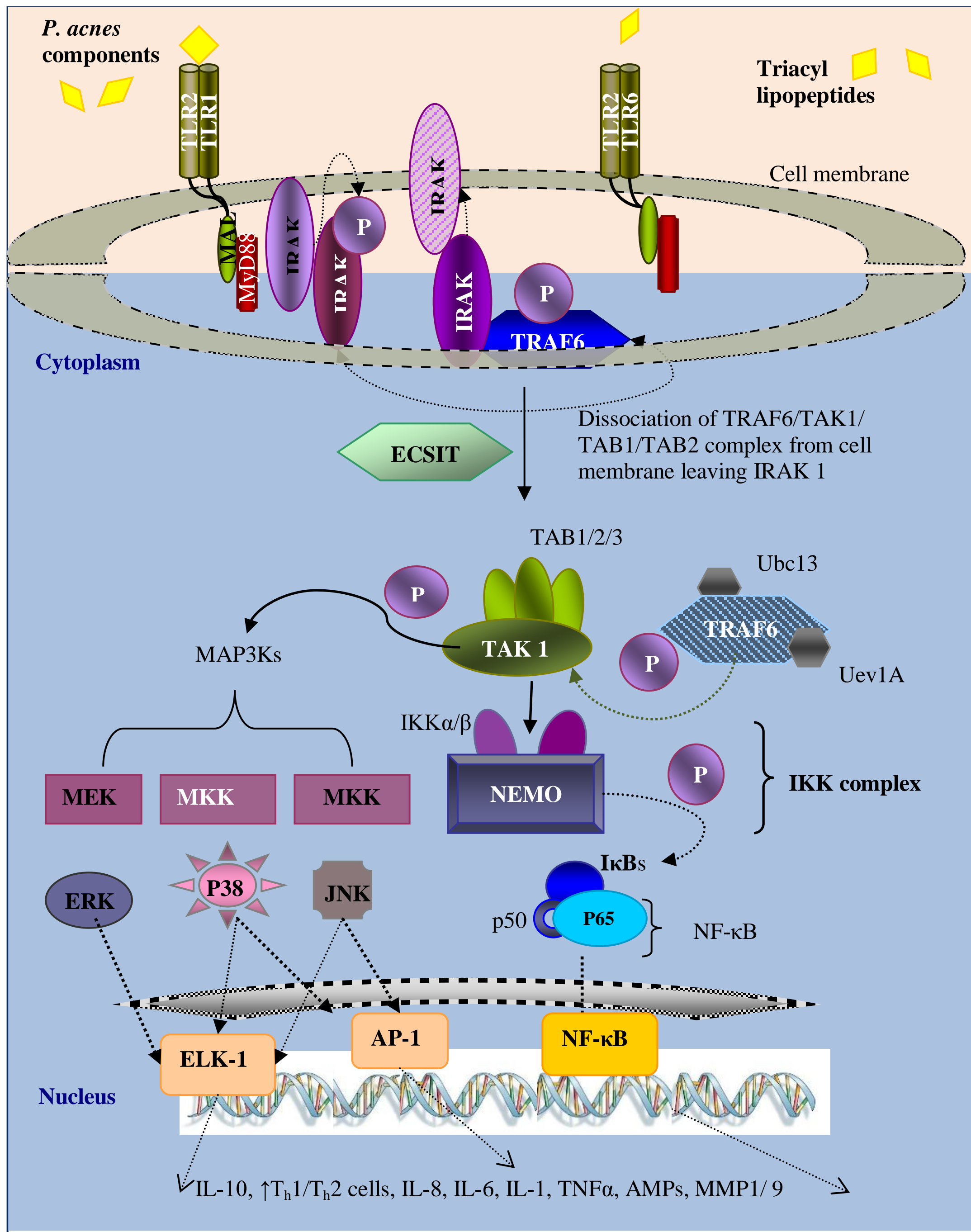


Figure 3.2: TLR2 Signalling. After TLR2 binds the stimulating ligand e.g. PGN from *P. acnes*, a number of membrane bound kinases (IRAKs) phosphorylate TRAF6. This eventually leads to phosphorylation of IκB, freeing NF-κB for nuclear translocation. Inflammatory gene transcription (e.g. IL-6, IL-8, IL-12 and TNFα) follows.

KEY: *AMPs*, antimicrobial peptides; *ECSIT*, evolutionarily conserved signalling intermediate in the Toll pathway; *ELK*, Ets-like protein 1; *ERK*, extracellular signal-regulated protein kinase **KEY:**

GPB, gram positive bacteria; *IκB*, inhibitor of κB; *JNK*, *c*-Jun N-terminal kinase; *MyD88*, myeloid differentiation primary response protein 88; *NEMO*, NF-κB essential modulator; NEMO = IKKγ; *P*, phosphorylation; *TAB2*, TAK binding protein; *TAK,* TGF-β activated kinase; *UEVA1/UBC*, ubiquitination enzymes. *Adapted from Liew et al. [283], Cell Signalling Technology [291], Takeda et al. [282] and Kaisho et al. [259].*

3.3.1.4 Control of TLR2 Signalling

TLR2 expression and signalling is regulated by extracellular, intracellular and trans-membrane mechanisms [283] at various stages during signal induction, transduction and gene expression .However, all of these checkpoints may not be functioning within the same tissue. One mechanism of preventing ligand binding is through the use of decoy receptors. Soluble splice variants of TLR2 and MyD88 act as receptor decoys. TLR2 soluble receptors mop up excess TLR2 ligands preventing excessive activation of the bound receptor [283] whereas a soluble form of MyD88 is preferentially recruited to IRAK4 rather than full-length MyD88 (**Figure 3.3**).

If ligand binding has occurred, soluble MyD88 and TOLLIP (Toll Interacting Protein), prevent phosphorylation of IRAK1, aborting the NF-κB pathway. TRAILR (TNF-related Apoptosis Inducing Ligand Receptor) is a trans-membrane protein which stabilises the NF-κB complex and prevents translocation to the nucleus. IL-10, another TLR2 regulator, is transcribed as a result of TLR2 signalling. Through negative feedback mechanisms, IL-10 can prevent NF-κB nuclear localisation and the release of IL-8, MMP-9 and MMP-2 in pro-inflammatory cells [292, 293].

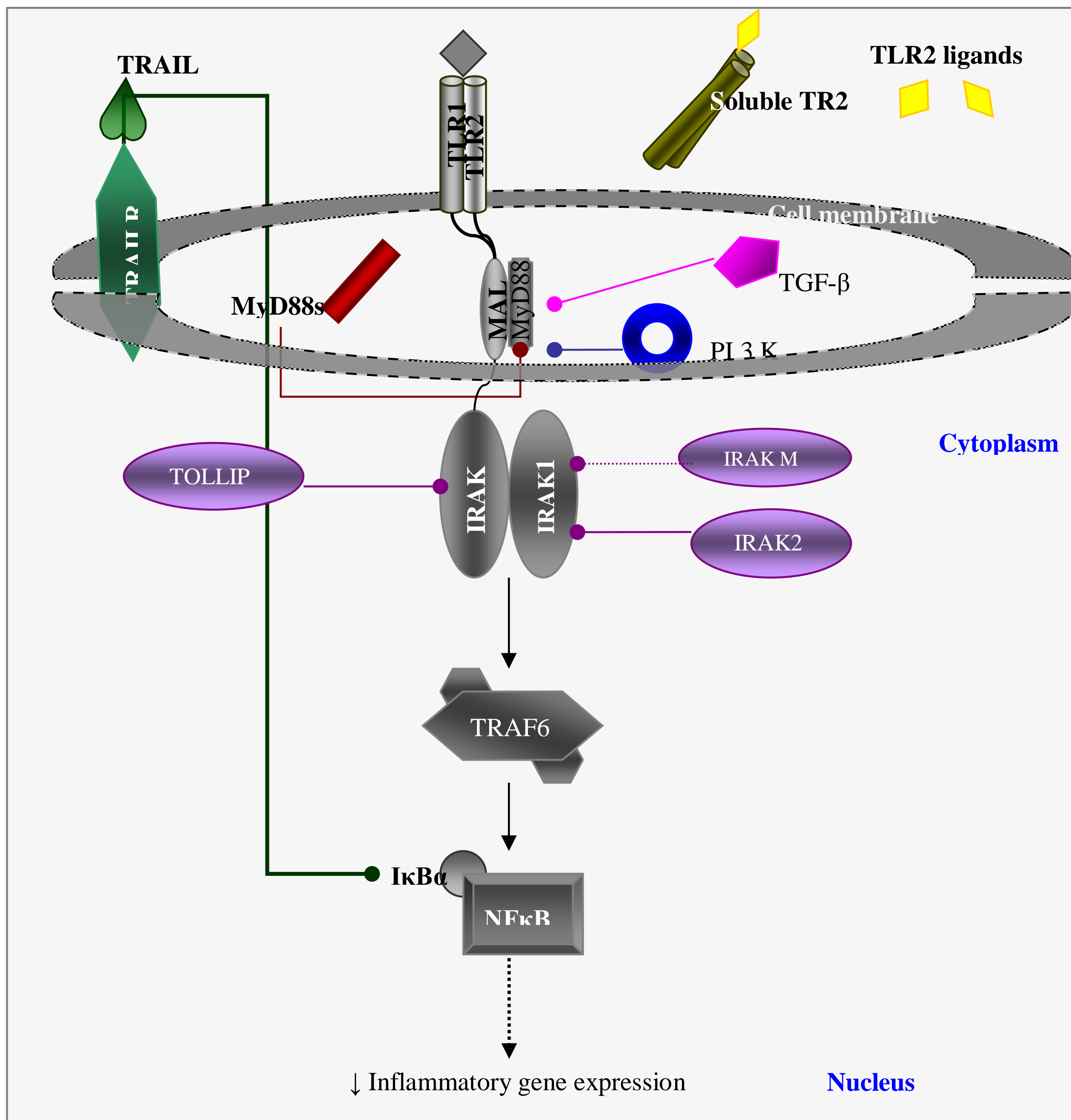


Figure 3.3: Negative Regulation of TLR2 Signalling. TLR2 signalling is regulated at several levels: ligand binding, signal transduction, NFκB activation and gene transcription.

KEY: *TOLLIP*, toll interacting protein; *TRAILR*, TNF-related apoptosis inducing ligand receptor (*Adapted from* Cell Signalling Technology [291] and Liew et al. [283]).

3.3.2 Interleukin–8 and *P. acnes*

Vowels et al. [294] found that *P. acnes* strain 6919 and its culture supernatant were able to significantly stimulate monocytic cell lines (U937 & ThP-1) to produce IL-1β, TNFα and large quantities of IL-8 protein. However, stimulation of peripheral blood monocytes from 10 acne and 5 normal subjects did not show any differences in cytokine release [294]. Basal et al. [295] and Caillon et al. [40] conducted similar but larger studies comparing acne patients with age and sex-matched controls. In both studies PBMC from acne patients released significantly higher levels of IL-8 compared with normal controls after *P. acnes* stimulation. In Caillon et al.'s cohort [40], higher concentrations of *P. acnes* (10 – 100µg/ml) vs. low concentrations (0.1µg/ml) stimulated significantly higher quantities of TNFα from acne-derived PBMCs.

Schaller et al. [65] looked at the cytokine profile produced by a keratinocyte cell line (TR146) after stimulation with live and heat-killed isolates of *P. acnes* harvested from comedones. IL-8 mRNA levels were far higher than those for IL-1α, IL-1β and TNFα. Interestingly, viable *P. acnes* induced a 5-fold increase in IL-1β expression and a 15-fold increase in IL-8 and GM-CSF when compared with heat-killed bacteria. The other cytokines showed no difference between the two groups. A similar study involving *P. acnes* stimulation of a monocyte cell line also revealed significant increases in IL-8 secretion [296].

Together, these studies agree that IL-8 release after *P. acnes* stimulation is dose-dependent, TNFα is up-regulated and heat-killed bacteria lose some of their immunogenicity. Therefore, photothermal destruction by IPL may be relevant.

3.3.3 TNFα in Acne

TNFα is a soluble pro-inflammatory protein which exists as a cell-bound 26 kDa protein or a soluble 17 kDa protein [297]. In its biologically active form, a TNFα trimer binds to its receptors (TNFR1 or TNFR2) present on virtually all nucleated cells [297]. TNFα binding to its receptors can have various effects but is heavily skewed towards an inflammatory response. TNFR1 activation stimulates pro-inflammatory and pro-apoptotic pathways [297, 298] whereas TNFR2-ligand binding results in the activation of inflammation via the AP-1 and NF-κB pathways. TNFα has NF-κB and AP-1 binding sites within its promoter region allowing for escalation and amplification of an incipient inflammatory response [298]. Hence, TNFα can be both pro- and anti-inflammatory, conferring complexity to its signalling cascade with a number of opportunities for cross-talk between adaptor molecules and transcription factors [298, 299].

Whole *P. acnes* and smaller polypeptide moieties are able to markedly increase the release of TNFα in acne patients [295]. In an *in vitro* study, SZ95 sebocytes incubated with TNFα synthesised more cholesterol and fatty acids [300]. TNFα can promote collagen breakdown through MMP-2 and MMP-9 release [300-302]. Thus, TNFα suppression may be beneficial in the treatment of inflammatory lesions, seborrhoea and acne scarring.

Direct TNFα inhibition as a therapeutic option for acne has been tried. Campione and colleagues published a case report of a young man with nodulo-cystic lesions that regressed after 24 weeks on etanercept, a TNFα receptor inhibitor [303]. Remission was maintained for the 3 month follow-up period, but this was too short to make a reasonable comment on any effects with regard to scarring. They did not comment on the patient's seborrhoea. Isotretinoin has also been found to inhibit TNFα's transcription factor, NF-

κB, via TRAIL (TNF-Related Apoptosis Inducing Ligand), *in vivo* and cultured sebocytes [304]. Another anti-acne agent, doxycycline, attenuates the increase in pro-MMP-2 released by dermal fibroblasts incubated with TNFα and *P. acnes* lysates [300].

3.3.4 Interleukin 10

Interleukin 10 (IL-10) is an immunomodulatory 18kDa polypeptide [305]. It was initially known as 'cytokine synthesis inhibitory factor' released by murine T-helper type 2 (Th2) cells [306]. This inhibitory factor was found to inhibit IFNγ release from Th1 lymphocytes. Macrophage activation, cytokine release and apoptosis are also inhibited as a result of IL-10 binding [307]. Later, it was discovered that IL-10 could also suppress Th2 lymphocyte function, enhance antigen presentation and MHC II expression, induce pro-inflammatory cytokines as well as regulate B- and T-cell development and maturation [292, 308, 309]. Thus, whilst being anti-inflammatory, IL-10 can also have pro-inflammatory actions which are dependent upon the timing of its release during an immune response, the target cell and, the portion of its receptor participating in the reaction [310].

3.3.4.1 IL-10 in Acne

IL-10 is produced by a number of cells including CD4+ and CD8+ T-cells [269], B-cells [311], keratinocytes, dendritic cells (DCs), macrophages, neutrophils [307, 312, 313] and mast cells [314]. However, IL-10 receptors (IL-10R) are most abundantly expressed by peripheral blood cells such as monocytes, B- & T-lymphocytes and macrophages [308, 315].

Using ELISA (enzyme-linked immunosorbent assay) to detect cytokine secretion from peripheral monocytes harvested from venous blood, Caillon et al. [40] showed that *P. acnes* stimulation of CD14+ mononuclear cells from acne patients reduced the secretion of IL-10 by 46% compared to normal controls ($p < 0.05$). Addition of IL-10 to the acne patient derived-monocytes improved their phagocytosing ability and reduced their secretion of IL-8 and TNFα [40]. The degree of IL-10 deficiency in the blood donors did not correlate with their acne severity. In contrast, using skin explants, Tenaud et al. [276] noted higher staining intensity for IL-10 in the epidermis of acne vs. normal skin. When incubated with adapalene, immunostaining for IL-10 fell significantly, along with that of TLR2 and Langerhans cells, which the authors assumed was related to its mechanism of action in acne [276].

Yellow spectrum IPL may show efficacy in this regard as it has been shown to up-regulate IL-10 expression in keratinocytes. Irradiation of HaCaT keratinocytes with yellow IPL (555 – 950 nm, 8 J/cm^2) increased IL-10 protein levels by almost 6-fold ($p < 0.05$). However, TNFα protein levels also rose by approximately 60% ($p < 0.05$) [255]. These conflicting results emphasise the need for an understanding of IL-10's signalling pathways to clarify IPL's cutaneous effects *in vivo*.

3.3.4.2 IL-10 Signalling via JAK-STAT

IL-10 is transcribed upon stimulation of the TLR2 receptor by its ligands [270, 312] primarily through activation of the p38 MAPK pathway [316-318]. When an IL-10 homodimer binds to its receptor (IL-10R, a tetramer of identical subunits of IL-10R1 and IL-10R2), this initiates cross-linking of IL-10R1 and IL-10R2 followed by recruitment of Tyk 2 (Tyrosine kinase 2) by IL-10R2 [315]. See **Figure 3.4**.

Tyk2 trans-phosphorylates the IL-10R1 subunit and JAK1 (Janus kinase 1), which is normally bound to the intracellular portion of IL-10R1 at rest [307, 315]. JAK1 then phosphorylates specific tyrosine residues (Y446 and Y496) on the intracellular portion of IL-10R1, allowing it to serve as a docking site for STAT3 (Signal Transducer and Activator of Transcription 3) [315, 319]. JAK1 also phosphorylates STAT3, which forms a homodimer. The STAT3 dimer translocates to the nucleus and binds to specific promoter sequences of IL-10 response genes which dictate genetic control of the cell-cycle and anti-inflammatory proteins. *In vitro* studies have demonstrated that STAT3 is partially responsible for many of IL-10's anti-inflammatory activities [307, 308, 320]. STAT1 and STAT5 are also implicated in IL-10 signal transduction. Like STAT3, they form homo- and hetero-dimers before translocating to the nucleus to dictate transcription of anti- and pro- inflammatory cytokines [308].

3.3.4.2.1 Anti-inflammatory Actions of IL-10

IL-10 exerts its anti-inflammatory effects through a number of molecules present in monocytes and macrophages. One of these molecules is suppressor of cytokine signalling 3 (SOCS3) that inhibits the JAK/STAT pathway, suppresses T-cell differentiation and regulation, and binds to the cytoplasmic portion of cytokine receptors leading to their inhibition [321, 322].

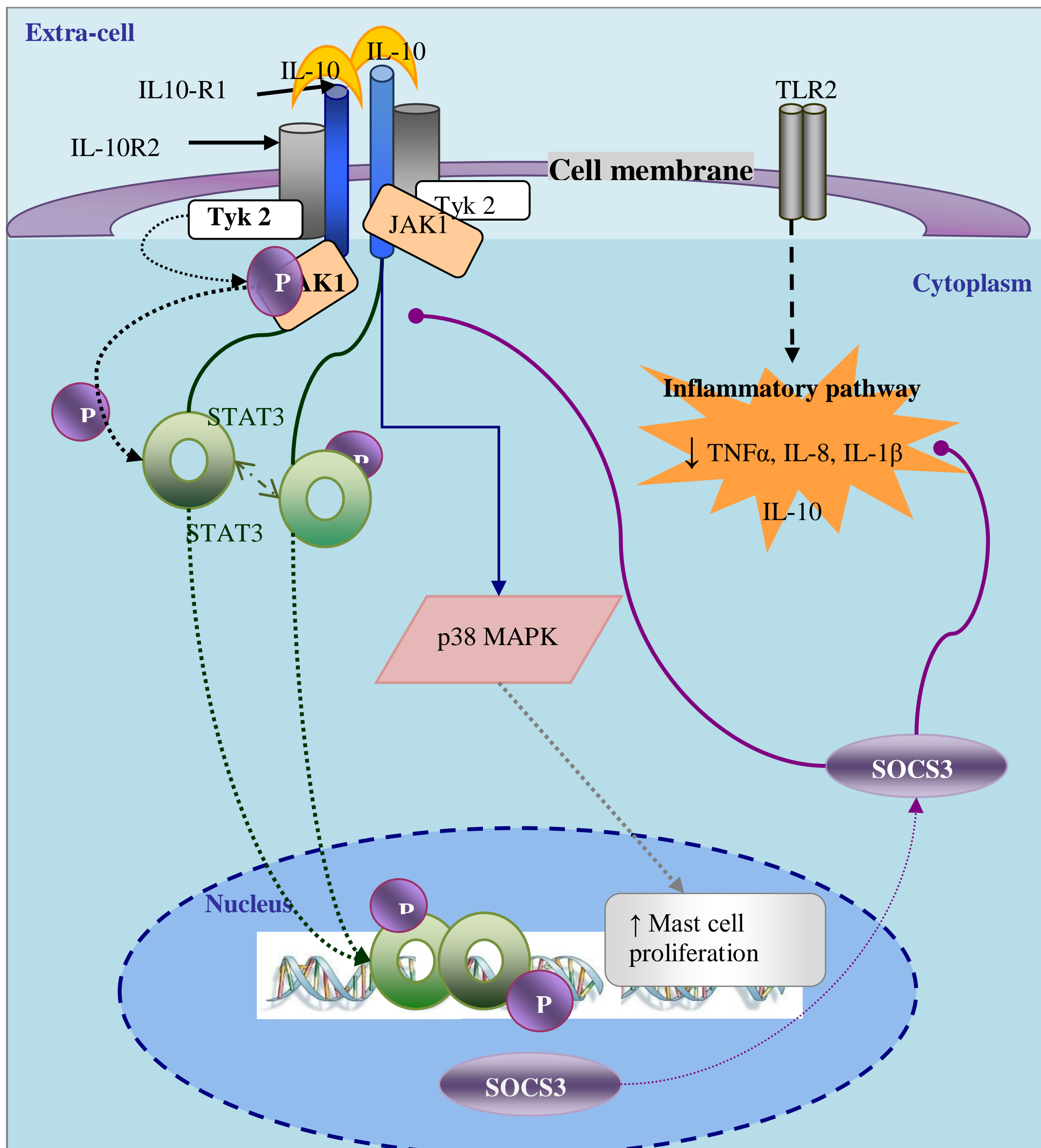


Figure 3.4: Model of IL-10 Signal Transduction in a Monocyte. At rest, the tetrameric IL-10 receptor associates with JAK1 (Janus kinase 1) on the intracellular domain of IL-10R1. An IL-10 homodimer binds to IL-10R1, recruiting Tyk2 (tyrosine kinase 2) to IL-10R2 with subsequent phosphorylation of JAK1. STAT3 binds to the phosphorylated docking site on IL-10R1 and is phosphorylated by JAK1. STAT3 homodimerises, translocates to the nucleus and binds to IL-10 response element genes to control gene transcription e.g. SOCS3. In inflammatory cells, SOCS3 negatively regulates the TLR inflammatory pathway and the JAK-STAT pathway (*adapted from* SABiosciences, www.SABiosciences.com on 26.09.2011 & Genego, Thomson Reuters, www.genego.com/map_531.php on 28.9.2011).

Regarding the suppression of TNFα activity, IL-10 reduces the transcription of TNFα, enhances soluble TNFR release and reduces the expression of surface TNFR on inflammatory cells such as monocytes and lymphocytes [323]. In keratinocytes, IL-10 can prevent nuclear localisation of NFκB1, inhibiting the transcription of pro-inflammatory cytokines [293], and hence participates in the suppression of TLR2 signal transduction.

3.3.5 Summary: Phototherapeutic Targets for Yellow IPL

Of the toll-like receptors, TLR2 preferentially binds *P. acnes*. In inflammatory acne, binding of *P. acnes* moieties leads to the transcription of inflammatory cytokines and amongst the pro-inflammatory cytokines, *in vivo* and *in vitro* studies have shown that IL-8 and TNFα are highly expressed after *P. acnes* stimulation. Transcription is largely regulated through the activation of distinct factors, NF-κB and MAPKs. Of these, the NF-κB pathway reliably leads to a pro-inflammatory response whereas MAPK activation may result in inflammatory and/or anti-inflammatory effects.

TLR2 signalling is tightly controlled *in vivo* by several mechanisms and cytokines including IL-10. The anti-inflammatory actions of IL-10 include suppression of TNFα release, down regulation of TNFR and inhibition of cytokine signalling via SOCS3. Acne patients may be IL-10 deficient [40] and this possibly contributes to the pro-inflammatory state seen in acne vulgaris.

Studies have shown that accepted anti-acne agents such as nicotinamide [290] and erythromycin [296], significantly reduce IL-8 expression possibly through inhibition of TLR2 signalling. Also all-trans retinoic acid significantly down regulates TLR expression [279, 324]. TNFα has been detected at very high levels in acne papules [325] and 400 –

420 nm IPL inhibits TNFα expression in the rat ear acne model [235] possibly in response to photodynamic destruction of *P. acnes*.

IPL (530 nm) has the potential to treat several arms of acne pathogenesis via photothermal/ photocoagulation, photodynamic and photoimmunological mechanisms. Photodynamic and photothermal destruction of *P. acnes* can result in a reduction of TLR2 activation and hence a reduction in IL-8 and TNFα transcription with subsequent down-regulation of TLR2 and TNFR. 530 nm IPL may also up-regulate IL-10 transcription *in vivo* [255] and photocoagulation can reduce the migration of inflammatory cells to the pilosebaceous glands causing an overall anti-inflammatory effect. These potential mechanisms are summarised in **Figure 3.5**. The receptors represented in this model are also present on monocytes, macrophages and sebocytes hence a similar mechanism is thought to take place in these cells, producing an overall anti-inflammatory effect.

The aims of the clinical and laboratory arms of the studies designed to determine yellow IPL's efficacy and clarify which photo-immunomodulatory mechanisms are taking place. These are listed in the final section of this chapter.

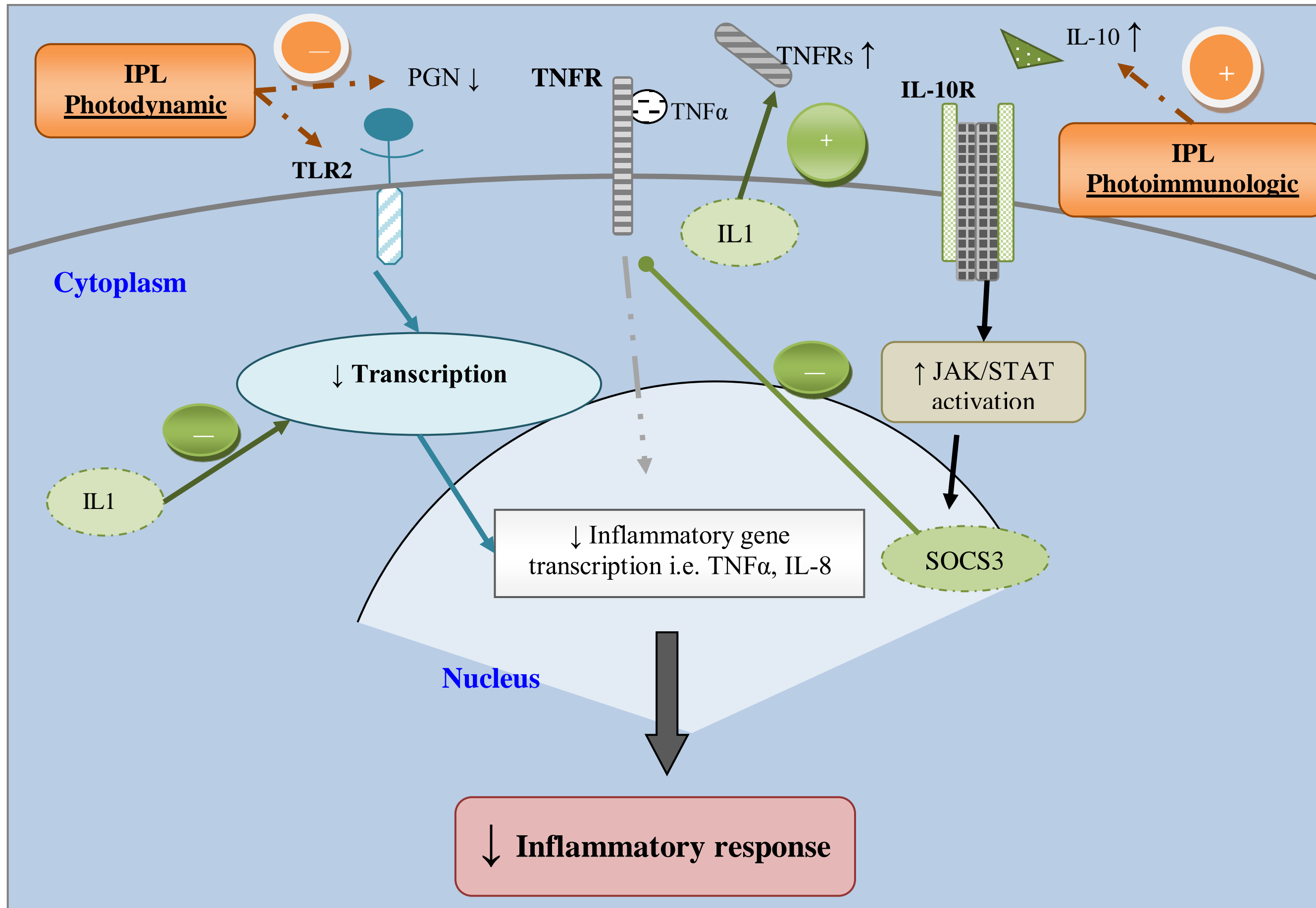


Figure 3.5: Hypothesised Anti-inflammatory Mechanisms for 530 nm IPL in Acne. IPL may cause photodynamic destruction of *P. acnes*, thus reducing availability of its peptidoglycan (PGN) moiety to stimulate TLR2. IL-10 transcription is increased, which antagonises NF-κB nuclear translocation and promotes TNFRs. IL-10 also acts to prevent cytokine signalling via SOCS3, as depicted for TNFR. Thus, inflammatory cytokine

3.4 Hypotheses and Aims

3.4.1 Clinical Study

Clinical trials have shown promising results when yellow coherent and incoherent light sources have been used to treat acne. They show a reduction in inflammatory lesions but have failed to objectively demonstrate an accompanying reduction in the SER. This, in addition to the expected anti-inflammatory action of 530nm IPL, has led to a hypothesis that it is an effective treatment for acne vulgaris. Thus, an open, prospective, single cohort trial was designed to determine whether IPL as monotherapy would be able to bring about observable changes in the clinical severity of acne.

3.4.1.1 Clinical Study Aims

To evaluate the clinical efficacy of IPL based on its ability to reduce:

a) Inflammatory lesion counts

b) Non-inflammatory lesion counts

c) Global acne severity

d) The sebum excretion rate

3.4.2 Laboratory Study

The direct and indirect photoimmunological effects of 530 nm IPL are hypothesised to be as follows:

1. IPL down regulates TLR2 expression
2. IPL reduces transcription of the inflammatory cytokines TNFα and IL-8
3. IPL increases IL-10 transcription
4. IPL reduces TNFα and TNFR due to increased IL-10 signalling

3.4.2.1 Aims for Laboratory Study

To determine whether 530 nm IPL exerts its effects by:

1. Down regulating TLR2 expression in acne patients
2. Increasing IL-10 expression
3. Reducing the expression of IL-8, TNFα and TNFR

Chapter 4

Methods and Materials

4. Methods and Materials

4.1 The Clinical Study

4.1.1 Rationale for Clinical Parameters

In keeping with several studies assessing the clinical efficacy of light therapy [52, 219, 326], indicators of acne severity such as a global score (the Leeds Revised Acne Grading System) [327], photographs, lesion counts and SER were used to assess the clinical effect of IPL. The back was selected as the treatment target because this could easily be protected from the sun thus eliminating incidental sunlight exposure as a confounder. It was also a more cosmetically acceptable site from which to take 3 biopsies. The Energist ULTRA VPL™ (**Figure 4.1a**) is a medical CE marked grouped pulse IPL whose high-intensity output is controlled by a computerised capacitor bank [183]. The spectral output of the Energist VPL™ has been shown to be consistent and reliable [184] and thus, this IPL device was used in our study.

4.1.1.1 Lesion Counts

The basic aim of acne therapy is to reduce the number and severity of acne lesions. Lesion counts are an objective way of determining whether lesions increase or decrease, however this method is subject to inter-observer variation [328]. It is also possible to use lesion counts as an indirect measure of severity by grouping lesions by their type. Therefore, a patient with 10 inflammatory cysts is considered far more severe than one with 10 comedones. Many global acne-grading systems utilise lesion type as well as absolute count to determine a severity score. Therefore, it seemed prudent to assign a global acne

score as well as determine the differential effect of IPL on inflammatory and non-inflamed papules.

4.1.1.2 Sebum Excretion Rate

Although correlation does not imply causation, the suggestion that a reduction in the SER results in an improvement of acne vulgaris has been well established. Patients with 'dry' skin have less inflamed lesions than persons with 'normal' skin [329]. 13-*cis*-retinoic acid (isotretinoin), known for its potent therapeutic activity against acne, long-term efficacy and for its intense drying effects on the skin and mucous membranes, reduces sebum output and glandular size by almost 90 % [75, 330] and continues to do so for 5–20 months after cessation of therapy. The clinical efficacy and/or mechanism of action of many anti-acne agents are judged on their sebo-suppressive effects (for example see article on zinc-erythromycin [331], tetracycline [332] and visible light [231]).

As previously mentioned, the pulsed dye laser has been used to treat sebaceous hyperplasia [244], which suggests yellow IPL may possess some ability to ablate or shrink sebaceous glands. If the sebo-suppressive effects are similar to those seen with photodynamic therapy [333] and isotretinoin [330], the anatomical alteration could be accompanied by a physiological reduction in sebum excretion.

The European Expert group on efficacy Measurement of Cosmetics and Other topical products (EEMCO) have outlined a number of methods to classify and measure sebum excretion [334]. The *sebum excretion rate* (SER) and *sebum casual levels* are the most useful methods to measure sebum excretion. The SER describes the quantity of sebum excreted by an area of skin over a predefined period and the sebum casual level is an

estimate of the skin surface lipids that have accumulated over a period of at least 4 hours. Casual levels represent an approximate measure of the oiliness of a person's skin. Other methods such as *follicular excretion rate*, *sustainable rate of secretion*, *instant sebum delivery and follicular density* do not always correlate with the SER or the presence of sebum and are not discussed further [334].

The more widely used methods to assess the sebum excretion rates include *photometric techniques* e.g. Sebumeter® (C+K Electronic, Köln, Germany) and Lipometer® (L'Oréal, Paris) and *lipid absorbent tapes* e.g. Sebutape® (Cuderm Corp, Texas, USA) and Sebufix® (C+K Electronic). The Sebumeter® (C+K Electronic) consists of a 64 mm^2 strip of lipid absorbent plastic, 0.1 mm in thickness, mounted on a spring-loaded probe delivering a constant pressure of 10N for each measurement [334]. The probe is placed gently against the skin and after a maximum of 30s, as measured by an internal timer, it is then inserted into the Sebumeter®. A light is passed through the tape twice, and the transmitted light measured by a photocell. The result is measured according to an internal standard and the microprocessor expresses the results in $\mu g/cm^2$ (range: 0 - 500 $\mu g/cm^2$) [335]. Tools like the Sebumeter® are unable to detect changes in lipid composition and can only measure changes in quantity [336]. Lipid absorbent tapes like Sebutape® can be attached to the study area and left for a predetermined amount of time, usually an hour. Sebum secreted from each follicular opening produces translucent spots on the tape, the number being proportional to the number of active follicles and each spot's size to the amount of lipid present. These spots can then be examined visually or using software for image analysis. Advantages of the tape include intra-individual reproducibility, the ability to extract and analyse lipid fractions if required and ease of application [337]. Disadvantages of the absorbent tape method include underestimation of surface lipids due

to interference by the adhesive, distortion of the follicular imprints on the tape over time [338] and the need for supporting software and equipment to analyse the tapes.

Investigators have compared the SER estimations produced by Sebutape® and the Sebumeter®. Pierard-Franchimont et al. [339] conducted an open study that aimed to quantify the effect of Effidrate® (La Roche Posay, Belgium) on seborrhoea. Both the Sebumeter® SM810 and Sebutape® were used in their study. The sebumeter was able to detect a significant difference in the SER from baseline but the results gained from alternative methods were equivocal. They surmised that the sebutape only assessed the follicular pool of sebum whereas the sebumeter also measured inter-follicular lipid, giving a value for skin surface lipid [339]. Serup [338] quantified the differences in the readings produced by the Sebumeter® and Sebutape® in 24 Danish medical students with acne. He found that there was a high correlation between the Sebumeter® and Sebutape® readings especially when measured 1-hour after degreasing. However, readings from the Sebumeter® had 50% less intra-individual variations than the Sebutape® measurements. In light of these reasons and the ready availability of the Sebumeter® SM815, this apparatus was chosen to assess changes in the SER.

4.1.1.3 Leeds Revised Acne Grading Scale

In assessing therapeutic efficacy, a reduction in lesion counts is not always accompanied by an improvement in perceived acne severity [340]. Therefore, there is a need for complementary acne severity scoring systems to give an overall picture of a treatment's efficacy. At least 25 acne-grading systems exist [341]. The more widely used methods include the Global Acne Grading Scale or GAGS [342], Allen & Smith's technique [343], The American Academy of Dermatology (AAD) Consensus Classification [344], Cook's

Grading scale [345], Burke & Cunliffe's Leeds Technique [346] and its later version, the Leeds Revised Acne Grading Scale (LRAGS) [327].

Some systems do not include comedonal acne in their scoring system (e.g. AAD classification), some only assess certain anatomical areas, usually the face (e.g. Leeds technique) and some assess all acne affected areas together to give a single score (e.g. GAGS), or utilize a photographic system such as LRAGS and Allen and Smith's [343].

In a trial setting, a grading system that utilises a standardised method of measurement, which allows for accurate record keeping and retrospective evaluation is advantageous. Therefore, a photographic method was chosen for this study. Of the two mentioned above, Allen & Smith's photonumeric system was unsuitable as it only assesses the face whereas the treatment area in this trial was the back.

The Leeds Revised Acne Grading System [327] (LRAGS/ Leeds) relies on subjective assessments of acne severity using a published photographic scale (**Appendix 2**), where the face, chest and back have separate grading systems. The LRAGS is a commonly used grading system and the Spanish version has recently been validated (2010) [341]. The grading system is such that grade 1 represents mild acne characterised by low numbers and densities of comedones and papules. As the number, density, extent and nature of the lesions worsen a maximum grade of 12 for the face, 8 for the chest and 8 for the back are assigned.

4.1.1.4 Energist ULTRA VPL™ as a Therapeutic Modality for Acne

Using the definitions in Section 2.3.6, the Energist Ultra VPL™ (Swansea, UK) currently being used in our centre is almost an 'ideal IPL'. Variable pulsing allows the operator to control the fluence delivered to the patient and hence, regulating the level of harmful lipid peroxide formation during IPL irradiation. However, the electrical discharge pulse shape is a grouped pulse rather than a square pulse and hence the time-resolved spectral output may not achieve the target's thermal relaxation time.

It is equipped with interchangeable applicators that have various dichroic filters (**Figure 4.1b, c**). Additional UV filtering is provided by the titanium- or cerium-doped quartz flashlamp envelope (the glass encasing for the xenon gas) [182] and a water-cooling system around the glass block absorbs infrared wavelengths above 950 nm, reducing non-specific epidermal heating. The unfiltered and filtered emission spectra for the 530 nm and 610 nm applicators are illustrated in **Figures 4.2** and **4.3**.

For the 530 – 950 nm applicator, its peak emission coincides with the first absorption peak for oxyhaemoglobin (**Figure 4.2**), CPIII has small excitation peaks within the 500 – 700 nm range and wavelengths within the 530 – 950nm range may penetrate up to 1.2 mm into the skin [33]. Thus, vascular photocoagulation, reduction of sebaceous gland size and photodynamic inactivation of *P. acnes* are its potential mechanisms of action [177, 179, 246].

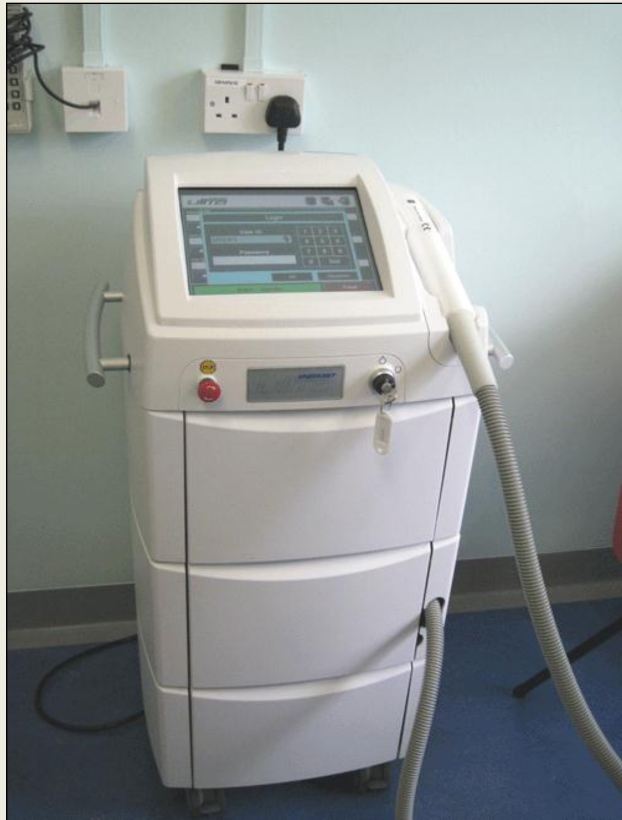


Figure 4.1b has been removed by author for copyright reasons

b

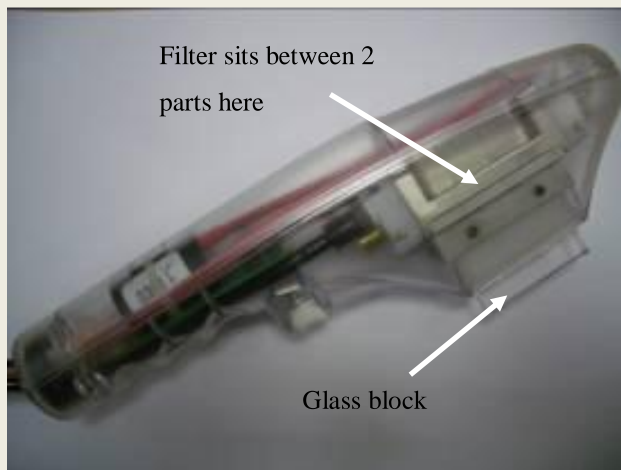


Figure 4.1: The Energist ULTRA VPL™ Device (a) Machine has a touch-screen interface (b) Applicators shown with the red (610 nm) and yellow (530 nm) cut-off filters in situ. (c) Applicator with a transparent cover to show components of the applicator & cut-off filters. *Figure 3.5a courtesy of: Sister H. Pugsley, Dept. of Dermatology, Cardiff University. Figure 3.5b retrieved from: http://www.endermologie.co.za/lpg-energist-vplmachne.html on Oct 8, 2010. Figure 3.5c courtesy of: Darren Thomas, Energist Ultra, Swansea, UK, © 2007.*

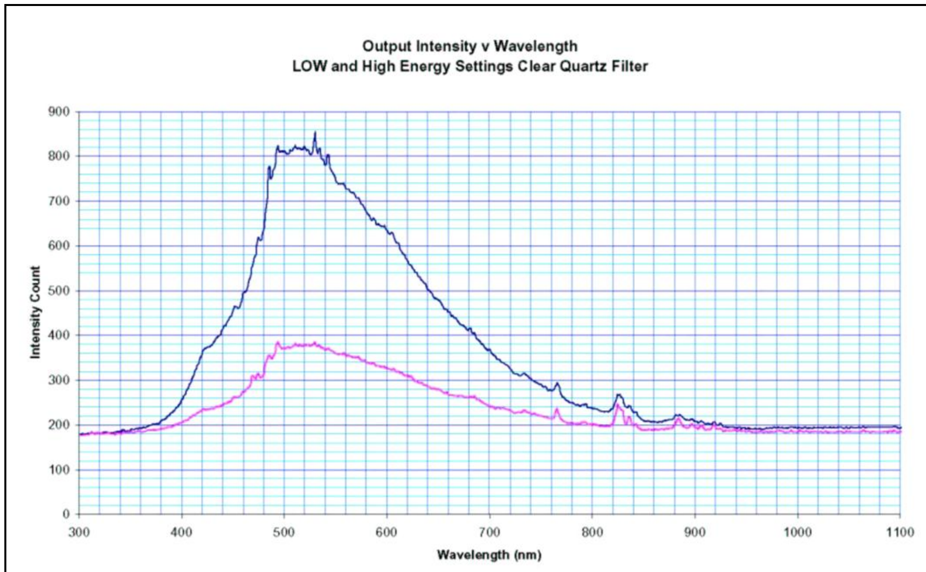


Figure 4.2: Unfiltered Spectrum of Xenon Flash Lamp. The spectral output at high energy (blue line) and low energy (purple line) settings. *Courtesy of Darren Thomas, Energist ULTRA, Swansea, UK, © 2007.*

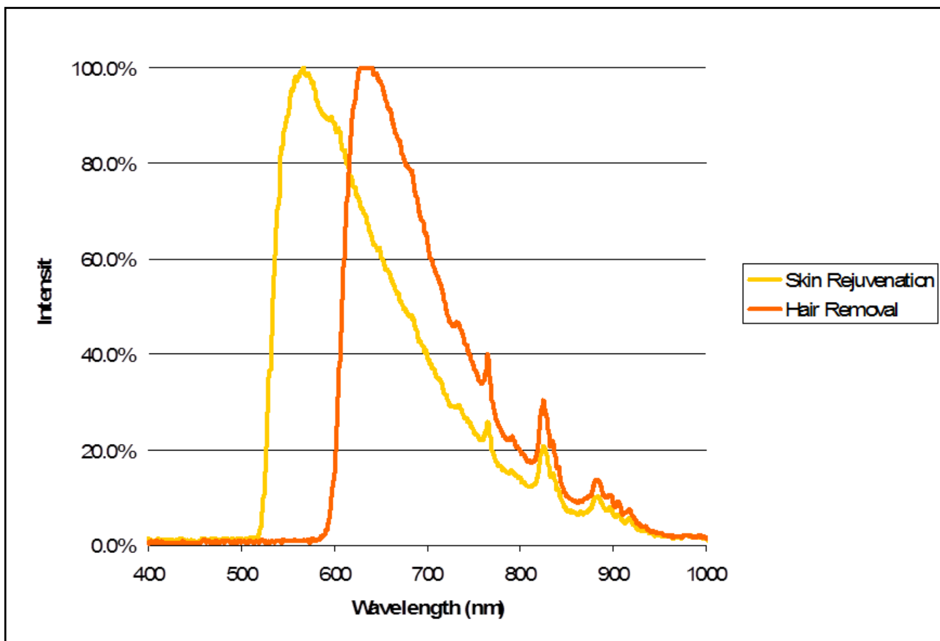


Figure 4. 3: Emission Spectra for the Yellow and Red Filters. Output from the yellow filter (530 – 950 nm) is labelled as 'skin rejuvenation' and from the red filter (610 – 950 nm) as 'hair removal'. *Courtesy of Darren Thomas, Energist ULTRA, Swansea, UK, © 2007.*

4.1.2 Clinical Methods

Ethical approval for this study was granted by the South East Wales Research Ethics Committee (reference number 08/WSE04/29) and Research & Development approval was granted by the Cardiff and Vale University Health Board, formerly the Cardiff and Vale NHS Trust in May 2008 (reference number 08/CMC/4196). The active intervention phase included 7 visits that spanned 10 weeks. Thereafter, participants were invited for 2 optional visits at 1 and 3 months after the last treatment to monitor the prolongation of their response to IPL.

4.1.2.1 Subjects and Recruitment

Male and female volunteers between the ages of 18 and 50 years with mild-moderate acne on the back were eligible for the study. Subjects were invited to enter the study through Cardiff University's online notice board announcements and at Halls of Residence between May 2008 and January 2009. Patients referred to the University Hospital of Wales for management of their acne were offered information about the study and general practitioners within the Cardiff area were asked to refer patients who they thought may be suitable for the trial.

All subjects gave informed consent (**Appendix 1**) before starting this study. Demographic data including each patient's age, weight, height, history of smoking and disease duration was also recorded. Before consenting to participate, patients were made fully aware of the number of biopsies to be taken. The inclusion and exclusion criteria as detailed in the study protocol are listed below.

4.1.2.1.1 Inclusion Criteria

1. Mild to moderate acne vulgaris with at least 15 inflammatory and non-inflammatory lesions, but no more than 3 nodulocystic lesions Thus, not exceeding Leeds grade 6 (for the back)
2. Patients willing to have ONLY their back treated
3. Skin phototypes I – III
4. Patients who are willing and able to provide written informed consent, after being informed of all the pertinent aspects of the trial
5. Patients who agree not to use sun-beds or undergo any UV light treatment for 4 weeks prior to entering the study and are willing to minimise the amount of exposure to direct sunlight for the duration of the study

4.1.2.1.2 Exclusion Criteria

1. 3 nodules and/or cysts present
2. Pregnancy
3. Use of anti-androgen containing contraceptives
4. Mental incompetence
5. Keloids or tendency to heal with keloids
6. Cosmetic treatment to their back:
    a. In the previous year with collagen, dermabrasion and laser resurfacing
    b. Alpha hydroxyl acids within 1 month
    c. Microdermabrasion within 3 months
7. Photosensitivity disorders e.g. solar urticaria

8. Porphyrias or allergy to porphyrins
9. Epilepsy
10. Systemic retinoid use in the past 12 months
11. Use of Vitamin A supplements > 2000 IU /day
12. Use of oral antibiotics and topical retinoids in the preceding 4 weeks.
13. Systemic medications such as steroids, immunosuppressant, statins and preparations containing St. John's wort[a]
14. Oral photosensitizers within last 4 weeks
15. Previous treatment with IPL or lasers to the areas of interest within the last 12 months
16. Severe systemic diseases such as impaired renal or liver function; regional enteritis or ulcerative colitis; a history of antibiotic-associated colitis; severe cardiovascular, neurological disease, or any other disease that may interfere with the evaluation of the study medications
17. Patients with psoriasis, acne rosacea, allergic rashes, bacterial, viral or fungal infections or other diseases of trunkal skin
18. Patients who are unlikely to be available for the duration of the follow-up
19. Persons involved in another clinical trial for the duration of this study

[a] Patients temporarily ineligible i.e. items 12,13 & 17, at the time of screening were enrolled after a wash-out period of 4 weeks or after resolution of their illness.

4.1.2.2 Photography

Photographs of the participants' backs were taken by the medical photography unit in the Dermatology Department using a specially designed protocol for this study (see **Appendix 2**). The photographs were then used for blinded Leeds grading as described below.

4.1.2.3 Leeds Grading

Leeds grades were assigned for the back, face and chest at the time of presentation. In keeping with the original publication, patients were graded in a well lit room with palpation of the lesions. Though the back was the primary treatment area, the face and chest were also graded in an attempt to track the natural history of each patient's acne in the absence of intervention. Hence the Leeds grades for the face and chest acted as internal controls. To circumvent problems associated with investigator bias, internal validity and reproducibility [329], Leeds assessments were performed by 4 independent dermatologists unaware of the treatment timings using full-back photographs taken at the baseline visit and 1 week after the final IPL session at the end of the study.

4.1.2.4 Lesion Counts

To ensure reproducibility, a 10 x 10.1 cm template on a transparent acetate film was placed on a defined area on the upper back of patients at the baseline and final visits to count non-inflammatory, inflamed lesions and resolving lesions [23, 25]. Each lesion was colour coded: red for inflamed lesions, blue for non-inflamed lesions and black for resolving lesions and static lesions. 'Static lesions' such as compound melanocytic naevi were used as landmarks for placement of the template.

4.1.2.5 Sebum Excretion Rate

Using the method described by Trivedi et al. [98] a SM815 sebumeter (Courage + Khazaka Electronic, Köln, West Germany) was used to measure sebum excretion rates (SER) at baseline and one week after the end of therapy. The upper back was cleansed with six swabs containing 70% isopropyl alcohol, 3 consecutive swabs per side (degreasing) and the time noted. Rode et al. [347] found that casual levels of sebum excretion returned to baseline 2 hours after degreasing and was not statistically different from the levels measured 1 hour after defatting. Based on the findings of Rode et al., one hour after degreasing, a cassette containing 0.1mm synthetic tape was applied to a predetermined area on the upper back for 20 seconds. The measuring head was then inserted into the sebumeter, where the transparency of the tape was determined. Light transmission through the tape is proportional to sebum content of the area. A microprocessor calculates levels of sebum at μg/cm^2 of skin. This process was repeated twice in adjacent areas and an average SER calculated. The area measured at the baseline visit was marked on a diagram to ensure measurement within the same region at the end of therapy.

4.1.2.6 IPL Treatments

The "back" was defined as the area encompassed by imaginary lines joining the superior aspect of the scapulae as the upper border and a horizontal line across the lower back at the level of the umbilicus was the lower border. The settings, 40 - 42 J/cm^2, 20 pulses, 5 on, 10-15 off, were loosely based on those used by Babilas et al. [189] who reported reduced pain during red light photodynamic therapy whilst retaining its efficacy using these settings in an identical device. After applying a thin layer of ultrasound gel (for optical

coupling), a 530 – 950 nm applicator for the VPL™ Energist Ultra® was used to administer non-overlapping pulses to the back of each participant. A second pass was then given perpendicularly to the previous pass. The back was simultaneously cooled using chilled air (*SmartCool*, Cynosure, USA) to further improve patient tolerability. One treatment was given as monotherapy at 2 week intervals for a total of 4 sessions.

4.1.2.7 Skin Biopsies

Four millimetre punch biopsies were taken from areas of clinically normal skin, within the treatment area under local anaesthesia (1–1.5 ml of 2% lidocaine with 1:1000 adrenaline). The timing of the biopsies was as follows:

1) At baseline (biopsy 1 or 'B1')
2) Forty-eight hours after the first IPL session (biopsy 2 or 'B2')
3) One week after the 4th IPL session (biopsy 3 or 'B3')

The subsequent processing of skin biopsies to examine changes in inflammatory and sebaceous markers is described in **Section 4.2.2**.

4.2 *Laboratory Studies*

4.2.1 Rationale

A number of techniques could have been used to determine the mechanism of action of IPL. Those chosen were based on previous work by researchers who had studied IPL mechanisms of action, inflammatory cytokine expression and the effects of acne treatment on gene expression.

TLR2 has a fundamental role in the induction and resolution of inflammatory acne. Through TLR2 activation, pro-inflammatory cytokines such as TNFα and IL-8, can be induced by bacterial colonisation, androgen-sensitive sebaceous glands and activated pre-comedonal keratinocytes [62, 260, 348]. Conversely, TLR2 negatively self-regulates its pro-inflammatory responses by stimulating IL-10 transcription [349]. Investigators such as Tenaud et al. [276] and Hunger et al. [350] evaluated the mRNA and protein expression of these molecules using polymerase chain reaction (PCR) and immunohistochemistry (IHC) techniques.

Byun et al. [255] determined the change in the expression of IL-10 and TNFα in IPL-irradiated HaCaT cells by measuring messenger RNA (mRNA) expression semi-quantitatively. They also utilised Western blotting to determine protein levels of expression. PCR techniques were used in this study to determine whether the expression of IL-10 and TNFα were respectively up- or down-regulated after treatment with IPL.

4.2.2 Biopsies

Four millimetre punch biopsies were taken under local anaesthesia (1 – 1.5 ml of 2% lidocaine with 1:1000 adrenaline) from the upper back. Jeremy et al. [23] reported that clinically normal skin from an acne patient still had a statistically significant increase in inflammatory infiltrate which was only detected by microscopy. Hence, it was decided that non-lesional skin was to be biopsied to overcome the problems of timing an acne lesion throughout its evolution. The biopsies were taken 2 cm away from an adjacent inflammatory papule or pustule, and at least 3 cm away from a nodule or deep cyst in an attempt to avoid a neighbouring inflammatory process. After obtaining patient consent, the skin biopsies were taken at baseline (B1), 48 hours after the first IPL session (B2) and at the final visit (B3) which was 1 week after the 4th IPL session. Therefore, 3 biopsies were taken from each subject, allowing comparison of IPL-induced changes before, during and after a course of treatment in the same patient.

These punch biopsies were covered with OCT embedding matrix (Raymond A. Lamb, Eastbourne, East Sussex, UK), snap frozen in hexane (Fisher Scientific, Loughborough, UK) previously cooled with dry ice, and then stored in liquid nitrogen until used. Seven micrometer sections were cut using a cryostat and mounted on Superfrost® Plus glass slides. These slides were then wrapped in aluminium foil and stored at -80 ºC until ready for use. Haematoxylin & eosin staining at intervals of 20 to 30 sections was carried out to identify sections containing portions of hair follicle and sebaceous gland.

4.2.3 Immunohistochemistry

An immunohistochemistry protocol for TLR2 adapted from Ku et al. [351] was used. Sections from 10 cases were allowed to reach room-temperature, fixed with 100% dried acetone (15 min) and then air-dried (15 min). The samples were then washed three times in phosphate buffered saline (PBS, pH 7.2) for 5 minutes each. Blocking of non-specific binding was carried out with 10% donkey serum made up in a diluent of 1% bovine serum albumin (Sigma Lifesciences, USA) and 1% Marvel (dried skimmed milk, Premier International Foods, Ireland) in PBS for 60 minutes. Overnight incubation at 4ºC with anti-TLR2 antibody (1:50; sc-10739; rabbit polyclonal; Santa Cruz BioTech, USA) was done in a humidified box. Negative controls were incubated with the diluent rather than the primary antibody. Serial washes in PBS were followed by 60-minute incubations with the secondary biotinylated donkey anti-rabbit antibody (1:200, Vector Laboratories, Peterborough, UK) and then 30-minute incubations in streptavidin conjugated to horseradish peroxidase (1:300, Vector Laboratories, Peterborough, UK).

Further washes in PBS were followed by visualisation with 0.1% hydrogen peroxide and 3, 3'- diaminobenzidine (0.01% DAB, concentration 1mg/ml). A brown colour is produced by a reaction between DAB and hydrogen peroxide catalysed by the conjugated horse radish peroxidase causing DAB to form an alcohol-insoluble brown precipitate. Haematoxylin was used as a counterstain (2 min). The last steps involved progressive dehydration in 70% vol/vol ethanol (x 1), 90% ethanol (x 1), 100% ethanol (x 3) and the sections were then cleared with xylene (3 washes). Sections were then mounted with a distyrene/ plasticizer (butyl, phthalate, styrene, BPS)/ xylene compound (DPX, BioChemika, Germany) and allowed to dry.

4.2.3.1 TLR2 Image Analysis

Where all experimental conditions are equal, a change in the intensity of immunolocalised material can be directly proportional to a change in the expression of that antigen. Intensity grading tends to be subjective and varies greatly depending upon the lighting, the section and the individual grading the section. Analysis using computer software leaves less room for subjective variation [352]. Image Pro Plus™ v6.0 (Media Cybernetics, Silver Spring, Maryland) has been used extensively to digitally grade staining intensity, cell counts and area [353-356].

For TLR2, digital images of four representative high power fields (x 20 objective) were taken using a Nikon camera mounted on a Carl Zeiss Axioplan microscope using Axiovision software. The internal optical density (IOD) per unit area (IOD/ Total Epidermal area) was determined for the four sections selected for each biopsy. The four values were averaged to give an overall IOD score for each biopsy [356].

4.2.4 Semi-quantitative Polymerase Chain Reaction

4.2.4.1 RNA Extraction

Total RNA was extracted from at least 600μm of each biopsy using TRIzol (Invitrogen, Paisley, UK) and 200 – 250 μg of glycogen (Roche Diagnostics GmbH, Germany). Glycogen is an inert substance that acts as a carrier molecule for the RNA, thus increasing the size of the pellet during precipitation steps [357, 358].

The steps are as follows:

1. **Cellular disruption at 15 -30°C**

    i. 750µl – 1000 µl of Trizol added to biopsy sections

    ii. Samples vortexed briefly and allowed to sit for 5 min

2. **Phase separation**

    i. 200µl chloroform added

    ii. Vigorously mixed for 15 – 20 secs and allowed to sit for 2-15 min

    iii. Centrifugation at 12,000g for 15 min at 2 – 8°C

    iv. After centrifugation, the mixture separated into 3 phases: Red (phenol-chloroform) phase, interphase and the upper aqueous (colourless) phase. RNA is only in the upper phase

3. **RNA precipitation**

    i. The aqueous phase was transferred to a clean tube and 200µg of glycogen added as per the manufacturer's instructions

    ii. The solution was briefly vortexed and 500 µl isopropyl alcohol (Fisher Scientific, Loughborough, UK) added

    iii. Incubated at room temperature for 10 min

    iv. Mixture was centrifuged at 12,000g x 10 min @ 2 - 8°C

    v. The RNA formed a tiny gel like precipitate at side or bottom of the tube

4. **RNA Wash**

    i. The supernatant removed and the pellet washed with 1 ml of 75% ethanol and briefly vortexed

    ii. Centrifuged at 7,500g for 5 min at 2 – 8° C

5. **Re-dissolving RNA**

    i. The ethanol pipetted off and the pellet air-dried

    ii. The remaining RNA pellet dissolved in 10 – 25 µl RNAase-free water depending on the quantity of RNA isolated

4.2.4.2 Reverse Transcription (RT)

After determining the concentration of RNA retrieved by measuring the optical density at 260nm (A_{260}) in a spectrophotometer (GeneQuant *pro*™ RNA/DNA Calculator, GE Healthcare, Buckinghamshire, UK), 1µg of RNA from cases 1-6, 9, 10, 12, 14, 18 (n =11) was used to generate cDNA templates. Each 20µl reverse transcriptase (RT) reaction consisted of 1µl AMV reverse transcriptase (Promega UK Ltd, Southampton, UK), 1µl Oligo dT (Promega UK Ltd., Southampton, UK) and 1µl RNAase inhibitor (Promega UK Ltd, Southampton, UK), 4µl 5x Buffer (Promega, Madison, USA), 4µl RNAse free water (Sigma Aldrich Lifesciences, Dorset, UK) and 4µl 10mM dNTPs (Roche Diagnostics, West Sussex, UK).

RNA and Oligo dT were first placed in a thin-walled 200µl tube and heated at 70ºC for 10 minutes and then cooled to 42ºC in a thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler, Waltham, USA). The rest of the RT-reaction mix was then added at 42ºC where it remained for 60 minutes followed by a final incubation at 70ºC for 10 minutes.

Though each RT-reaction was conducted using 1µg of total RNA, each sample's RNA concentration differed necessitating the use of reaction volumes from 20 to 50 µl. This meant that the more dilute RNA concentrations required larger reaction volumes. In an attempt to normalise the cDNA concentrations, the optical density of each sample was measured using a GeneQuant pro™ RNA/DNA calculator (GE Healthcare) or a Nanodrop™ Spectrophotometer (Fisher Scientific, Loughborough, UK). A concentration of 350ng/µl was chosen which represented the lowest cDNA concentration in any one sample in a 1:10 dilution.

4.2.4.3 Primers

Primer sequences were designed from complete cDNA sequences listed on the University of California's Santa Cruz genome bioinformatics website (http://genome.ucsc.edu). They were designed so that they were 20 – 21 nucleotides in length, had 50 – 60% guanine and cytosine bases and ended with a G or C at the 3' end where possible (Table 8.1). The primers were synthesised by Sigma-Aldrich (Dorset, UK) and PCR reactions optimised for maximum amplification by varying the magnesium chloride concentration ($MgCl_2$, Qiagen Ltd., West Sussex, UK), annealing temperatures and the presence or absence of Q-solution (Qiagen Ltd., West Sussex, UK). Q-Solution is a betaine additive that allows more efficient amplification of GC-rich sequences or templates which have a high secondary structure.

4.2.4.4 Preparation of PCR Mix and Procedures

For each reaction, the 50 µl PCR mix consisted of 1µl of cDNA (Qiagen Ltd., West Sussex, UK) , 1 µl dNTPs (10mM, Qiagen Ltd., West Sussex, UK), 2µl of forward and

reverse primers (0.1µg/ml; Qiagen Ltd., West Sussex, UK), 0.25µl of HotStarTaq® DNA polymerase (5 units/ml, Qiagen Ltd., West Sussex, UK), 5µl of 10X CoralLoad PCR buffer [contains 15 mM $MgCl_2$, Tris-Cl, KCl, $(NH_4)_2SO_4$, gel loading reagent, red dye, orange dye, 10X CoralLoad concentrate; pH 8.7; Qiagen Ltd., West Sussex, UK] and 22.5 µl of RNAse free water (Sigma-Aldrich, Dorset, UK). Additional $MgCl_2$ (5mM, Qiagen Ltd., West Sussex, UK) and Q-Solution[b] (5X concentrated, Qiagen Ltd., West Sussex, UK) were also added if required (**Table 4.1**). CoralLoad PCR buffer and concentrate contains gel tracking dyes allowing PCR products to be directly loaded on to an agarose gel without adding a loading buffer.[c] To prevent evaporation during cycling, a heated lid was used on the thermal cycler (*GeneAmp® PCR System 9700*, Perkin-Elmer, Norwalk, USA and *MJ Research® PTC-200 DNA Engine Thermal Cycler*, Waltham, USA).

4.2.4.4.1 Cycling Parameters

The annealing temperatures were calculated using the formula:

$$(4 \times GC) + (2 \times AT) - 5°C$$

The elongation times were adjusted according to the amplicon size (30 seconds for each 500 bp). The settings for each primer are listed in **Table 4.2.**

[b] HotStar Taq *Plus* PCR Handbook, 02/2008, pgs. 10-11.

[c] HotStar Taq *Plus* PCR Handbook, 02/2008, pgs. 10-11

Table 4.1: Oligonucleotide Sequences and Additive Requirements for Semi-qPCR Experiments

Gene	Accession Number	Primer Sequences Forward (above) Reverse (below)	Size	25mM $MgCl_2$ / 50µl	Q/ 50µl
IL-8	NM_00584	CTTGGCAGCCTTCCTGATTTC CACTGTGAGGTAAGATGGTGG	845 bp	6µl	10µl
IL-10	NM_000572	CAGCTCAGCACTGCTCTGTTG GTCGCCACCCTGATGTCTCAG	549 bp	4µl	10µl
TLR 2	NM_003264.3	GGGTTGAAGCACTGGACAATG GCAGCCTCCGGATTGTTAACG	970 bp	4µl	10µl
APRT	NM_001030018	GCTGCGTGCTCATCCGAAAG CCTTAAGCGAGGTCAGCTCC	250 bp	-	10µl
β-actin	NM_001101	ATAGCACAGCCTGGATAGCAA AGAAAATCTGGCACCACACCT	174 bp	-	10µl
GAPDH	NM_002046.3	GGTGGTCTCCTCTGACTTCAACA GTTGCTGTAGCCAAATTCGTTGT	127 bp	-	10µl

Table 4. 2 Cycling Parameters for Gene Quantification Using Semi-qPCR

Gene	PARAMETERS					
	HotStar Taq Activation Step	**Denaturation Step**	**Primer Annealing**	**DNA Synthesis**	**Cycle Number**	**Final Elongation Step**
TLR2	95 ºC x 5 min	94 ºC x 30s	55 ºC x 30s	72 ºC x 1 min	40	72 ºC x 10 min
IL-8	95 ºC x 5 min	94 ºC x 30s	61 ºC x 1 min	72 ºC x 30s	40	72 ºC x 10 min
IL-10	95 ºC x 5 min	94ºC x 30s	57 ºC x 30s	72 ºC x 45s	40	72 ºC x 10 min
GAPDH	95 ºC x 5 min	94ºC x 30s	55 ºC x 30s	72 ºC x 30s	35	72 ºC x 10 min

The number of cycles (40) to see a result was based upon extinction experiments which indicated that the initial quantities of cDNA for the desired genes were present only in minute amounts. Hence, even after 35 cycles, the amplicon was only faintly visible if at all. The agarose gel images for TLR2 and GAPDH (endogenous control) depicting this are shown in **Figure 4.4** below. The experiments illustrated were performed using cDNA from Case 5 baseline biopsy (B1), which was chosen because it produced the most obvious bands for TLR2 during primer optimisation.

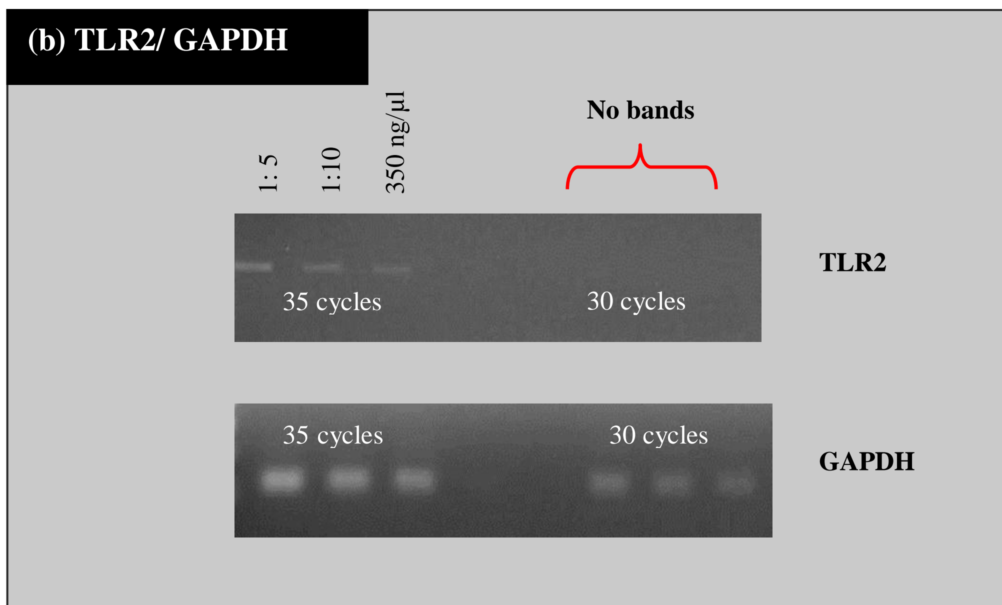


Figure 4. 4: Effect of Cycle Number and cDNA Concentration on Semi-qPCR Band Intensity. Agarose gel images showing rapid extinction of TLR2 and GAPDH band intensity with a reduction in the number cycles. Concentration of 1:10 was approximately 300.1 ng/µl.

4.2.4.4.2 Agarose Gel Electrophoresis

A sample (5 µl) of each PCR product was resolved on a 2% (w/v) agarose gel. One gram of high resolution standard agarose (Geneflow Ltd., Fradley, UK) was dissolved in 50ml 2X Tris-Acetic Acid-EDTA buffer (50X TAE contained 4.84 g tris hydroxymethyl-aminomethane + 11.4 ml of 17.4M glacial acetic acid + 3.7g of EDTA disodium salt + 160 ml deionised water) by heating for 1 minute in a microwave. Ethidium bromide (5 µl) was then added to the cooled mixture (1µg/ml) and poured into the electrophoresis tray (Model AGT-1, VWR International, Leicestershire, UK) to set. Each PCR product (5 µl), which already contained loading buffer, was pipetted into a well of the agarose gel and run for 30 minutes at 50V in 2X TAE buffer. The DNA product size was determined by running a 100 bp ladder (New England Biolabs, Herts, UK) in an adjacent well.

4.2.4.4.3 Housekeeping Genes

PCR has the ability to amplify low levels of gene transcript, making it a very powerful method of detecting very subtle changes in gene expression [359, 360]. This also means that small differences in RNA extraction, efficiency of the RT-reaction and pipetting technique amount to many-fold alterations in the final PCR result. Therefore, it is important to use a stably expressed gene that remains unaltered by the experimental conditions, against which these changes can be compared. 'Housekeeping genes' (HKG) are often used in this way.

Semi-quantitative PCR (semi-qPCR) using three HKGs, adenine phosphoribosyl transferase (APRT), β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was tested on pre- and post-IPL samples from two patients using varying concentrations

of cDNA. As illustrated in **Figure 4.5**, GAPDH was stably expressed at a reasonable level and therefore used as the HKG of choice.

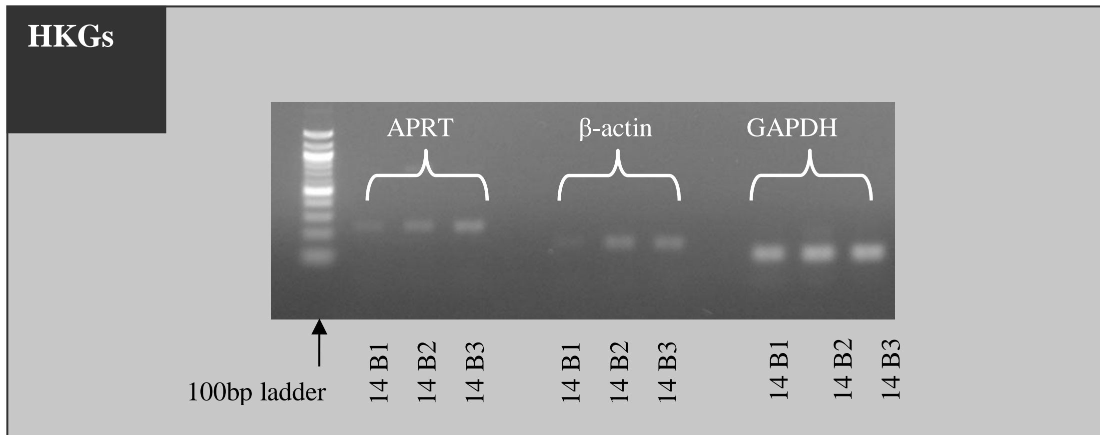


Figure 4.5: The Effect of IPL on Housekeeping Gene Expression. DNA agarose gel showing the relative band intensities of three housekeeping genes GAPDH (127 bp), APRT (250 bp) and β-actin (174 bp) after 35 cycles in biopsies taken at baseline (B1), after 48 hours (B2) and at the end of therapy (B3).

4.2.4.4.4 DNA Sequencing

To verify that the correct gene was being amplified by the PCR, products producing strong clean bands of the expected size were sequenced using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Here, the DNA was precipitated with PEG mix (26% polyethylene glycol 8000, 6.6 mM $MgCl_2$, 0.6M NaOAc, pH 5.2) at room temperature for 10 min and centrifuged for 25 minutes at room temperature. After 2 washes with 70% ice-cold ethanol (15,000 rpm, 2 min, 4°C), the pellet was mixed with the primer, Big Dye® buffer and Big Dye® mix. After 28 thermal cycles (denaturation at

96ºC for 10 seconds, annealing at 50 ºC for 5 seconds and DNA elongation at 60 ºC for 5 minutes) the mixture was centrifuged again and the remaining pellet washed in 70% ethanol. The sequencing reactions were run by the Central Biotechnology Services (CBS) Lab using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). The sequence obtained was then checked against the published cDNA sequences obtained previously from the University of California's Santa Cruz genome bioinformatics website and using the Basic Local Alignment Search Tool (BLAST) on the National Centre for Biotechnology Information (NCBI) website.

4.2.4.5 Densitometry

The agarose gels were photographed with 302 nm UV light using the AlphaImager HP® Imaging System (Alpha Innotech, Cell Biosciences, Santa Clara, CA). Relative amounts of PCR products were determined by measuring the intensity of each band using the 'spot denso' utility in the AlphaImager software which was called an '*integrated density value'* (IDV).

IDVs were recorded for each experiment (done in triplicate) and then averaged to produce a single, mean intensity value for each biopsy. This was called the mean or 'μ' IDV [361-363] . An IDV value for the no-template control was also measured and subtracted from the IDV value of the desired amplicon to correct for the inherent background signal emitted by each gel.

The formula for Gene A's expression in biopsy α taken from Patient Y is:

= **Gene A μIDV** in **Case Y biopsy α – Gene A μIDV** in **No-template control**

Finally, despite controlling for it, there may have been slight variations in the quantity of total genetic material in Patient Y's biopsies. This variation would be mirrored by a difference in the intensity of the bands for the housekeeping gene, GAPDH.

Hence each IDV has to be normalised against the starting quantity of genetic material:

$$= \frac{\textbf{Gene A μIDV in Case Y biopsy α – Gene A μIDV in No-template control}}{\textbf{GAPDH μIDV for Case Y biopsy α}}$$

Therefore, as an example, the normalised densitometry value for TLR2's expression in Case 7 biopsy 1 would be:

$$= \frac{\textbf{TLR2 μIDV (Case 7 biopsy 1) – TLR2 μIDV (No-template control)}}{\textbf{GAPDH μIDV (Case 7 biopsy 1)}}$$

4.2.5 Quantitative PCR

RT and QPCR experiments were performed by Dr. Ayman Hawrani, Central Biotechnology Services (CBS) using a SYBR® Green assay (Agilent Technologies, USA).

The SYBR® Green assay rests on the ability of an asymmetric cyanine fluorescent dye (SYBR® Green I) to bind to the minor groove within double-stranded DNA (dsDNA) [364]. Unbound, SYBR® Green produces no fluorescence. Therefore, as DNA amplification progresses the fluorescent signal increases [364]. The amount of product made followed a sigmoid distribution, where the reaction rate peaked in the exponential phase and then plateaued as depleted substances in the reaction mix limit the reaction. This saturation level is approximately the same for all products in a typical qPCR experiment and therefore does not give an accurate reflection of the initial amount of product. At best, it can assess the presence or absence of a transcript [364]; however it is often at this very point at which semi-qPCR experiments are visible on an agarose gel.

4.2.5.1 RNA Extraction

In an attempt to maximise the quality of the extracted RNA, sets of 3 biopsies from cases 1, 2, 3, 4, 7, 11 were first preserved in *RNALater® -ICE Frozen Tissue Transition Solution* (Ambion Ltd., Huntingdon, UK) prior to RNA extraction (as described above). In spite of following the manufacturer's instructions, the RNALater® - ICE formed a gelatinous material in association with the OCT-embedded biopsies which degraded the RNA to undetectable levels. For the succeeding samples, RNA was extracted using TRIzol (as described in **Section 4.2.4.1**). RNA extracted from Cases 2, 4, 9, 10, 15 -17, 20-22, 24 - 29 and 30 were transported to CBS on dry ice and stored at -80ºC until used.

4.2.5.2 RNA Analysis

Phenol from the RNA extraction process can inhibit PCR amplification and therefore all samples were 'cleaned' using RNeasy MinElute Clean-up Kit (Qiagen). 'Cleaning' involves mixing the RNA with ethanol and a guanidine-isothiocyanate–containing lysis buffer which enables RNA to stick to the silica membrane and the impurities to be washed away.[4] RNA concentrations and integrity was checked using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, USA) and Agilent 2100 Bioanalyser (Agilent Technologies UK Ltd., Berkshire, UK). The quality of RNA affects the efficiency of amplification, so poor quality RNA may not amplify efficiently. RNA integrity was expressed as an RNA integrity number (RIN) [365]. The Agilent Bioanalyser electrophoretically separates and analyses the characteristics of 28S and 18S ribosomal RNA to assign the RIN, which was expressed on a scale of 1 to 10 where 1 represents totally degraded RNA and 10 represents intact RNA. RIN values of less than 6 have been shown to give spurious results [366].

Only samples with a clear, clean peak at 28S and 18S RNA, no genomic DNA contamination, an A_{260}/A_{280} ratio of 1.8 – 2.0 and a RIN of 6 and above were considered to be suitable for qPCR. Full sets of RNA that met the above criteria were present for cases 12, 14-17, 20-22, 24-27, 29 and 30 (n =14). As these experiments were limited by the concentration of RNA available (other than for case 12), they were not duplicated using semi-quantitative PCR.

[4] Retrieved from RNeasy MinElute Cleanup Kit (04/10/2010): http://www.qiagen.com/products/rnastabilizationpurification/rneasysystem/rneasyminelutecleanup.aspx#Tabs=t1

4.2.5.3 Primers

Design, optimisation and quality control of the study primers were carried out by CBS (data not shown). The primer sequences are listed in **Table 4.3** and GAPDH was the HKG used as a control.

4.2.5.4 QPCR Procedure

Based on the standard operating procedures for a SYBR® Green Assay (A. Hawrani, CBS), 10 µl of the SYBR® Green PCR Master Mix was added to 4.8 µl of RNAase-free water, 0.6 µl each of the forward and reverse primers (300 nMol) making the final volume 16 µl. This 16 µl aliquot of master mix was placed in each well of a 96-well plate and 4 µl of cDNA sample (1:5 dilution of stock) was added. After the plate was sealed and centrifuged for 1 minute at 1000 rpm, it was placed in the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Cheshire, UK) for thermal cycling. The instrument automatically detects and records the fluorescent signals from each well. The data was exported in an Excel format.

Table 4.3: Oligonucleotide Sequences of Primers Designed for qPCR Experiments

Gene	Sequence 5' → 3' Forward (above) Reverse (below)	Amplicon Size
IL- 8	CCAGGAAGAAACCACCGGA GAAATCAGGAAGGCTGCCAAG	91 bp
IL-10	GCCAAGCCTTGTCTGAGATGA TCACATGCGCCTTGATGTCT	90 bp
TLR 2	CTCTCCAAGGAAGAATCCTCCAA GCCCTGAGGGAATGGAGTTT	100 bp
GAPDH	TGCACCACCAACTGCTTAGC GGCATGGACTGTGGTCATGAG	87 bp

4.2.6 TaqMan® Low Density Arrays

As discussed in the upcoming results section, gene amplification was sub-optimal using semi-QPCR and QPCR techniques which consumed the majority of the available cDNA. Therefore, an alternative technique which had the ability to use minute amounts of cDNA with relatively high specificity was sought. This was the TaqMan® Low Density Array. A Taqman Low Density Array (Applied Biosystems, Cheshire, UK) is a 384-well microfluidic card that enables parallel analysis of a panel of 96 genes by RT-PCR using one-quarter the concentration of RNA required for other QPCR techniques.

Like QPCR, TaqMan® assays use fluorescent technology but also include TaqMan® probes, engineered to be complementary to a specific sequence in the target gene between the forward and reverse primers [367]. The 5' end of the probe is linked to a 'reporter dye'

and the 3' end linked to a non-fluorescent 'quencher'. During PCR, the probe anneals to its specific sequence, where the DNA polymerase (AmpliTaq Gold®) cleaves the quencher from the 3' end, allowing the reporter dye to fluoresce. As more probe-specific product forms, the fluorescent signal increases. Unlike SYBR Green®, non-specific fluorescent signals do not occur, since there is no fluorescence unless the probe is cleaved [367].

TaqMan® Low Density Arrays have been successfully used with archived tissue [368] and a similar method of gene expression profiling to quantify inflammatory gene expression in acne has been described [325]. For this study, cards were pre-loaded with specific probes and primers for a panel of 90 genes involved in human inflammation including IL-8, IL-10, TNFα and TNFR. TLR2 is not included in this panel. Out of a possible 6 HKGs, GAPDH and colony stimulating factor 1 (CSF-1) were used as the endogenous controls. Seven cases (2, 14, 22, 24-26 and 30) were analysed with TLDA and the data obtained with both HKGs is presented and discussed in parallel.

These experiments were carried out by CBS. As per the protocol, 10 µl of cDNA was mixed with 40µl of RNAase free water and 50µl of TaqMan® Gene Expression Master Mix (Applied Biosystems, Cheshire, UK). This mixture (100µl) was then pipetted into each fill reservoir, allowing the analysis of 48 genes for each aliquot. The card was then covered and centrifuged twice at 1,596 rpm for 1 minute to ensure each well was adequately filled. The card was then placed in the ABI Prism 7900HT Sequence Detection System and allowed to cycle 40 times with the following parameters: 50ºC x 2 min, 94.5 x 10 min, 97.0 x 30s, 59.7 x 1 min. Each experiment was run in triplicate, yielding a data set of 21 experiments for each gene where the dCt values were used for statistical analyses.

4.3 *Statistical Analysis*

4.3.1 Statistical Analysis for Clinical Study

Based upon statistical advice, a sample size of 20 was chosen to enable the detection of a shift of 0.63 times the standard deviation for within-subject differences giving a power of 80% at the conventional 5% alpha level (p-value < 0.05). This calculation applies to all of the inflammatory markers studied. Testing the mean of differences between the pre- and post-treatment data using probability-probability and quantile-quantile plots revealed that they had a Gaussian distribution. Hence, the paired sample t-test was a suitable method for analysing the data. Correlations between changes in the variables were analysed using the Pearson's product-moment coefficient. The data was encoded within the statistical software package, SPSS version 16. The 2-tailed level of significance was set at 0.05. The data is presented as the mean ± standard deviation (S.D.) where applicable.

Leeds grading is a subjective quantitative scale and prone to marked variations depending on the observer [369]. To measure the agreement between the 4 blinded raters, and hence usefulness of the scale, the intra-class correlation was used [370]. When compared to the kappa coefficient, another statistical technique that is used to calculate inter-rater reliability, the intra-class technique was deemed to be the better instrument for quantitative, scaled data like the Leeds scores. Intra-class correlations (ICC) have been used in the validation of the Leeds Revised Acne Grading System [341]. Kappa coefficients are accepted as more useful for non-quantitative classifications [370]. The ICC has a maximum of 1 where 0.7 represents a satisfactory agreement and ≥ 0.9 is considered to be very good agreement.

A value of 0 describes a correlation that is not above chance. The data is presented as the average measure, 95% confidence interval and significance.

4.3.2 Statistical Analysis for Laboratory Study

A repeated measures analysis of variance (ANOVA) with Greenhouse-Geisser correction, where applicable, was chosen to determine whether IPL caused a statistically significant change in the mean expression of each marker at 3 time points (B1, B2 and B3). If the ANOVA *P* value (expressed as '*P*' in this thesis) was significant, it was then appropriate for further pair-wise testing between time-points e.g. B1 vs. B3. In these pair-wise tests, Bonferroni adjustments corrected for multiple means testing [371]. Values are presented as mean ± S.D and the two-tailed significance was set to $p < 0.05$ (expressed as 'p' in this thesis). SPSS version 18 (IBM, Chicago, USA) and Microsoft Excel were used to perform statistical analyses.

Chapter 5

RESULTS

5. Results

5.1 The Clinical Effect of 530nm IPL on Inflammatory Acne

5.1.1 Participants' Baseline Characteristics

A total of 190 persons were screened. Of these, the eligible cohort included in the study consisted of twenty-eight healthy adult volunteers (18 – 35 years) with mild to moderate acne on their backs and Fitzpatrick skin phototypes I - III. Of 28 patients, 5 were used as pilots and 2 dropped out before the final assessment (reason unknown). Consequently, 21 patients were used for statistical analysis of the lesion counts, Leeds scores and SER. Of this cohort, 12 (57.1%) were male and 9 (42.9%) were female. The average age was 24.6 ± 5.3 years and they had suffered with acne for an average of 9.6 ± 6.8 years. The average Leeds score at baseline was 1.99 (open assessment).

5.1.2 530 nm IPL Significantly Reduces Inflamed Lesions

As illustrated in **Figure 5.1**, the lesion counts for case 13 were far outside of the norm. In fact, there was a 5.8 fold increase in lesion counts, whereas the mean percentage change in lesion counts of the other 20 participants was 0.3-fold. After seeking statistical advice, it was decided that the data for this patient was an outlier and so the results were expressed with and without case 13. Cases 13 and 9 had lesion counts in excess of 100 at either time point, where the mean lesion count at baseline was 24.05 ± 22.41 and 23.10 ± 34.32 at the end of therapy. Thus to illustrate individual trends more clearly, these cases were omitted in **Fig. 5.1b**.

At the end of the active treatment phase, the mean change in inflamed lesion counts fell significantly (p = 0.023) by 28% (**Fig. 5.2**). When the outlier was included, there was only a 4.0% reduction in inflamed lesions which was not significant (p = 0.88).

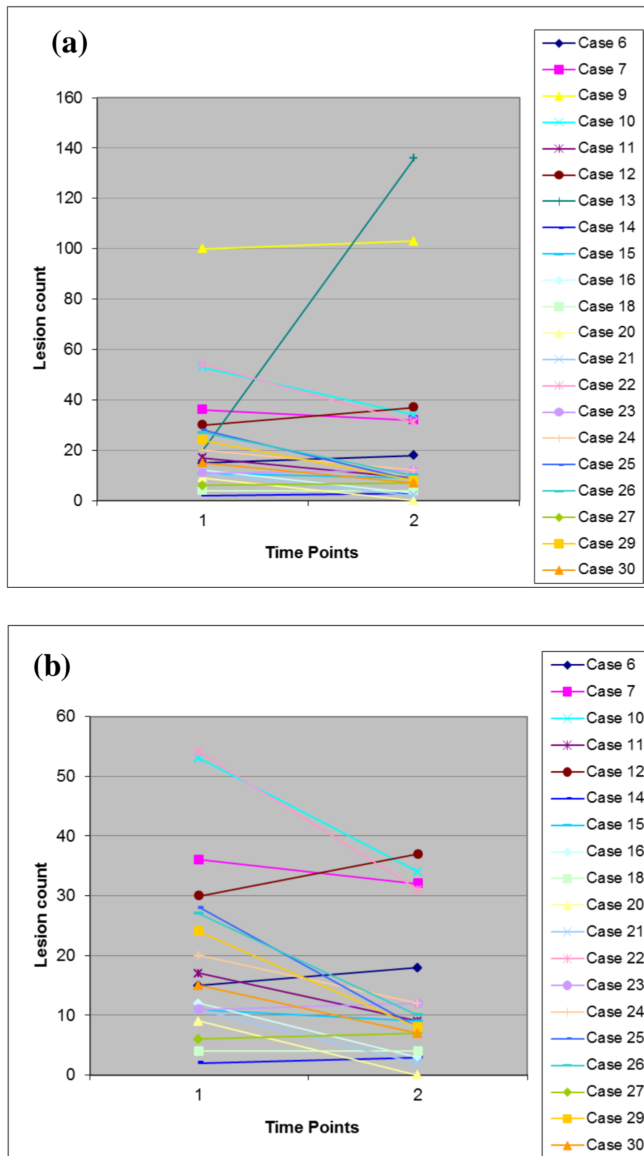


Figure 5.1: Change in Inflamed Lesion Counts Before and After IPL (a) Case 13 shows a marked increase in inflamed lesions, far in excess of that seen for the other cases, (b) Cases 9 and 13 have been omitted to illustrate individual trends for the rest of the study cohort.

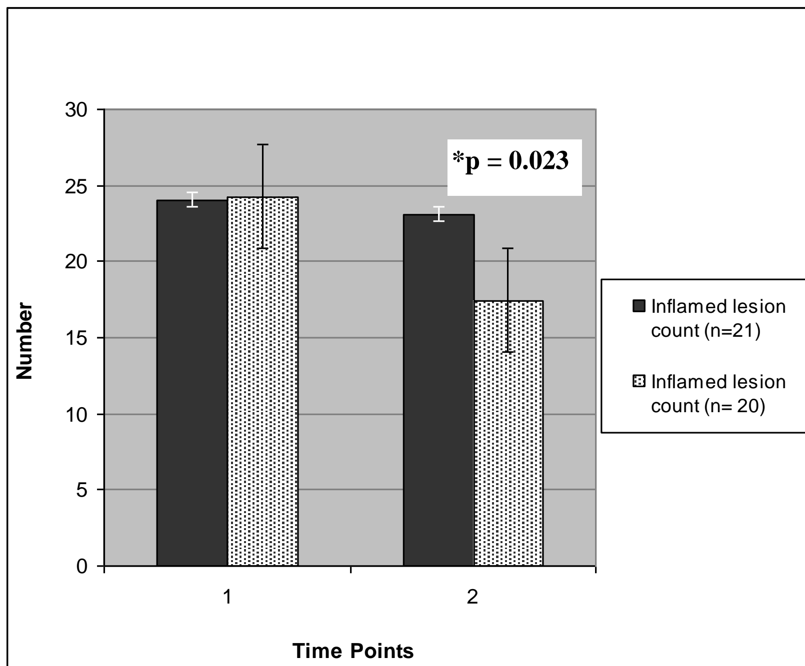


Figure 5.2: Mean Changes in Lesion Counts between Baseline ('1') and End of Therapy ('2'). Data with and without the outlier is shown. IPL significantly reduced inflammatory lesion counts in the final study cohort.

5.1.3 530 nm IPL Does Not Significantly Affect Non-inflamed Lesions

The decrease in non-inflammatory lesions and increase in resolving lesions were not significant. A comparison of the mean changes in lesion counts and SER are graphically represented (**Figure 5.3**). Individual rates before and after a course of IPL are illustrated in **Figure 5.4**.

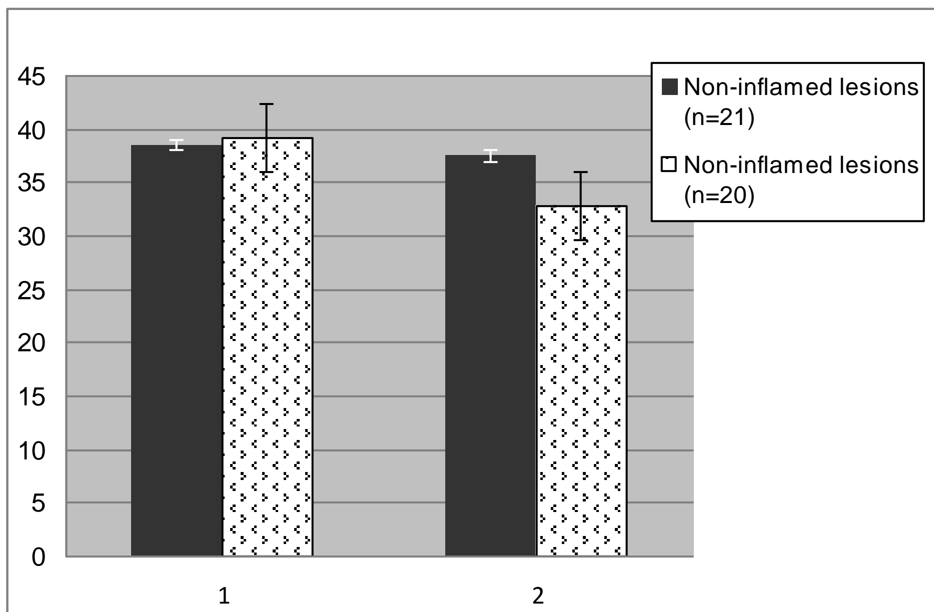


Figure 5.3: Mean Non-inflamed Lesions Before and After IPL. This graph illustrates the mean reduction in non-inflamed lesions after IPL therapy, which was not statistically significant.

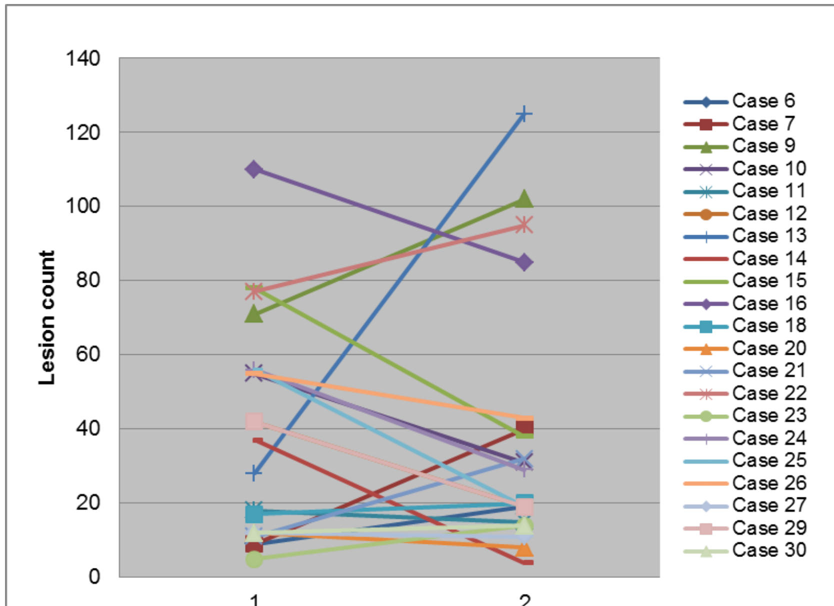


Figure 5.4: Change in Non-inflamed Lesions After IPL. Graph showing change in non-inflamed lesion counts for the entire cohort. Note the steep rise in non-inflamed lesions for the outlier, Case 13.

5.1.4 The Effect of 530nm IPL on Sebum Excretion Rate

After IPL therapy, the sebum excretion rate fell in 9 patients, remained stable in 9 and increased in the remaining 3 cases. The mean SER fell by 35.86% in the cohort of 21 and by 33.03% in the cohort of 20 patients (**Figures 5.5** and **5.6**). Neither reduction was statistically significant (p = 0.23).

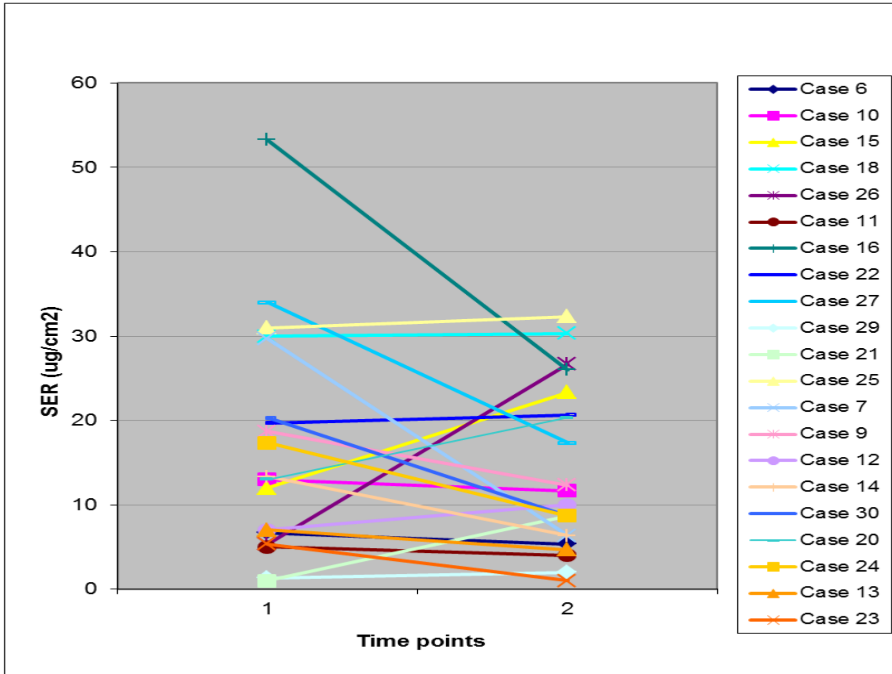


Figure 5.5: Individual Variations in SER at Baseline and at the End of Therapy.

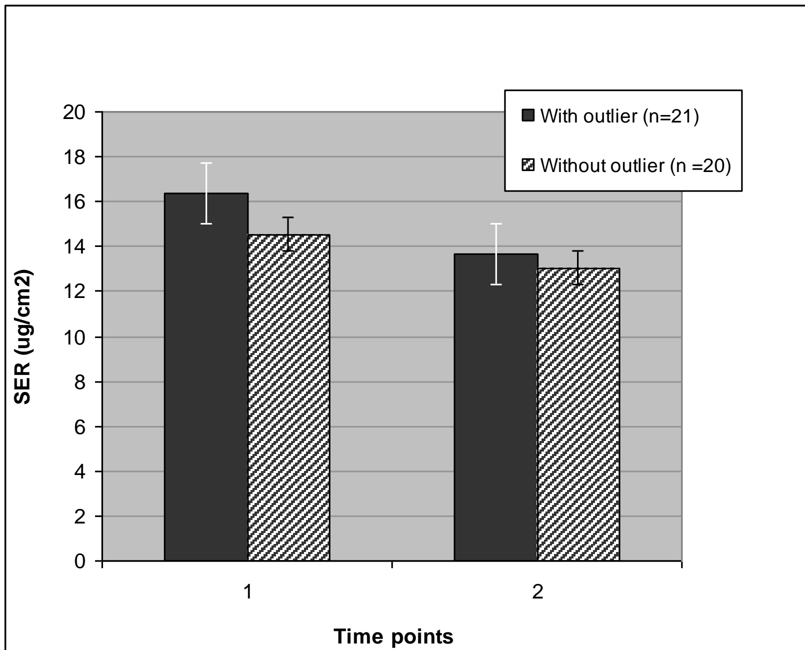


Figure 5.6: Change in Mean SER Before and After IPL. There was a small but non-significant reduction in SER.

5.1.5 IPL's Effect on the Revised Leeds Score

5.1.5.1 *Inter-rater Reliability of Leeds Scores*

There was a very good agreement between each blinded assessor for the Leeds grades for each photograph (Appendix 2). The correlations for both the baseline and post therapy scores came in well above 0.7 and with a significance of p < 0.001 indicating that the scores given by the raters were consistent with each other (**Table 5.1**).

Table 5.1: Inter-rater Reliability for Leeds Scores. The correlations between the scores of the four blinded raters show very high levels of concordance.

	Baseline Score	**1 week after Therapy**
Intra-class Correlation (average measures)	0.903	0.843
95% Confidence Interval	0.812 – 0.956	0.698 – 0. 929
Significance (p value)	p < 0.001	p < 0.001

5.1.5.2 *Blinded vs. Open Assessments*

In the cohort of 21 patients, the differences between the pre- and post-IPL Leeds scores for the back did not achieve statistical significance for either the four blinded, (p = 0.667) or the candidate's open assessments (p = 0.081). The mean improvements in the Leeds score with and without the outlier for both the clinical (open) and photographic (blinded) assessments are listed in **Table 5.2**. Using blinded assessments, 19.04% improved, 23.4%

worsened and 12 subjects (57.1%) remained the same. For the open assessments, approximately 61.9% experienced an improvement, one seventh (14.3%) deteriorated and a quarter (23.8%) of the cohort showed no change. Both sets of scores show that irrespective of the method used, IPL did not significantly improve the acne severity as determined by the Leeds grading system. The chest emerged as a suitable internal control in the open assessments as the percentage change for the back and chest were very similar as were the levels of statistical significance.

Table 5.2: Mean Reductions in Leeds Scores. The average changes in the Leeds scores are listed below for the open and blinded assessments. The assessments without the outlier (n = 20) are highlighted in blue italics (* *denotes statistical significance).*

	Blinded **BACK**	**Open** **BACK**	**Open** **CHEST**	**Open** **FACE**
Mean change in Leeds Grade from baseline	0.05 ± 0.5 *0.05 ± 0.51*	0.23 ± 0.42 *0.31 ± 0.56*	0.25 ± 0.29 *0.24 ± 0.29*	0.27 ± 1.46 *0.29 ± 1.5*
% Reduction from baseline	- 9.41 % *- 9.88 %*	35.84 % *33.04%*	36.84 % *34.45%*	19.49% *20.91%*
2-tailed Significance	0.667 *0.667*	0.081 *0.023**	0.018* *0.018**	0.457 *0.458*

5.1.5.3 Photographic Assessments

Three cases illustrated in **Figure 5.7** represent the overall typical clinical response. Case 9 (**Figure 5.7a, b**) and case 25 (**Figure 5.7c, d**) show mild improvement in the inflammatory appearance of their acne. If the biopsy sites are excluded, there is a small but noticebale reduction in the number of lesions. Case 15 (**Figure 5.7f, g**) appears to have an increased number of inflamed lesions at the end of therapy. When cases 15 and 25 opted to return 1 month after the final IPL treatment, which involved abstaining from sun-exposure and any acne therapy, case 15 continued to improve (**Figure 5.7h**) whilst case 25 relapsed (**Figure 5.7e**). Case 9's third image was not included as she did not opt for further follow up after the final IPL irradiation.

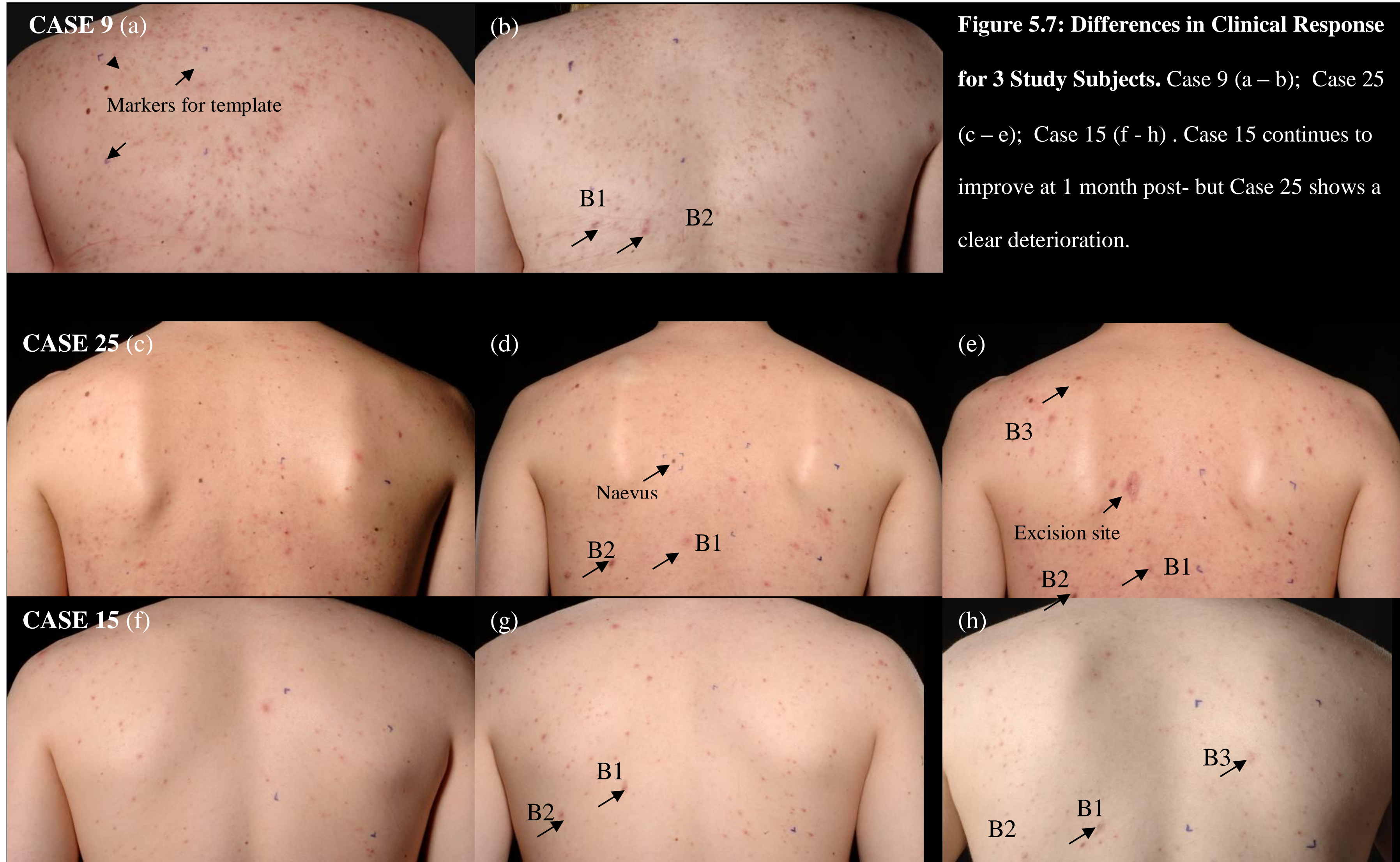


Figure 5.7: Differences in Clinical Response for 3 Study Subjects. Case 9 (a – b); Case 25 (c – e); Case 15 (f - h) . Case 15 continues to improve at 1 month post- but Case 25 shows a clear deterioration.

5.1.6 Summary of Primary Clinical Outcomes

Though yellow IPL was able to significantly reduce inflammatory lesions, it did not have a clinically appreciable effect on the Leeds score or the sebum excretion rate. The final efficacy data is summarised in **Table 5.3**.

5.1.7 Medium-term Endurance of Yellow IPL

Within the analysed cohort, 14 subjects returned for follow-up 1 month after their final IPL treatment. Of these, 3 subjects (21.4 %) relapsed, while another 3 (21.4%) had an improvement in their Leeds score and the remainder experienced no change in their scores. Of the 8 subjects evaluated 3 months after their final treatment, 2 maintained their scores over the period, and the remainder relapsed. Therefore at the end of 3 months, the medium term benefit of IPL therapy was preserved in 2 of 14 subjects (14.3%). Due to the small numbers of patients that returned for follow-up, this portion of the study failed to be adequately powered, and hence was not statistically tested.

5.1.8 Gender and Acne Duration Do Not Influence IPL Response

As expected, the duration of the participants' disease was positively associated with their age ($p < 0.001$). There was no relationship between the patients' Leeds scores, inflamed lesion counts or non-inflamed lesion counts. Correlations between BMI, changes in the SER and the open Leeds score (back) were not found. Likewise, there were no relationships between gender, lesion counts, SER or the Leeds score.

Table 5.3: Summary of Clinical Outcomes. The mean reductions without the outlier (n= 20) are italicised in blue. * *denotes statistical significance*

Parameters	Inflamed Lesions	Non-inflamed Lesions	SER (µg/cm^2)	Leeds Score BLINDED	Leeds Score OPEN
Baseline Assessments (mean ± S.D.)	24.05 ± 22.41 *24.25 ± 22.98*	38.67± 29.25 *39.2 ± 29.91*	16.38± 13.12 *14.53 ± 10.28*	1.36 ± 0.59 *1.38 ± 0.60*	1.99 ± 1.09 *2.04 ± 1.10*
Final Assessments (mean ± S.D.)	23.10 ± 34.32 *17.45 ± 23.13*	37.24 ± 34.31 *32.9 ± 28.5*	13.65 ± 9.72 *13.03 ± 9.54*	1.40 ± 0.55 *1.43 ± 0.55*	1.74 ± 1.11 *1.73 ± 1.13*
Mean Reduction ± S.D.	0.95 ± 28.1 *6.8 ± 8.69*	1.43 ± 31.2 *6.35 ± 22.11*	5.87 ± 9.70 *4.80 ± 8.58*	0.05 ± 0.5 *0.05 ± 0.51*	0.23 ± 0.42 *0.31 ± 0.56*
Mean % Reduction from Baseline	3.95 % *28.04 %*	3.70 % *16.2%*	35.86% *33.03%*	- 9.41 % *- 9.88 %*	35.84 % *33.04%*
2-tailed Significance (p value =)	0.878 *0.002**	0.836 *0.214*	0.272 *0.501*	0.667 *0.667*	0.081 *0.023**

5.1.9 Adverse Events

Side-effects included mild to moderate discomfort during treatment, which was easily remedied by adjusting the intensity of the air cooling. One subject had a mild infection at their first biopsy site and therefore their 2nd biopsy was omitted to allow complete resolution of the first.

5.1.10 Summary of the Clinical Findings

1. Yellow IPL reduced inflammatory lesions by 28%, which was significant at $p = 0.002$, when the outlier was excluded from the analysis (**Section 5.1.2**). The 16.4% reduction in non-inflamed lesions after IPL was not significant ($p = 0.214$, **Section 5.1.3**).

2. The reduction in the SER of 35.1% was not statistically significant (**Section 5.1.4**). The reduction in inflammatory lesions was proportional to the reduction in SER for the cohort of 21. Exclusion of case 13 resulted in the p value > 0.05 (**Section 5.1.4**).

3. There was no significant change in the Leeds score following a course of yellow IPL. Quoting the scores from the open assessments, the 33% improvement in the Leeds score for the back ($p = 0.023$) was mirrored by a 34% improvement in the score for one internal control, the chest ($p = 0.018$). Using blinded assessors (**Section 5.1.5**), the Leeds score worsened by 9.9% ($p = 0.67$).

4. Of the 14 patients that remained in follow up, only 2 maintained remission 3 months after the final IPL session. Therefore, any statements about the upper limit of the

duration of yellow IPL's therapeutic effect will have to be restricted to 3 months after the final treatment (**Section 5.1.6**).

5. There was no association between gender and the change in inflamed or non-inflamed lesion counts (**Section 5.1.7**).

5.2 Elucidating the Anti-inflammatory Actions of Intense Pulsed Light

5.2.1 Data Presentation

A combined total of 25 cases were analysed using three molecular assays: semi-quantitative PCR, SYBR® Green qPCR and TaqMan® low density arrays (TLDA). The number of cases where the main target genes were successfully amplified using each technique is listed (**Table 5.4**). Functioning positive controls verified that the experimental conditions and primers used were correct. To maximise the data, compensation for 'RNA degradation-related shifts' in the Ct values [372] and reduced data loss through repeated averaging, statistical testing was carried out on the threshold values (dCt) rather than the relative quantities (RQ) of the gene expressed. Unless otherwise stated, the TLDA data presented was based on GAPDH as the house keeping gene (HKG) in all 7 cases. Using geNorm (a software algorithm used to determine the most stably expressed gene amongst a panel of target genes) [373], analysis of the data showed that colony stimulating factor 1 (CSF1) was also stably expressed in these samples (**Appendix 3**). Where applicable, the CSF1 values are presented alongside GAPDH's as evidence of the data's reliability.

Table 5.4: Target Genes Successfully Amplified in Each Molecular Assay

Technique (Duplicated Cases/ n Cases)	TLR2	IL-8	IL-10
Semi-qPCR (2/11)	11	0	0
QPCR (2/14)	7	3	8
TLDA (7/7)	Not available	3	7

5.2.2 530nm IPL Down-regulates Epidermal TLR2 Expression

TLR2 was found to be expressed throughout the epidermis (**Figure 5.8**) with some background staining within the dermis. Of the 10 cases studied, 48 hours after IPL (biopsy 2) epidermal TLR2 expression increased by 5 – 46% in five cases. The other 5 cases showed a reduction in epidermal TLR2 expression of 4 – 13% in biopsy 2. Overall there was a mean increase in TLR2 expression of 8.57% ($p = 0.003$).

Statistically, though the mean difference between the integrated optical densities (IOD) between the baseline and final biopsies was 2.63%, it was significant at $p < 0.001$. TLR2 expression fell from baseline in 7 cases by 0.1 – 14% and increased in Cases 2, 6 and 14 by 7%, 51% and 67%. The repeated measures ANOVA *P* value with Greenhouse-Geisser correction was significant ($P < 0.001$). .

Figure 5.8 illustrates the IHC images of 2 representative cases (2 and 10). Note that there is a clear up-regulation of TLR2 expression when measured 48 hours after IPL irradiation

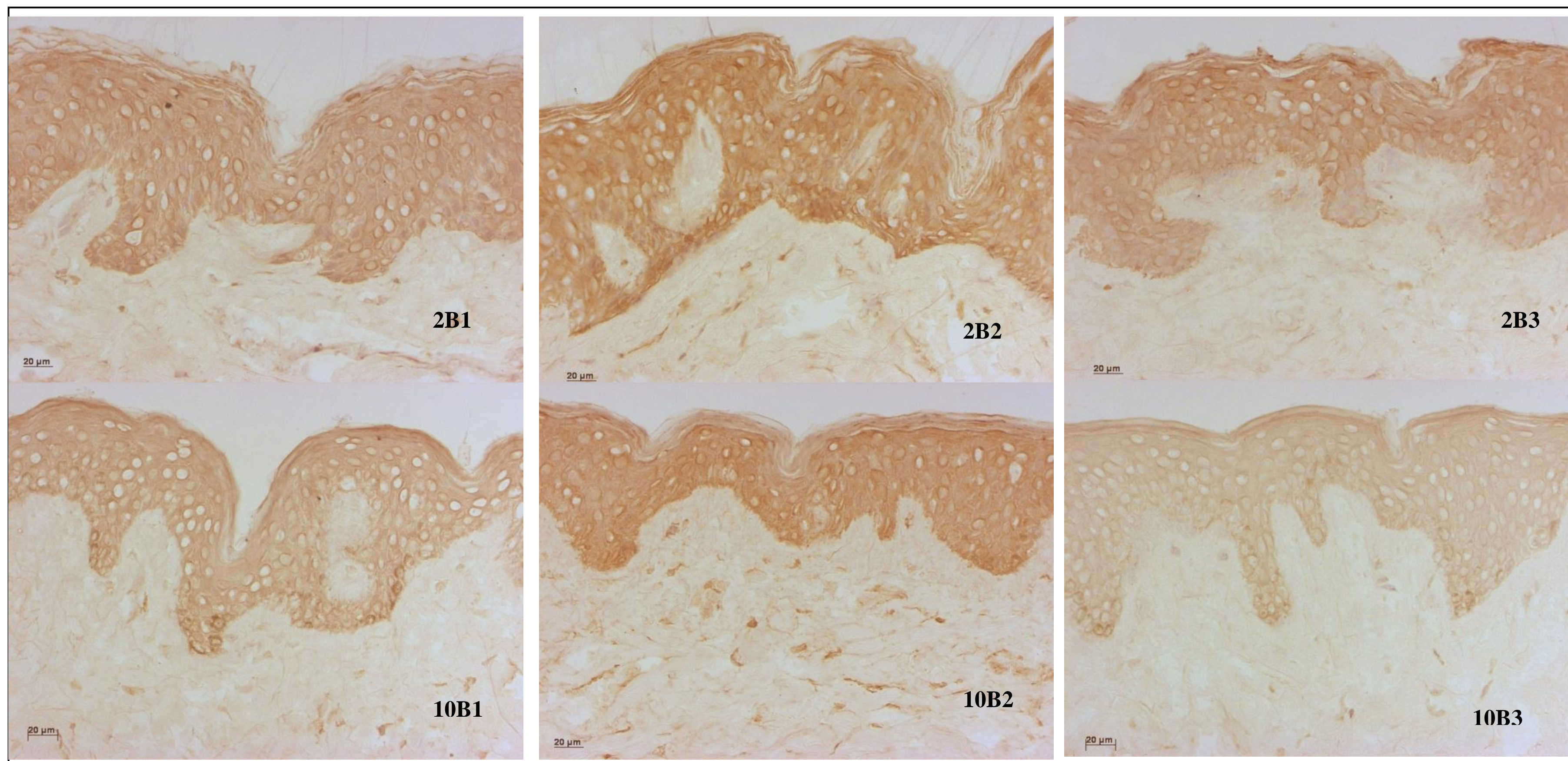
(B2) that appears to return to baseline levels in Case 2 and falls below baseline expression in Case 10 at one week post-therapy (B3).

5.2.2.1 Correlation between IHC and PCR Results

The 10 cases selected for IHC were also evaluated with semi-quantitative PCR (**Figure 5.9**). Integrated density values (IDV) demonstrated a 10.2% ($p = 0.84$) increase in mean TLR2 mRNA expression from baseline at the end of therapy (B3). There was also a 34.6% ($p = 0.50$) increase in TLR2 expression in the 2nd biopsy and an 18.1% ($p = 0.84$) fall a week after the final irradiation (**Figure 5.9**).

Comparing IHC image analysis data with the semi-qPCR IDV values, there was agreement in the up- and/or down regulation of TLR2 in 5 cases (cases 5, 6, 9, 12, 14) and partial agreement in the other 5 cases. The mean data for both methods (Semi-quantitative PCR and Image analysis) correlated well. Both show an overall increase in TLR2 expression when measured 48 hours after IPL irradiation and an approximation to baseline levels 7 days after the final irradiation (**Figure 5.10**). The raw data is listed in **Appendix 3**.

Figure 5.8: Effect of IPL on TLR2 Expression in Human Epidermis by IHC. Two representative cases, 2 and 10, are shown. Note increased expression in B2. *Scale bar: 20µm*



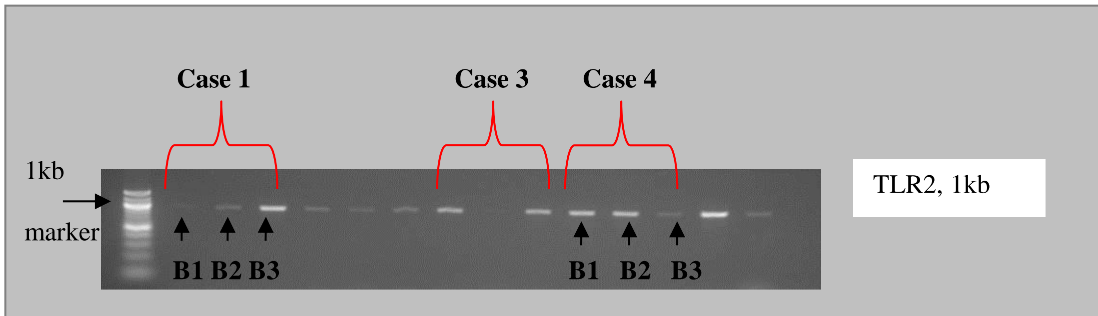


Figure 5.9: Agarose Gel Images Showing the Effect of IPL on TLR2 Expression. PCR product analysis of biopsies (B1-B3) from cases 1–5 were analysed on a 2% (w/v) agarose gel and stained with ethidium bromide and visualised under UV light. Each case is represented as a set of 3 bands (B1, B2 and B3). Note: *kb = kilobase*.

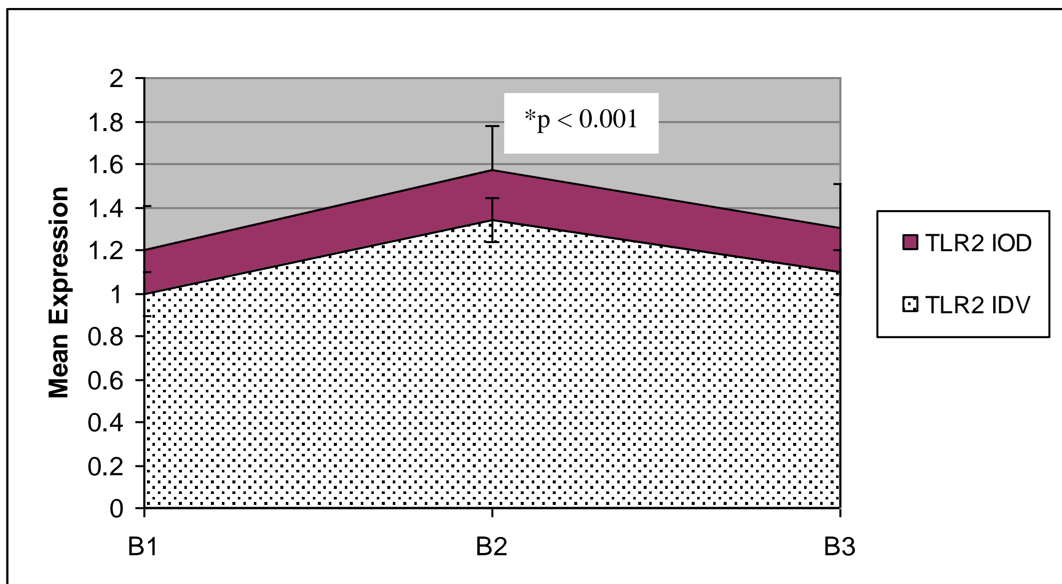


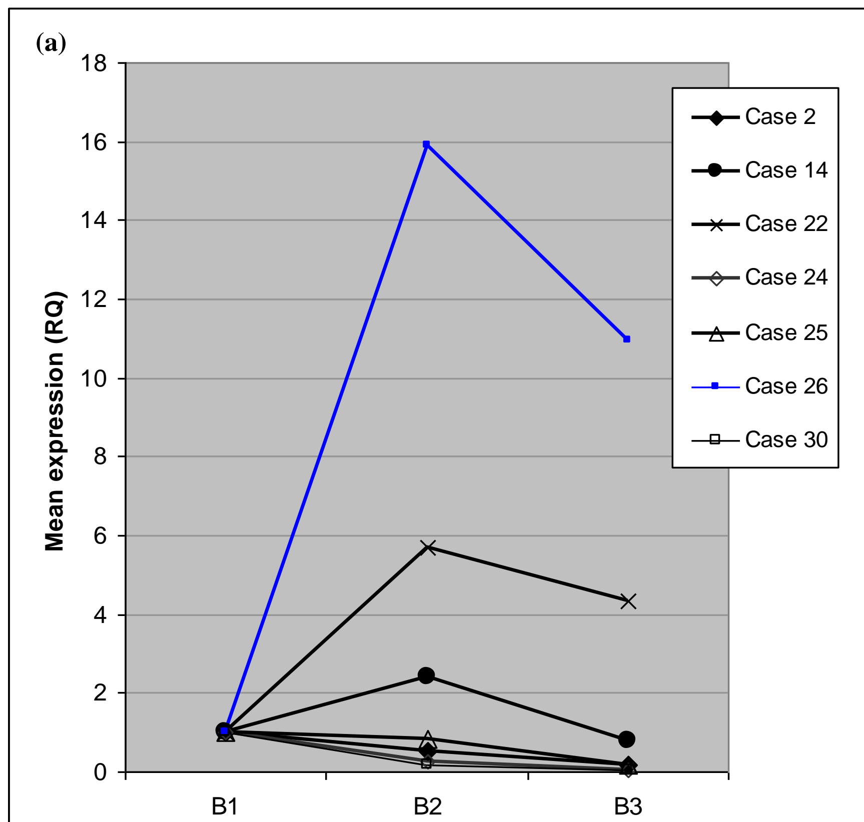
Figure 5.10: Mean Changes in TLR2 Expression Measured by Semi qPCR and IHC. IDV curve represents Semi-qPCR data and IOD, IHC image analysis data (mean of data for Cases 5, 6, 9, 12, 14). Average TLR2 expression increases by 10 – 20% in B2 and returns to baseline levels in B3 using both methods. *Error bars* = SEM*; IDV*= integrated density values*; IOD* = integrated optical density

5.2.3 530nm IPL's Effects on Inflammatory Cytokine Expression

5.2.3.1 IPL Does Not Significantly Change IL-8 Expression

Regarding individual changes, the relative quantities of IL-8 for case 26 were significantly higher than the rest of the cohort, but the trend was similar to changes observed in other members of the sub-group (**Figure 5.11a**). Mean IL-8 expression increased in biopsy 2 and fell below baseline levels in 4 cases (Cases 2, 24, 25, 30). In biopsy 3, IL-8 fell to just above baseline levels in 2 cases (Case 26 and 22) and to baseline in one case (Case 14), and below baseline levels in Cases 2, 24, 25 and 30.

IL-8 expression (mean of all data) increased by an average of 66.42% ($p > 0.50$, ANOVA $P = 0.179$), when measured 48 hours after IPL irradiation (biopsy 2) but fell to approximately 7.9% below baseline ($p = 0.434$) when assessed 1 week after the fourth and final treatment (biopsy 3). See **Figure 5.11b**.



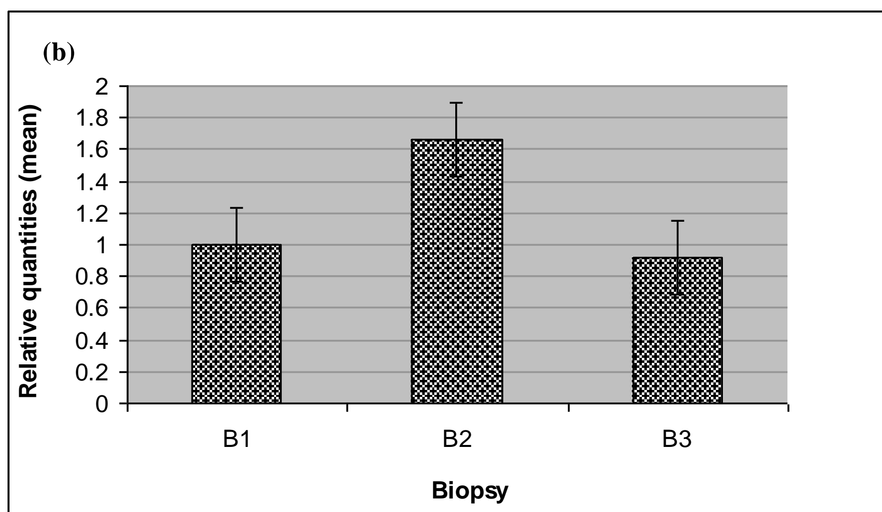


Figure 5.11: Effect of IPL on IL-8 Expression. (a) Effect of IPL on IL-8 expression in individual cases; (b) Mean change in IL-8 expression over a course of IPL in all cases (e*rror bars = SEM,* n= 7*).*

5.2.3.2 *IPL Down-regulates TNFα and TNF Receptor Expression*

TNFα expression fell by 17.6% (p = 0.031, ANOVA *P* = 0.006) by the end of therapy. Also, TNFR expression fell by 37.6% (p = 0.017, ANOVA *P* = 0.11) by the end of therapy (see **Figure 5.12**).

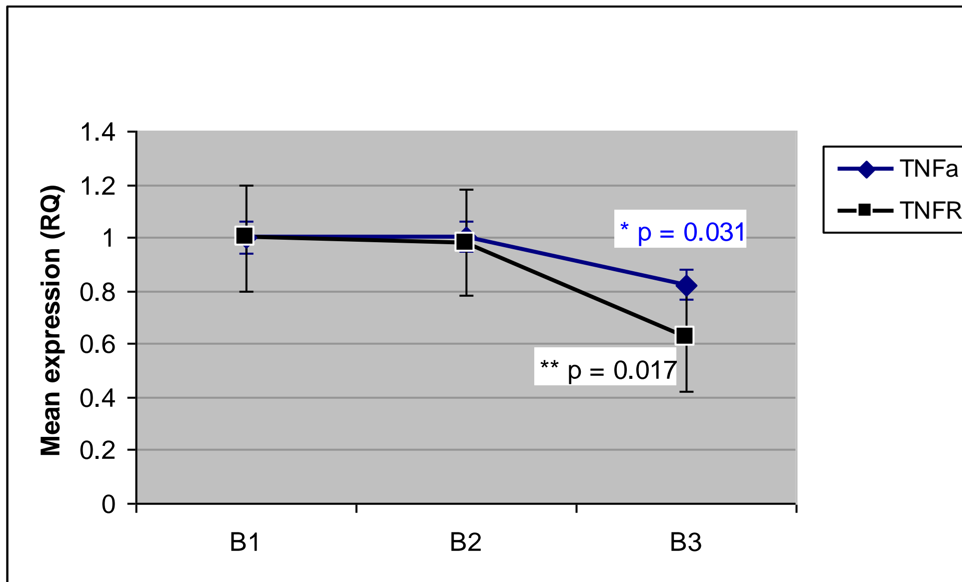


Figure 5.12: Effect of IPL on TNFα and TNFR Expression. Graph showing down-regulation of TNFα and TNFR expression in acne prone skin after treatment with IPL. Data represents a mean of all values obtained for the cases examined (*Error bars = SEM, n =7).*

5.2.4 IPL Does Not Significantly Affect IL-10 in Acne Prone Skin

IL-10 was detected in all 7 cases using TLDA and in 8/13 cases analyzed with SYBR Green® QPCR. For the SYBR Green® assay, of the 8 cases that amplified, only 3 cases had detectable levels of IL-10 in all three biopsies and 5 cases (12, 14, 20, 22 and 30) produced data from biopsies 1 (baseline) and 3 (1 week after 4th and final treatment) using

this method. For this reason, only SYBR Green® data for biopsies 1 and 3 are presented. The TLDA data is also shown in **Table 5.5**.

Where GAPDH was used as the house-keeping gene, the TLDA data showed that IL-10 expression increased by 97% ($p = 0.96$) in B2 which was maintained in B3. Using SYBR Green®, IL-10 expression increased from baseline by approximately 114% ($p > 0.05$). Both molecular assays showed a doubling of IL-10 expression after IPL irradiation, but this was not statistically significant (**Table 5.5**).

Table 5.5: Relative Changes in IL-10 Expression. SYBR Green and TLDA (GAPDH) data show that the percentage increase in B3 is comparable using both methods despite different patient cohorts. CSF-1 and GAPDH exhibit similar trends in IL-10 expression using TLDA (*RQ* = relative quantity).

IL-10		**B2 – B1**	**B3 – B1**	**B3 – B2**
Technique (HKG)	**Cases Analysed**	**% RQ change (p value)**	**% RQ change (p value)**	**% RQ change (p value)**
SYBR Green® (GAPDH)	12, 14, 20, 22, 30	N/A	+ 113.67 % $P > 0.05$	N/A
TLDA (GAPDH)	2, 14, 22, 24 - 26, 30	+ 96.78 % $P = 1.00$	+ 94.99 % $P = 0.75$	- 0.91 % $P = 1.00$
TLDA (CSF-1)	2, 14, 22, 24 - 6, 30	+ 68.19% $P = 1.00$	+58.06% $P = 0.71$	- 6.02% $P = 1.00$

5.2.5 Relationship between In vitro and In vivo Findings

A key question was whether the clinical change in inflamed acne lesions correlated with the change in the expression of inflammatory mediators. Those that achieved statistical significance as determined by the ANOVA *P* value and/or pair-wise comparisons were evaluated i.e.TLR2, TNFα and TNFR. A weak negative relationship was found between the change in lesion counts and TNFα expression (see **Figure 5.13**, $R^2 = 0.253$). A linear relationship was not observed between the percentage change in lesion counts and TNFR ($R^2 = 0.007$) or TLR2 ($R^2 = 0.008$) expression (*data not shown*).

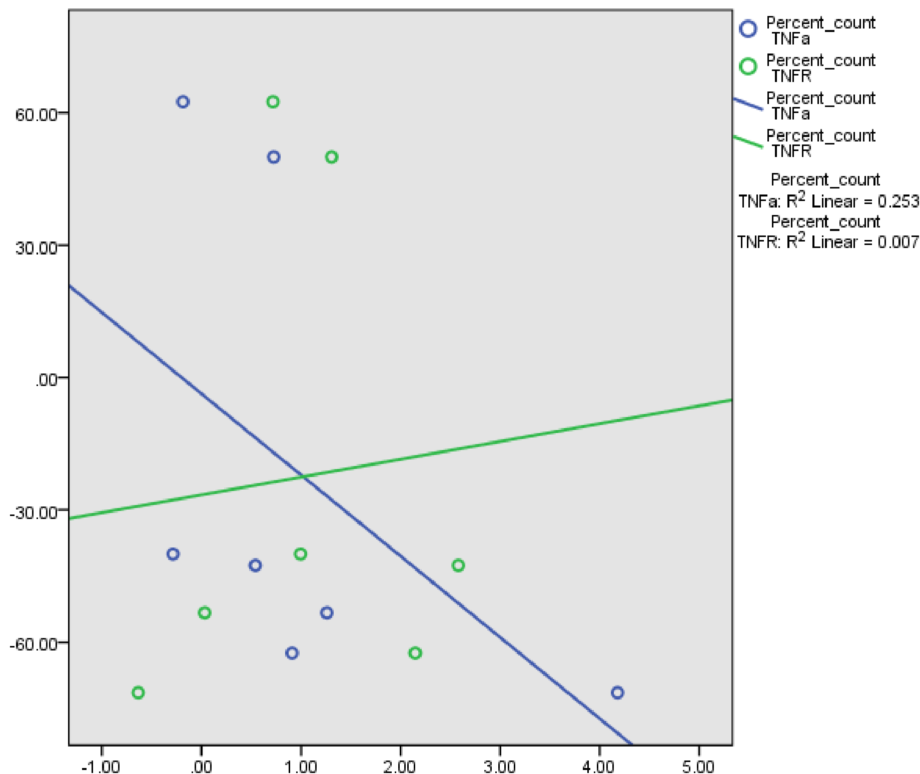


Figure 5.13: Correlation between Lesion Counts and TNFα and TNFR. The percentage change in lesion counts were plotted against the change in cycle threshold values for each gene (dCT B3-B1).

5.2.6 Summary of Results from Laboratory Study

A number of pro- and anti-inflammatory molecules were examined to determine the mechanism of IPL's anti-inflammatory effect. The findings are summarised below.

1. Immunolocalisation of TLR2 to the epidermis decreased by a small but significant increment of 2.6% ($p < 0.001$) by the end of therapy. When measured 48 hours after the initial exposure (biopsy 2), epidermal expression of TLR2 increased by 8.6% ($p = 0.003$). TLR2 mRNA transcript levels from whole skin biopsies partially agreed with the trends noted in the IHC results but the changes were not statistically significant (see **Section 5.2.2**).

2. IL-8 expression increased by 66.4% in biopsy 2 and fell slightly below baseline levels in biopsy 3. These changes were not statistically significant (see **Section 5.2.3**).

3. TNFα and TNFR expression remained stable when measured in B2, 48 hours after the first IPL treatment. However, both markers then fell significantly at the end of the course of IPL with 18% reduction in TNFα expression (ANOVA $P = 0.006$, $p = 0.031$) and 38% in TNFR expression (ANOVA $P = 0.107$, $p = 0.017$), see **Section 5.2.3**.

4. IL-10 mRNA expression increased by approximately 100% in B2 and B3. Though mean QPCR and TLDA data correlated well, this change was not statistically significant (see **Section 5.2.4**).
5. Clinical correlation using the data from this subgroup of patients suggests that there is a weak negative relationship between percentage change in inflammatory lesions and

TNFα expression (see **Section 5.2.5,** R^2 = 0.253). Thus, those who had deterioration in their lesion counts after therapy also tended to have higher TNFα expression post-IPL.

5.3 Study Critique

5.3.1 Clinical Trial

The data, though able to produce valid conclusions, may have been coloured by a number of inherent biases in the conduct of the study as well as unavoidable patient factors, which are discussed in detail below.

5.3.1.1 Clinical Study Strategy

1. The lesion counts were limited to a 101cm^2 area rather than the entire treatment area. Inclusion of the entire back may have produced more dramatic changes, approximating to that described in the literature. However, based on assessment of the degree of variation between trained and untrained lesion counters by Lucky et al. [374], they concluded that the use of a trained assessor to count within a defined area reduced variability, leading to an average intra-observer concordance rate of 0.8. Therefore, the use of a template to count lesions within a defined area was advantageous. Further evidence is provided by this study's finding of a more significant reduction in inflamed lesions when compared to the Leeds score, making it unlikely that any improvement caused by exposure to IPL was under-represented by choosing a defined area for lesion counts.

2. The large changes in significance after exclusion of the outlier for lesion counts and un-blinded Leeds scores probably indicates that had the sample size been larger, its effect would have been smaller. The outlier, was using a topical zinc-erythromycin combination before entering the study. The marked deterioration in her acne may have been a pre-menstrual flare (history not obtained during the study), a late rebound response despite completing a 4 week wash-out period or IPL may have had a pro-inflammatory effect which is alluded to in the final chapters.

3. Clinical Leeds assessments estimated a 33% change in the Leeds grade vs. - 9% for the blinded photographic grading.
    a. Utilising medical professionals trained in Leeds grading reduced the bias in the scores for each patient [375]. However, in order to maintain blinding, the biopsy scars were not highlighted on each patient's full back photo which may have resulted in the patient being given a higher Leeds score than if absent. This potentially reduced the observable difference between pre- and post-IPL photographs. This would have been compounded by the fact that this cohort's mean baseline acne severity was 2.04 ±1.10 (open assessment,Table 5.3), which is mild and in the absence of complete clearance post-IPL, may not have warranted a reduction by a full grade. In a larger commercial trial setting or collaborative study, Leeds grading by blinded practitioners at the time of presentation rather than with the retrospective use of photographs would have been preferable.

    b. A weakness of the Leeds Revised Acne Grading System is that it favours inflammatory lesions and does not account for their depth, which is only

possible through palpation [327]. Thus, in cases where comedones or scattered nodules predominate, a photographic evaluation would naturally give a lower score than that on clinical examination. This, in addition to investigator bias, is the most likely reason for the difference in the percentage change in the scores. In spite of this, both clinical and photographic grading yielded the same conclusion. Although there was a 30% reduction (approximate) in inflamed lesions, IPL was unable to improve the severity of acne to a point that was appreciable to the eye as being significant enough to reduce the Leeds score by a grade of ≥ 1.

c. As shown by this study and several others before, the reliable identification of a trend utilising a visual scale is possible but the use of a subjective instrument to do an objective measure will always be imprecise. This disparity highlights the need to use both types of assessment for a truly representative picture of improvement or deterioration.

4. The sebum excretion rate is affected by external and internal factors.
   a. As pointed out by the EEMCO guidance, patients with excessive amounts of skin surface lipid could saturate the tape, making the reading inaccurate and the roughness and relief of the measured area could also impair proper contact with the skin [334].

   b. Measurement could have been confounded in the one hour waiting period by inadvertent removal of sebum by clothing, movement etc. which could not be controlled for or guaranteed as constant at baseline and final measurements.

c. SER is affected by ambient room temperature, circadian rhythms and hormonal status at the time of measurement [112, 376]. Thus, although measurements were done in the same room, measurements were done according to patient availability and therefore circadian rhythms were not controlled. This difficulty has been described by other researchers like Blume et al. [377] who measured sebum excretion rates in male and female acne patients using Sebutape®. However, Rode et al. [347], who also assessed 12 non-acne patients, did not find any day-to-day variation when the same individuals were measured using the Sebumeter® over 2 days.

d. Though assumed to be representative of the entire back, the measured area may not be equivalent to other areas on the back. Different areas on the face have varied sebum outputs and this may be the case for the back.

e. In response to therapy, the lipid composition can change without a corresponding change in the excretion rate [376], therefore SER may not be an accurate reflection of IPL's effect on the sebaceous glands.

5. This trial utilised intra-individual controls, which carries with it inherent biases. Though the use of internal controls has its advantages, a more objective approach would be to have a placebo controlled or a parallel assignment study comparing IPL with an already accepted standard of therapy such as adapalene 0.1% gel. In fact, such a study was designed in conjunction with the project presented in this thesis, but the lengthy process involved in conducting such a trial dictated that my remit be narrowed to that described in this thesis. The randomised controlled double-blind clinical trial

comparing the efficacies of photodynamic therapy (IPL + methyl-aminolaevulinic acid), IPL only (IPL + sham cream) and adapalene is being undertaken by other researchers [378].

5.3.1.2 Treatment Parameters

1. The device settings may have been too gentle to cause a considerable change and probably required a longer ‘on time’ e.g. 5 pulses ‘on’ and 5 ‘off’ rather than 5 pulses ‘on’ and 15 ‘off’. A suboptimal energy delivery was unexpected as these settings were clearly sufficient to clear actinic keratoses when used in conjunction with photodynamic therapy (Babilas et al., 2007) and did not seem unreasonable when discussed with a co-author of that paper especially where patient tolerability was a real concern. The patients who were treated with shorter off times (10 ‘off’) and higher fluences (42 J/cm^2) visibly experienced more pain.

2. Covering the treatment area with ultrasound gel was deemed useful as it reduced total internal reflection thus increasing the effective energy absorbed and hence treatment efficacy. However, air bubbles within it can cause light scatter and dissipate delivered energy [184]. In the context of this study, inadequate time between each discharge could have led to heating of the ultrasound gel resulting in bubble formation and dissipation of energy.

3. Darker skin types have an increased risk of post-inflammatory hyperpigmentation where high temperatures are induced by IPL [379]. Cooling the skin increases the energy threshold at which epidermal damage occurs and reduces the pain associated with the treatment. Operator controlled cooling however, introduces

variability in the amount of energy absorbed by the skin. In the course of treatments in this study, patients were cooled prior to and during the IPL sessions. Hence, a reduction in the energy absorbed by the skin may have contributed to the low complication rates but treatment efficacy possibly suffered as well.

Town et al. [184] found that the measured pulsed durations were as stated on the Energist ULTRA™ 610 nm device and the percentage deviation of the cut-off filter was small at 2.1%. The device however, was operating only at 77% of the maximum fluence stated by the manufacturer. No data was given for the 530 nm applicator, but a similar level of energy loss is very likely as the only difference between the applicators is the filter. Though the active treatment phase spanned 10 months, just prior to undertaking the study, calibration and servicing was performed by the company, hopefully this mitigated any additional deterioration in device performance.

4. A narrower light spectrum may have been more efficacious as the spectral output of the clinically effective wavelengths would be greater for any given wavelength. The IPL applicator spanned an emission spectrum of 530 – 950 nm. Bjerring et al. [380] found that 525 – 750 nm IPL was better in improving redness and 555 – 950 nm IPL was better for pigmentation. Hence, energy distribution across a narrower spectrum may have led to increased energy delivery to the skin and possibly a more specific, and therefore more identifiable, phototherapeutic response.

5. Unavoidable inter-individual variation:

a. Our skin's irregular shape and surface in addition to its appendages being embedded in a non-homogenous dermis makes its optical properties complex and varied and hence, dosimetry estimations are difficult.

b. Physiological factors such as menses, gender, acne duration and baseline severity influence the lifespan of inflamed lesions [381]. Of course, other studies of this nature would also have been affected by these general patient factors.

5.3.2 Laboratory Investigations

The changes in TLR2, TNFα, TNFR, IL-10 expression in response to IPL irradiation represents a small subgroup of the entire study cohort. Also, the TNFR ANOVA *P* value fell just outside the threshold of significance and IL-10 mRNA expression after IPL irradiation showed a clear increase without statistical import. Thus, an important biological phenomenon may have been missed and conclusions will have to be made cautiously. A number of factors may have contributed to this:

1. The difficulties of extracting high quality RNA from complete sets of 3 biopsies per case resulted in small numbers being suitable for molecular evaluation. Hence, the data has very likely suffered from several type II errors (finding no statistical significance when in fact there is one). A larger cohort of suitable samples may have uncovered greater consistency between the ANOVA and pair-wise testing p values.

2. The high threshold values (Ct = 35- 40) required to observe any amplification of this study's inflammatory target genes above background for semi-qPCR, qPCR and TLDA demonstrate that these cytokines were expressed in very minute quantities, which is not unusual for clinically normal skin. However, the relative success of the TaqMan® probes, which required a quarter of the cDNA required for SYBR Green® attests to the superiority of this assay and the specificity of the probes despite published literature attesting otherwise [364, 382]. In hindsight, the 63 biopsies assessed using SYBR Green® and Semi-qPCR would have been far better served if they were assessed via TLDA from the outset.

3. It would have been useful to correlate the changes in TLR2, TNFα and TNFR expression in the same subset of patients. However, due to the factors described above, this was not possible.

4. RNA was extracted from whole skin biopsies (this included dermis and subcutis) whereas immunohistochemical assessment of TLR2 expression was confined to the epidermis. Thus, molecular evaluation of a whole skin biopsy may have precluded accurate detection of an epidermal phenomenon accounting for the lack of absolute correlation between the two assays.

5. In this study, the biopsy taken 48 hrs after the 1st irradiation was to give a snapshot of the early biological effect of IPL and the 3rd biopsy taken 7 days after the last irradiation aimed to show the cumulative effect of IPL on acne-prone skin. However, during the intervening time points, inflammatory cytokine and receptor expression may have varied significantly. Measurement at these time points may

have provided a more accurate account of IPL’s effect on acne prone skin. However, this would have required more than 3 biopsies in each patient, making this an unfavourable and unrealistic alternative.

6. Yellow IPL probably has differential effects on various structures within the skin. An alternative approach would have been to determine IPL’s effect on the epidermis, pilosebaceous follicle, dermis and subcutis. Though technically challenging, immunohistochemical identification of these cytokines or microdissection of each structure followed by RNA extraction could have provided these answers.

7. Byun et al.’s study [255] also found that IL-10 expression measured with semi-quantitative PCR did not reveal a statistical difference after yellow IPL. ELISA however, was able to detect a significant increase in IL-10 protein [255]; hence protein quantification rather than mRNA transcript levels may have also yielded significant results in this study.

Chapter 6

DISCUSSION

530nm IPL: A Therapeutic Alternative for Acne Vulgaris

6. 530nm IPL: A Therapeutic Alternative for Acne Vulgaris

6.1 Clinical Study Discussion

The clinical aim of this project was to examine the ability of IPL to cause a discernible change in acne severity by assessing its effect on inflamed and non-inflamed lesion counts, SER and the Leeds scores.

6.1.1 Lesion Counts

The observed 28% decrease in the number of inflamed lesions was significant despite a static Leeds score. The degree of improvement seen in this trial was similar to a blue-light study previously done in this Department (Cardiff University) [52]. Using *Clear Light™ (*Lumenis Ltd., Yokneam, Israel; 407–420 nm; 20 $mWcm^{-2}$ or 75 Jcm^{-2}) twice weekly for 4 weeks, Ammad et al. [52] noted a 26% reduction in inflammatory lesions that was statistically significant. Of note, they also observed a significant reduction in non-inflamed lesions which was not seen in this study. Of course, the two light sources are not directly comparable as they have different action spectra. In a more closely related study, Barikbin and colleagues [383] conducted an open split-face trial in 15 Persian females (Fitzpatrick skin types II – III) with moderate – severe facial acne. They aimed to assess whether the pulse duration (55 ms and 101 ms) of a 572 nm IPL (Kemedical Hair & Skin IPL, λ= 400 – 900 nm, Peak λ= 572 nm, 35 J/cm^2) affected treatment efficacy. Treatments were administered weekly for 5 weeks. Both pulse durations produced a 30% reduction in

inflamed and non-inflamed lesions (p = 0.0003 and p = 0.005 respectively) when assessed at week 5. Though our study did not detect a statistically significant reduction in non-inflammatory lesions, both studies report very similar percentage improvements in inflamed lesion counts, in spite of different treatment sites and baseline severities.

Compared to other studies utilising yellow IPL, this trial is, to some extent, an improvement. In a split face trial by Yeung et al. [10], an Asian cohort was treated with full-face adapalene and half-face 530–750 nm IPL +/- MAL-PDT. Another cohort reported by Chang et al. [14] was treated with full-face benzoyl peroxide and half-face IPL. Neither yellow IPL–adapalene nor yellow IPL-benzoyl peroxide as dual therapy gave any additional improvement when compared to adapalene or benzoyl peroxide alone. The lack of a significant effect after yellow spectrum IPL can be attributed to the co-administration of active therapy or to a sufficiently minute clinical effect of IPL that can be masked by partially efficacious topical agents.

Conversely, Sami et al. [218] and Choi et al. [181] report improvements of 41% and 66% in inflamed lesions, exceeding that noted in this study. Here, patients received a month of weekly 550 -1200 nm IPL sessions and inflamed lesions were counted one month after the final IPL irradiation [218]. Taking their treatment regimes into account, both of these studies report cumulatively higher energy deliveries to the skin either through more frequent treatments (Sami et al.) or higher energies (Choi et al.) than those used in this trial.

6.1.2 Sebum Excretion Rate (SER)

The 35.9% mean reduction in SER was not significant (p = 0.50). Orringer et al. [219] were also unable to objectively find a marked reduction in the SER after yellow light irradiation as was the case for Pollock et al. [238], who used a 532 nm light source with ALA-PDT to treat 30 cm^2 areas on the backs of 10 acne patients. The success of incoherent red light in significantly reducing the SER [333, 384], with or without ALA-PDT, suggests that yellow light's inability to substantially affect the SER may be related to its depth of penetration.

Studies have suggested that the relationship between SER and acne severity is not linear. Sebum production can be strongly influenced by dihydrotestosterone, a metabolite of testosterone through the action of the enzyme 5α reductase (see Chapter 1). Leyden et al. [385] conducted preliminary studies evaluating the sebo-suppressive effects of a 5α-reductase inhibitor (*Compound A*). They found that *Compound A* reduced SER levels by 45% in normal individuals. A follow-on double-blind randomised clinical trial comparing the efficacies of *Compound A* + minocycline to minocycline alone, did not find a statistically significant difference between the two groups [385]. Unfortunately, SER levels were not reported for this portion of the study. Walton et al. [386] measured the SER in 20 pairs of identical and fraternal twins. Interestingly, they found that whilst the SER was equivalent in identical twins (but not in non-identical twins), the severity of their acne did not significantly correlate. Serup [338] also reported that the SER did not correlate with acne severity in his cohort of 24 Danish medical students.

A recent publication from Choi et al. [387] attempted to dispel this view. They measured the facial casual sebum levels using a Sebumeter® (SM815, C+K, Köln, Germany) in 914

Korean acne patients. A significant and positive correlation between sebum casual levels and lesion counts (inflamed and non-inflamed) was found. Though significant, the *r* values for inflamed and non-inflamed lesions when measured in the U-zone of the face were $r = 0.120$ and $r = 0.079$ respectively. This study was relatively large suggesting that it was sufficiently powered to detect a small but statistically significant difference. Pearson's correlation coefficients (*r* values) range between +1 and -1. Thus, even though these results are intuitive, *r* values less than $\pm$ 0.20 are not considered to show a relationship above chance and hence are negligible [371]. Hopefully, investigators will continue to study this subject in order to confirm how SER correlates with acne severity.

The authors of a small study (n = 6) published in 1969, Beveridge and Powell [332], proposed that while tetracycline does not affect the sebum secretion rate, it alters the sebum composition, which may then account for the clinical improvement seen in these patients 8 weeks after starting therapy. Powell and Beveridge (1970) [388] published another comparative study, which evaluated the differences in SER and sebum composition between males with and without acne vulgaris. As before, they reported that the SER did not differ significantly between the two groups but acne subjects had higher levels of aliphatic alcohols in their sebum [388]. UVA can alter the composition of skin surface lipids by photo-oxidising squalene and producing mono-hydroperoxides, which have an uncertain role in pathogenesis [389]. Thus, in responders, it is possible that yellow IPL may exert its anti-inflammatory effects by altering the sebum composition, but in the absence of definitive studies, this only remains a theory.

These investigations propose a few scenarios about the role of SER in acne pathogenesis and clinical efficacy evaluations. SER, though linked to acne pathogenesis may not be

directly related to acne severity. Other conditions associated with excessive sebum production e.g. Parkinson's disease, are not clinically associated with acne and, despite the same SER levels in identical twins, there is no correlation with acne severity. Rather, sebum composition appears to play a significant role in acne pathogenesis and could be the true target of light and antibiotic therapies. The modulatory properties of diet and the relative 'linoleic acid deficiency' proposed as contributors to the onset of inflammatory acne support this theory. Alternatively, where light therapy is able to induce a reduction in the sebum excretion rate, the accompanying improvement in acne may be a reflection of a reduction in the substrate for bacterial fatty acid production. Studies utilising newer, refined techniques for sebum assessment are required to clarify this debate.

6.1.3 Leeds Score

The Leeds scores of our cohort did not reflect a significant improvement in their global acne severity. As noted with the earlier Leeds technique (1984) [346] and the Leeds Revised Acne Grading System, problems arise when grading patients with large numbers of comedones and isolated lesions. This has probably added a significant amount of variability to these clinical trial results as the assessors were unable to appreciate a change in the depth of the lesions and were not informed of the biopsy scars in order to preserve blinding. In fact, Gibson also encountered this problem when comparing clinical and photographic methods [340].

Nonetheless, where blinded assessments showed a deterioration of 9.4% in the Leeds score, open assessments did not reveal any improvement over the control area (chest), indicating this trial was not significantly disadvantaged by using this technique. It was also observed that patients with higher Leeds scores remained relatively worse. Likewise,

Leheta et al. [390] surmised that patients with milder acne had more appreciable improvement in their acne after six PDL (RegenLite Laser, 585 nm, 350 μs, 7 mm spot, 3 J/cm^2) treatments. When compared to either 25% TCA peels or a topical tretinoin and benzoyl peroxide combination, Leheta et al. concluded that there was no statistical difference between the benefits gained from either modality.

6.1.4 Long-term Efficacy

For any treatment regime, a major concern is the duration of its efficacy. In all the studies that included an extended follow-up (Sami et al. [218], Goldberg et a.l [230], Elman & Lask [240] and Choi et al. [181]), an on-going reduction in inflamed lesion counts 8 weeks after the final treatment was noted. Conversely, Papageorgiou et al. [229] and Santos et al. [391] did not. Though the number of patients followed at the end of this study was too small for statistical testing, 79% of these patients maintained or had an improvement in their Leeds score (open assessment) 4 weeks after the final IPL session, but by the 3rd month 75% of the patients had relapsed.

6.1.5 Therapeutic Endpoints for 530nm IPL

Del Rosso [328] advocates the use of 'efficacy, tolerability and safety' as therapeutic endpoints for acne rather than 'clear' or 'almost clear'. Assessing tolerability and safety, IPL scores highly. The single adverse event in a cohort of 28 patients was a mild skin infection caused by the biopsy procedure and not the IPL treatment. However, regarding the main parameter (efficacy), an average of 40% of patients had an improvement in their open Leeds score, but only 19% improved when assessed blindly (lesion counts fell by 28%). Lee et al.'s letter [392] describes the treatment of 18 patients with facial acne using

a 560 nm IPL and 3% liposomal ALA-PDT. When measured a week after the final treatment, inflammatory lesion counts fell by 36%, representing a minimal improvement on our results particularly because an exogenous photosensitizer was used to augment the effect of the IPL.

Whilst Barikbin et al. [383] quoted similar reductions in lesion counts (30%, p= 0.0003) to that found in this study, Choi et al. [181] and Sami et al. [218] both used yellow spectrum IPL with greater success. They cited reductions in inflamed lesions of 41% ($p < 0.05$) and 55% ($p < 0.05$) respectively. It must be noted however that Sami et al.'s cohort received a mean total of 10 irradiations and lesion counts were measured 1 month after therapy whereas in Choi et al.'s study, lesion counts were assessed 1 week post-therapy. In our study, 79% of patients assessed 1 month after treatment either maintained their improvement or continued to improve, suggesting a sustained cutaneous anti-inflammatory process. Pulsed dye lasers (585 and 595 nm) have wide variations in their efficacies. They have been ineffective in a non-purpuric mode [219], reduced lesion counts by 49% as monotherapy [393] or, boasted efficacies ranging from 38% with ALA-PDT [394] to 84% when combined with a 1,450 nm diode laser [247]. These figures suggest that there may be a ceiling to yellow-spectrum light's efficacy in acne, even when administered at high intensities. However, in combination with other modalities such as heat energy [240], high levels of acne clearance is achievable. If 530 nm IPL is used as an adjunct to conventional therapy, responders may enjoy up to 3 months of remission but also require re-treatment at regular intervals to maintain improvement.

6.2 Laboratory Study Discussion

The laboratory arm of this project aimed to determine whether IPL exerted its photodynamic and photoimmunologic effects through down-regulation of TLR2, IL-8, TNFα, TNFR and up-regulation of IL-10. At the end of therapy, there was a small (2.6%, $p < 0.001$) but significant decrease in TLR2 expression accompanied by significant reductions (17.6%, $p = 0.006$ and 37.6%, $p = 0.017$) in TNFα and TNFR expression respectively. The 97% rise in IL-10 expression was sustained in B2 and B3 but this was not significant. SYBR green data from another subset of patients also showed a 2-fold but insignificant increase in IL-10 expression at the end of therapy.

The increase in mean TLR2 expression in biopsy 2 juxtaposed to the lack of a corresponding peak in TNFα expression would suggest that TNFα suppression was not a direct result of IPL's effect on TLR2 signalling. Thus, considering the original hypotheses, the ability of IPL (at 530 nm) to reduce inflammatory acne lesions is probably through a photo-immunomodulatory rather than a photodynamic mechanism. The correlation between percentage changes in lesion counts and TNFα ($R^2 = 0.253$) but not TLR2 expression, suggests that this theory may be plausible. IL-8 is a cytokine regularly blamed for the main inflammatory events noted in acne but it was not significantly affected by IPL treatment. This observation offers one potential reason for the nominal efficacy of IPL (at 530 nm) in inflammatory acne and highlights, within the scope of these results, that IPL does not produce blanket immunosuppression. The findings are discussed in more detail below, followed by a modified hypothesis for the mechanism of action of IPL and suggestions for future study.

6.2.1 Effect of 530 nm IPL on TLR2 Expression

6.2.1.1 Variation in TLR2 Expression between Individuals

The mean effect of IPL was to reduce TLR2 expression but across the entire cohort there were variations in response. Koreck et al. [395] looked at four loss-of-function mutations in the TLR2 and TLR4 genes in 63 Caucasian patients with acne and 38 without. In 101 of these patients, they found no difference between the frequencies of these mutations in these two groups, neither could they link the presence or absence of the mutation with clinical severity. No mention is made of statistical advice prior to this study, so the sample may have been too small to pronounce a verdict on the entire population of acne sufferers. However, they did admit that other unidentified polymorphisms may be responsible for the difference between subjects with acne and those without [395].

As highlighted in Chapter 3, numerous molecules and sub-pathways are involved in TLR2 signalling. Variation in any of the proteins within these complex pathways can explain the variability in TLR2 expression after IPL irradiation noted in this study. Where warranted, step-wise investigation of the pathway in suitable models may elucidate other sources of polymorphism within the population.

6.2.1.2 TLR2 Up-regulation

At the 48-hour juncture, TLR2 expression was modestly but significantly increased in our study cohort. Is this because IPL is pro-inflammatory or does it generate TLR2 ligands from photodynamically destroyed *P. acnes*? Does IPL uniformly cause an elevation in TLR2 expression at the same time point after each treatment and of similar magnitude?

Complete explanations for many of these questions remain undefined but probable answers can be deduced from evidence currently available in the literature.

6.2.1.3 TLR2 Expression is Inducible – Photothermal Actions of IPL

It has been previously shown *in vitro* that the consequence of TLR2 stimulation is a reciprocal up-regulation in its expression. An et al. [396] incubated mouse dendritic cells with lipopolysaccharides isolated from *E. coli* strains and they observed that TLR2 as well as TLR4 and TLR9 expression were increased when measured 2 hours after exposure to LPS. This up-regulation was short lived as its expression started to fall 3 hours after exposure, returning to baseline levels 6 hours after LPS exposure [396]. Chang et al. [397] performed similar experiments using a human cervical epithelium cell line (HeLa) and *Trichomonas vaginalis* as the ligand. TLR2 expression was up-regulated after exposure to *T. vaginalis*. In both studies, co-incubation of their chosen cell lines with NF-κB (pyrrolidinecarbodithoic, PDTC) and MAPK (PD98059, SB203580) inhibitors successfully suppressed TLR2 expression [396, 397].

These observations suggest that TLR2 is inducible, through both NF-κB and MAPK pathways. This induction is usually followed by a down-regulation of its expression at a later time point. Hence our *in vivo* finding of TLR2 up-regulation followed by down-regulation at a later time point after IPL irradiation is in keeping with the literature. But, a challenge remains in defining how 530nm IPL might be generating a TLR2 ligand. One likely explanation is IPL photothermal induction of heat shock proteins. HSP60 and HSP70 are both able to activate the Toll/IL-1 receptor signalling pathway in PBMCs and murine macrophages [398-400] so their up-regulation may explain this effect.

6.2.1.4 TLR2 Down-regulation without IL-10 Up-regulation

When assessed one week after the fourth and final IPL irradiation, TLR2 epidermal expression was 2.6% ($p < 0.001$) below that measured at baseline, which was surprisingly significant in spite of the small margin. So, is this reduction biologically noteworthy and is it related to the reduction in inflammatory lesions?

In the absence of a statistically significant change in IL-10 mRNA expression, it is difficult to attribute TLR2 and TNFα suppression to IL-10 up-regulation. Equally, if TLR2's pro-inflammatory pathway was activated by 530 nm IPL, the increased TLR2 expression in B2 should have been accompanied by a similar increase in IL-8 and TNFα expression but TNFα expression remained stable in B2.

Studies involving IL-10 protein assays after UV light irradiation have described an increase in IL-10 expression [41, 401]. Barr et al. [401] exposed 71 patients with skin types I and II to simulated solar radiation. Protein assays of suction blister fluid taken at different time intervals revealed significantly up-regulated IL-10 levels at the 15 hour mark coinciding with increases in soluble TNFR, a negative regulator of TNFα signalling [401]. Whilst it is understood that the mechanism of action of UV light differs from 530 nm pulsed light, the expected sequence of IL-10 up-regulation, accompanied by transient TNFα up-regulation followed by a significant down regulation of TNFα does not occur in our study. Whilst, the small numbers involved in this study may have impacted on the lack of statistical significance obtained for IL-10 expression in this study, it is also possible that another mechanism, not identified by these methods, may be responsible for the observed anti-inflammatory response.

6.2.2 IPL's Effect on TNFα Expression

In this study cohort, TNFα expression fell by 17.6% ($p = 0.006$) when measured a week after the final IPL session. This was accompanied by a down-regulation of TNFR by 37.6% ($p = 0.017$) in the same cohort of patients. Byun et al. [255] exposed HaCaT cells to 555-950 nm IPL and found that 48 hours after exposure, TNFα mRNA levels did not increase significantly. Thus, the absence of a reciprocal rise in TNFα expression after exposure to IPL in biopsy 2 is in keeping with their *in vitro* findings.

On the other hand, Byun et al. [255] also reported an increase in TNFα protein expression as measured by ELISA as well as an increase in IL-10 protein. Thus, clarification of changes in TNFα levels in biopsy 2 could be achieved by extending the experiment to include quantification of protein expression through ELISA or western blotting. If this showed an increase in TNFα protein expression, patients may experience an initial worsening of their acne prior to the later improvement. This study did not evaluate interim lesion counts, Leeds scores or record subjective assessments of their acne severity and hence this may be an avenue for future study. Alternatively, TNFα's static expression could be multifaceted where IPL activates both stimulatory (via TLR2) and inhibitory (via IL-10 and TGFβ1) signalling cascades.

Yellow IPL as an anti-TNF agent, may have potential to prevent and/or reduce acne scarring caused by MMP release in addition to its action against inflammation. Any anti-sebocytic effect is a more distant possibility as we, along with previous investigators, did not detect a significant reduction in SER.

6.2.3 Transforming Growth Factor-β as the Unidentified Anti-inflammatory Mediator

Earlier (see Chapter 3), TGFβ was identified as a potential IPL-modifiable target. TGFβ is a well-known immuno-regulatory molecule implicated in the modulation of TNFα [402], toll-like receptor [403] and heat shock protein [404] signalling pathways. Yellow light sources such as the pulsed-dye laser [13, 405] and IPL [406] have been shown to increase TGFβ expression in the context of acne and photo-rejuvenation. Hence, the results noted in this study could be secondary to IPL's ability to manipulate this molecule.

6.2.3.1 TGFβ and TLR2

The mechanism by which TGFβ1 regulates TLR2 activity and expression is still being studied. TGFβ not only counter-regulates TLR2 by stimulating MyD88 degradation [407] but may also be pro-inflammatory [408].

Mikami et al. [403] conducted a series of *in vitro* and *in vivo* experiments in a human cervical cell line (HeLa) and BALB/*c* mouse tissues in an attempt to determine whether TGFβ receptor stimulation also regulated TLR2 expression. They found that TGFβ1 positively regulated TLR2 expression via two mechanisms: (i) TGFβ receptor I/ II (TGFβR I/II) signalling through Smad3 and Smad4 stimulating the NF-κB pathway and (ii) TGFβR signalling suppressing p-38 MAPK signalling, which in turn normally acts by suppressing TLR2 transcription [403]. Therefore, 530nm IPL could also indirectly up-regulate TLR2 expression by initially inducing TGFβ1 [403] accounting for the rise in TLR2 mRNA noted in biopsy 2.

Unfortunately, effects of TGFβ on TLR2 signalling is far from simple as it depends upon the context within which it was triggered and the cell type. In odontoblasts, TGFβ1 suppresses TLR2 expression [409] and in endometrial tissue, TGFβ1 inhibits neutrophil degranulation stimulated by TLR ligand binding [410]. Sumiyoshi et al. [402] tried to determine whether TNFα and IFNγ's pro-inflammatory effects in Th2 cells could be antagonised by TGFβ1 supplementation. They found that TGFβ1 was able to suppress pro-inflammatory cytokine production, especially via Smad3 signalling TGFβ's stimulatory effect on TLR2 expression through p38 MAPK inhibition in cervical epithelial cells as described by Mikami et al. [403] also contributes to the confusion, as TGFβ seems to antagonise its own up-regulation. In macrophages, TLR2-dependent IL-10 transcription is activated via the p38 MAPK pathway [318] and IL-10 can increase TβRII expression on activated T-cells enhancing their responsiveness to TGFβ [411]. Thus, comparisons between TGFβ publications have to be restricted to similar cell types to reduce ambiguity in their interpretation.

6.2.3.2 TGFβ, Heat Shock Proteins and IPL's Anti-inflammatory Action

Using the skin biopsies from this study, a colleague has been examining the effect of IPL on all three isoforms of TGFβ (TGFβ1, 2 and 3) and their signalling molecules, Smad1, 2 and 3 [412]. Thus far, in agreement with the literature [402], Mohammad Ali et al. [412, 413] have found that 530 nm IPL significantly increased TGFβ1 via Smad3 signalling but appears to have no effect on TGFβ2 or TGFβ3. This up-regulation of TGFβ1 could be through a direct mechanism or indirectly via HSP60 induction, based on studies by Zanin-Zhorov et al. [404]. In their experiments, $CD4^{+}$ $CD25^{+}$ T-reg cells were cultured with HSP60, which activated the TLR2 signalling pathway within the T-reg cells, stimulating the release of TGFβ and IL-10. Dermal fibroblasts isolated from human foreskin

irradiated with 560 – 1200 nm IPL secreted more TGFβ1 (measured 48 hours after irradiation) at higher IPL fluence (72 J/cm^2) than at 27 J/cm^2. Through western blotting, it was observed that steadily increasing fluence caused significant inhibition of MAPK phosphorylation [414]. This corresponded with the increase in TGFβ1 secretion at higher fluence.

In a study by Huang et al. [414], use of a MAPK/JNK inhibitor (SP600125) resulted in a 35.6% reduction in TGFβ1 secretion and a 3-fold increase in MMP-1 secretion whereas, use of a p38-MAPK inhibitor (SB203580) caused a 2.6 fold increase ($p < 0.05$) in TGFβ1 secretion[414]. Overall, these results suggest that activation of the p38-MAPK pathway by IPL irradiation is partially responsible for increased TGFβ1 secretion [415].

Our results demonstrated up-regulation of TLR2 48 hours after IPL irradiation at an average fluence of 40 J/cm^2. Hence IPL may preferentially activate the TLR2 – MAPK signalling pathway rather than the TLR2 – NF-κB pathway leading to TGFβ1 up-regulation. This theory may account for the lack of a significant increase in inflammatory cytokine mRNA transcripts despite an increase in TLR2 expression.

6.2.4 Summary

Based on the arguments made above, the laboratory findings from this study may be explained as follows:

1. The initial up-regulation of TLR2 could be a result of IPL's ability to induce HSP 60, HSP70 and possibly other HSPs. The transient peak may represent the natural history of TLR2 expression when induced by light irradiation.

2. TLR2 induction does not seem to be related to photodynamic destruction of *P. acnes* but objective studies would be required to confirm this.

3. The down-regulation of TLR2 noted 7 days after the fourth and final IPL session is not associated with IPL's anti-inflammatory activity.

4. The absence of IL-10 induction could be due to the chosen technique used to detect its up-regulation. Nonetheless, the absence of meaningful changes in IL-10 expression could be one of the underlying mechanisms for the limited efficacy noted in this trial.

5. Initial stability in TNFα mRNA levels could be artificial and may be associated with increased protein expression without an increase in transcription. Alternatively, the observed stability could be secondary to simultaneous activation by TLR2 and suppression by TGFβ1.

6. TGFβ1 has pleiotropic properties. Based on the quoted studies, TGFβ has been shown to induce and suppress TLR2 expression. In complementary but separate studies on this cohort, TGFβ1 is significantly up-regulated. The observed increase in TGFβ1 post-IPL is the most probable candidate for the observed reduction in TLR2 and TNFα expression at the end of therapy.

7. IPL's ability to suppress TNFα transcription may be the reason for the reduction in inflamed lesions. Through further investigation, IPL parameters may be manipulated to improve seborrhoea and scarring by extending its anti-TNFα properties.

6.2.5 IPL's Mechanism of Action: An Evolving Hypothesis

These results show that 530 nm IPL irradiation may significantly reduce TNFα and TLR2 expression in an acne patient. The molecules inciting these changes were not identified in the experiments outlined but are theorised to involve HSPs and TGFβ1, thus an updated view of IPL's theorised mechanism of action is given below. It is described in three stages, similar to the description of the evolution of an inflammatory acne papule in Chapter 1.

6.2.5.1 Initiation

In an acne prone patient, IPL irradiation of the epidermis leads to induction of heat shock proteins in the keratinocytes and dendritic cells. These HSPs bind to TLR2, already up-regulated in response to *P. acnes* moieties. HSP binding to TLR2 activates both the NF-κB and MAPK pathways, especially the p38 MAPK pathway. The p38 MAPK pathway is the preferred route for TLR2's anti-inflammatory activity inducing TGFβ1 and IL-10 secretion (though not shown to be significantly up-regulated). This might explain the suppression of the expected TNFα up-regulation in response to TLR2 activation. HSPs may also induce TGFβ1 secretion directly through an undefined mechanism.

6.2.5.2 Progression

Within 4 - 48 hours, HSP activation of the TLR2-MAPK signalling pathway results in a positive feedback mechanism, further increasing TLR2 expression – protecting the skin against further injury. At this juncture, TGFβ1 protein expression is maximal within the epidermis and Langerhans cells. TGFβ1 binds to its receptor activating the Smad3 and MAPK pathways. Through the Smad pathway, NF-κB, the pro-inflammatory arm of TLR2 signalling is inhibited. A possible route is through binding of Smad3 to the AP-1 promoter

region, a transcription factor shared by TGFβ, TLR2 and TNFα. Simultaneous stimulation the NF-κB pathway maintains TNFα transcription, reflected as stable expression levels. However, HSPs also stimulate the JNK-MAPK pathway, limiting TGFβ induction. This results in an attenuation of TGFβ1's anti-inflammatory properties. But, where TGFβ induction is sufficiently high and TNFα inhibition is maximal, inflamed acne papules are pushed towards their resolution phase clinically manifested as a reduction in inflamed lesions. Several treatments are required to achieve this effect with a suitable treatment-free period in between to prevent significant tissue injury and a pro-inflammatory, pro-apoptotic response.

6.2.5.3 Resolution

Days after irradiation, TLR2 and TGFβ signalling is abolished through de-phosphorylation of the kinases within their pathways, proteasomal degradation of activated factors and removal of their transcription factors including p38 MAPK, Smad3 and NF-κB from the nucleus. Thus, TLR2 and TGFβ1 expression return to baseline levels. Suppression of the inflammatory cascade within keratinocytes and other inflammatory cells, results in a sustained reduction of TNFα transcription and its receptor, TNFR.

The modified hypothesis is summarised in **Figure 6.1**. The details of the signalling pathways, NF-κB, MAPK and Smad, are omitted for clarity.

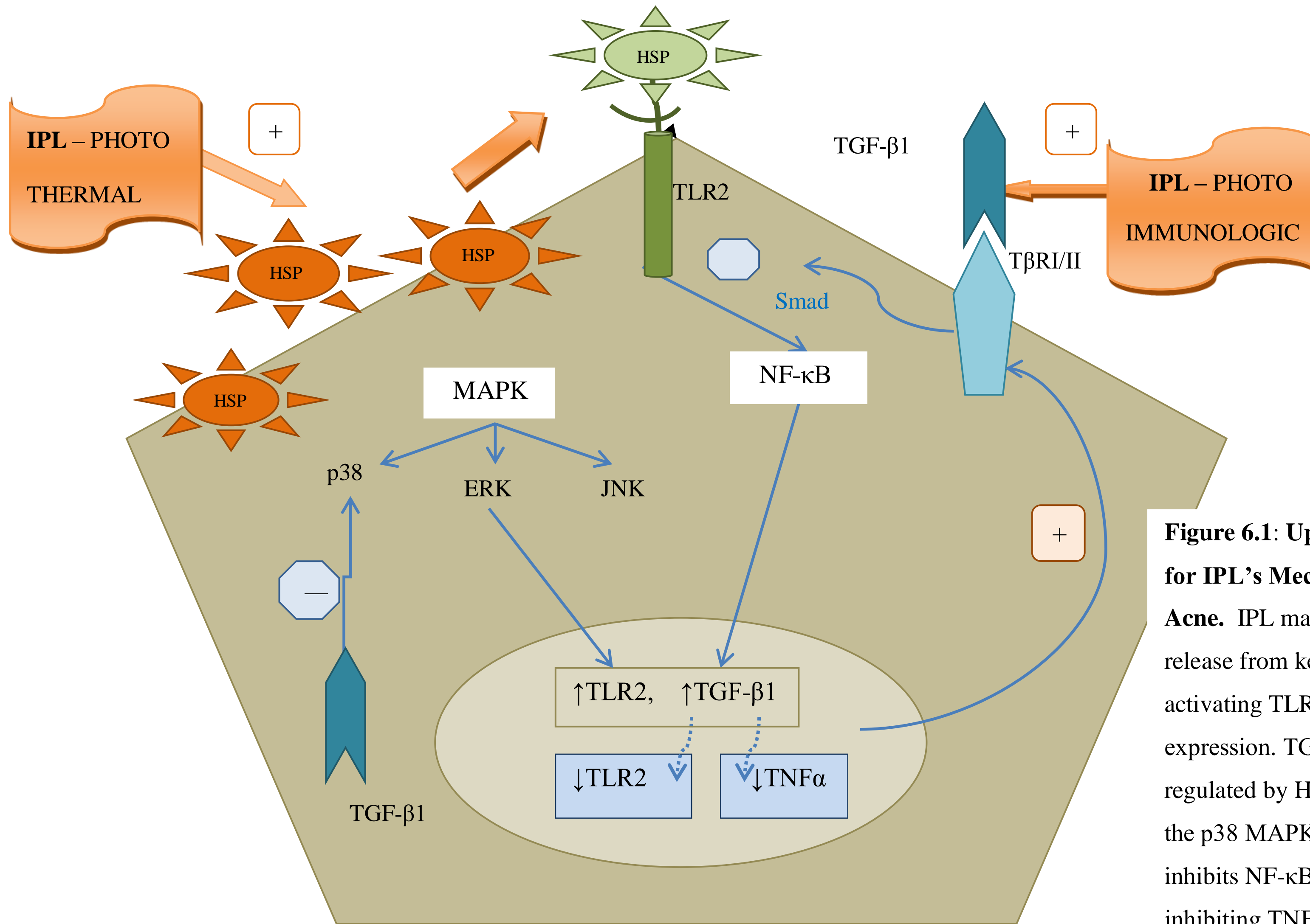


Figure 6.1: **Updated Hypotheses for IPL's Mechanism of Action in Acne.** IPL may stimulate HSP release from keratinocytes, thus activating TLR2 signalling and TLR2 expression. TGFβ1 is also up-regulated by HSPs primarily through the p38 MAPKs. TGFβ1 via Smad3, inhibits NF-κB nuclear localisation, inhibiting TNFα expression. Through p38 MAPK, TGFβ1 later suppresses TLR2 expression.

6.3 Future Studies

Although this study only showed partial benefit from yellow IPL, further investigation into the use of IPL as an anti-acne agent is warranted. Any future studies should aim to clarify the questions raised by this project, especially with respect to the IPL's hypothesised mechanisms of action as suggested below:

1. Lee et al.'s study [392] suggests that the addition of a photosensitizer augments the efficacy of 560 nm IPL. Thus, an extension to this study would be to determine whether the addition of a photosensitizer (e.g. 5-ALA, liposomal ALA or MAL) would increase the efficacy 530 nm IPL.

2. The possibility of IPL altering sebum composition was discussed. Assessment would require simultaneous quantitative and qualitative sebum analysis which is possible using the Sebutape® method [146, 389, 416]. Robosky et al. [417] promoted the use of nuclear magnetic resonance imaging in favour of high performance liquid chromatography for sebum composition analysis due to its higher sensitivity.

3. There was doubt whether IPL was able to photodynamically destroy *P. acnes*. Therefore, measuring the effect of 530 nm IPL on *P. acnes* using methods such as fluorescence photography [239, 418] and bacterial colony counts. Currently, there is some controversy regarding whether the follicular orange-red fluorescence observed represents a sebaceous component or coproporphyrin products from *P.*

acnes [68, 419]. This uncertainty advocates a heavier reliance on reproducible high-yield techniques to isolate *P. acnes* for colony counting.

4. Laboratory studies showed that TLR2 was initially up-regulated without a significant change in IL-10 or IL-8 cytokines. However, TLR2 and cytokine expression were measured using two different techniques. Thus, the inability to confirm the absence of IL-10 up-regulation and IL-8 down-regulation at the protein level, dictates that quantification of IL-10 and IL-8 protein expression in response to IPL irradiation is paramount.

5. If these cytokines are significantly altered, then IPL-induced reduction in TLR2 expression may have a greater impact on acne resolution than illustrated in these experiments. Later, possibly through *in vitro* block-and-replacement studies, using inhibitors of the TLR2 pathway may help to clarify whether IPL's modulation of the TLR2 pathway is essential for its anti-inflammatory activity. If it is involved, is it via the NF-κB and/or the MAPK signalling pathways? The updated hypothesis proposes that the MAPK pathway is more heavily involved in IPL's anti-inflammatory activity. Use of specific NF-κB and MAPK inhibitors could help to locate its preferred signalling pathway i.e. IKK, p38, JNK or ERK.

6. In a similar manner, strong circumstantial evidence suggests that TGFβ1 up-regulation is crucial for IPL's anti-inflammatory actions, but this remains unproven as TGFβ1 up-regulation may be contributing solely to its mechanism of action in photo-rejuvenation. Studies confirming the dependence of IPL's anti-inflammatory actions (in acne) on TGFβ1, possibly through the use of TGFβ signalling inhibitors,

measurement of downstream cytokines, Smad nuclear localisation and acne models, would aid in this clarifying this question.

7. Heat shock proteins were hypothesised to be initiators of IPL's anti-inflammatory response through the MAPK pathway. Thus, studies to confirm whether heat shock proteins are induced by IPL in acne patients would be useful. Following clarification, do these HSPs have a direct role in regulating TLR2, TNFα and TGFβ1 expression in acne? Does this extend to the control of TNFR and TGFβ receptor expression? These molecules have already been studied in acne patients, and indeed, in our study cohort. Therefore, immunohistochemical techniques, western blotting and DNA-shift assays (to assess nuclear translocation) are techniques that could be used to address these questions.

8. Isotretinoin can inhibit TNFα expression in cultured sebocytes [304]. In this study, IPL was shown to have anti-TNFα properties, and caused a 35% reduction ($p > 0.05$) in the SER. Thus, it would be interesting to determine whether IPL also reduces TNFα expression in sebaceous glands.

6.4 530nm IPL's Fate in Acne Management

Inevitably, many questions about acne remain. Though unfailingly common, why does acne affect some people and not others? Why does it persist until middle age in a minority of patients? Why do some people develop scars and others don't? Is acne a constellation of diseases rather than a single entity? Why is *P. acnes* so heavily implicated in acne vulgaris in favour of other skin commensals [50, 420]? A reduction in bacterial counts is not directly correlated with treatment efficacy and the development of an attenuated *P. acnes* vaccine [421] has not gained popularity. As shown in this trial and several others [219, 332, 422], a reduction in the sebum excretion rate (SER) is not necessarily associated with a clinical improvement in acne. Even with isotretinoin, SER returns to baseline levels after a few months but the anti-acne effect is still sustained [423]. Androgenic suppression reduces SER, but as a monotherapy it is not very useful in acne [385]. Broad spectrum anti-inflammatories like prednisolone tend to provide only transient relief and encourage acneiform lesions. Even genetic susceptibility provides only partial evidence as identical twins with equivalent SERs can have varying acne severities [386].

In light of the partial successes in treating acne, 530 nm IPL was proposed as a possible therapeutic alternative, sufficiently unexplored to warrant further investigation into its likely molecular mechanisms of action. In this thesis, the laboratory studies described suggest that IPL is anti-inflammatory as it was able to down-regulate TLR2 and TNFα expression. However, we also observed counter-productive properties of IPL such as an inability to suppress IL-8 expression and significantly induce IL-10. The absence of a significant involvement of these two cytokines suggests that other signalling pathways are responsible for the observed effects on TNFα and TLR2. Heat shock proteins and TGFβ1 were offered as reasonable candidates that primarily utilised the anti-inflammatory MAP

kinase pathways rather than the pro-inflammatory NF-κB pathways. Their signalling pathways are intertwined, complex and not likely to be clarified in a single set of simple experiments. Thus, each stage probably needs to be re-examined separately to assess their relevance and importance in IPL's photo-immunomodulatory functions.

Considering the implications of translational medicine's 'bench-to-bedside' ethos, the physician and the patient are primarily concerned about IPL's clinical efficacy. Here, it seems reasonable to conclude that the modest improvements seen with yellow IPL as monotherapy reflect that it is only marginally helpful for inflammatory acne. Webster's commentary [424] on a randomised trial assessing the efficacy of PDL in acne [393] expressed concern that the observed clinical improvement, though not insignificant, would disappoint many patients [424]. Likewise, it has not been demonstrated that yellow IPL is a viable treatment option as monotherapy. If presented as an alternative to patients, suitable candidates should have mild inflammatory acne, have exhausted other conventional forms of treatment and/or use it in combination with an accepted anti-acne agent. Thus far, advice about long-term remission will have to be confined to a maximum of 3 months until further studies are done to confirm or disprove this. Unlike UVA and UVB [169, 425], no long-term sequelae from the repeated irradiations with IPL or other visible light sources have been reported. Nonetheless, the frequency of repeated therapy should be undertaken carefully with a clear management strategy and a defined end-point.

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Appendices

Appendix 1

- Patient Information Sheet
- Consent Form
- Advertisement
- Online Questionnaire

Patient Information Sheet

<u>An analysis of the action of intense pulsed light on inflammatory markers and sebaceous gland function in mild to moderate acne vulgaris lesions</u>

Dear Sir or Madam,

You are being invited to take part in a research study to find out how intense pulsed light (IPL) clears 'spots' (called 'acne'). Before you decide, it is important for you to understand why the research is being done and what it will involve. Take the time to read the following information carefully and discuss it with others such as your family or family doctor. If you wish, Dr. Marisa Taylor & Sister Anne Thomas, who are both involved in this project, would be happy to speak to you. Their telephone numbers and email addresses are written on the last page of this leaflet.

Thank you for taking the time to read this.

What is Intense Pulsed Light (IPL)?

The visible or 'white' light that we see consists of all the colours of the rainbow. Laser machines produce only one colour of light. Intense Pulsed Light (IPL) is NOT a laser machine. Its light is made up of different colours or shades of the same colour of light. This is referred to as being 'broad-spectrum'. This light is produced in short bursts or pulses hence the term "intense pulsed light''.

IPL acts on (i.e. 'targets') specific things in the skin like pigments, chemicals and collagen. Depending on the machine settings, IPL can target the dark pigment found in hair, and hence it is useful in hair removal. It can also target the red pigment in blood and therefore can help to reduce the appearance of red spots and veins on the skin.

IPL machines can give out blue, green, yellow and red light. The machine's settings can be changed to produce only the colours that are most beneficial to you. In acne, blue light acts on chemicals called porphyrins. These porphyrins are made by the bacteria that cause acne. Through blue light's action of these porphyrins, the bacteria are either weakened or killed. Red light can go deeper in the skin than blue light, and in some studies it has been shown to reduce the number and size of the oil-producing glands. These oil-glands are also involved in causing acne.

It is important to note that unlike sunlight, IPL machines have filters that remove ultraviolet (UV) light. Therefore the bad effects of UV light that cause skin wrinkling and cancer are removed.

What is the purpose of the study?

Over the past few years, doctors, mostly in Europe and the United States, have said that light therapy is helpful in treating 'spots' - which are also called 'acne'. Also, studies looking at the skin cells affected by acne have said that there are high levels of substances within and around the cells that cause the redness, pain and oiliness associated with acne. However, there is not much known about *how* IPL acts on these substances to bring about the changes that we see on the skin.

Why have I been chosen?

You have been chosen because you have been identified by your doctor as having acne on your back. Or, because you answered our advertisement, we have sent more information to you about this project.

Do I have to take part in the study?

Participation in this study is entirely up to you. If you decide to take part, you will be given this information sheet to keep and asked to sign a consent form. However, if for any reason, you would like to withdraw from the study, after agreeing to join, you are free to do so at any time. Withdrawal will not affect your standard of care or ability to receive treatment from any hospital or your GP.

Reports from this study will not contain any personally identifiable information about you, so it will remain completely confidential.

What will happen to me during this study?

We expect you to be involved in this study for no longer than 9 weeks.

The light (IPL) treatments may cause a mild stinging or burning sensation. Some say that it feels similar to the sensation of an elastic band being snapped against the skin. During the treatment, we will place a cooling gel on skin that is going to be treated before giving the light. This should help to reduce any discomfort you may experience. Only your back will be treated.

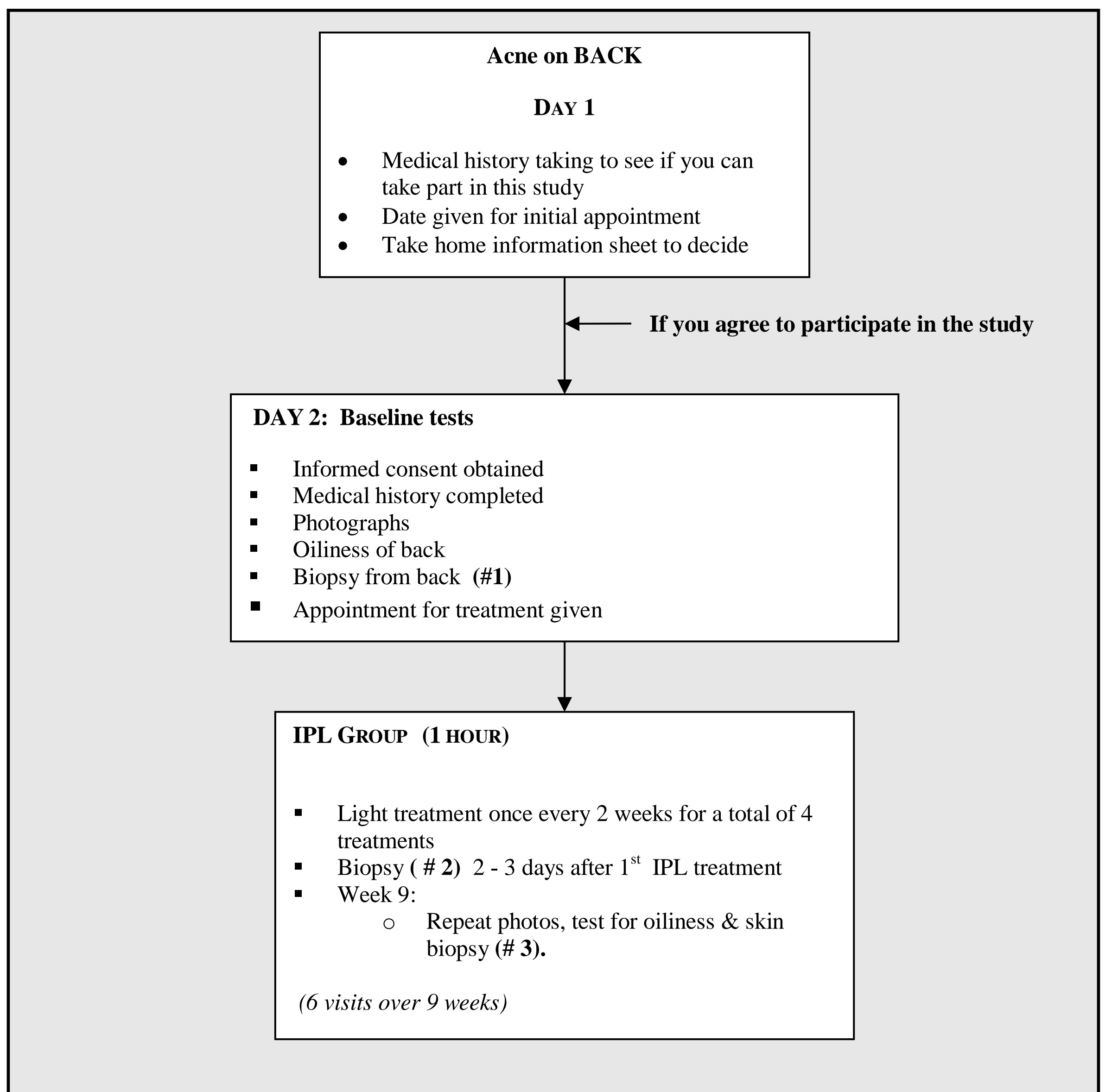
Your acne or 'spots' are caused by different cells and chemicals. These cells and chemicals cause blackheads and red and painful bumps to come up. It is possible to see these cells and chemicals under the microscope. We are going to look to see if treating your spots with IPL makes any difference as early as one day after your treatment starts. Also, we will look at these cells again at the end of treatment.

A small sample of skin, called a 'biopsy', will be taken from your back before, during and after your IPL treatments. The area will be fully numbed before the biopsies are taken and dressed. This procedure is usually quick and almost pain-free. You should not have any pain after the pain-killer has worn off.

After it has fully healed, you may find it has left a small scar that does not tan. Please note that 3 biopsies will be taken over the duration of this study.

There is a simple diagram and notes below to help us explain to you in what order

things will happen and how long they are expected to take.



What do I have to do?

Please protect your back from getting tanned while you are helping us in this study. You can do this easily by wearing a shirt that covers your back during the day time and staying out of direct sunlight when possible. Extra measures include wearing a sunscreen formula that says 'non-comedogenic' and has a sun protection factor (SPF) at least 15 to your back before going out in the sun.

Being in the sun or on a sunbed causes your skin to produce more pigment, which we call a 'tan'. Also, the pigments in sun-tanning lotions leave extra pigment in your skin. As said before, IPL works by 'targeting' pigments in and on the skin. Hence, IPL treatment of your skin while you have a deep tan *may* result in much worse side-effects e.g. a bad 'sun-burn' effect, blisters, darkening or lightening of the skin that was treated. Therefore, please do not sunbathe while you are helping us in this study, even if you are wearing a sun protection cream.

Certain medications may cause you to be more sensitive to light, which can affect your response to the treatments used in this study. Therefore, please make us aware of any medications or herbal supplements that you are currently taking and any that you might be prescribed during the course of your treatments

What do I have to do after the biopsy has been taken?

After your biopsies are taken, please keep the dressing on and keep the area dry for 24 hours. A separate information sheet about how to care of your biopsy site will given to you. Sometimes a stitch may be needed after the biopsy is taken. If you do get one, please have it removed in 7 to 10 days at your GP's office.

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What are the alternatives for treatment?

There are many treatments that are now available from your local pharmacy or through your doctor. If you are using creams such as benzoyl peroxide, azelaic acid, nicotinamide on your face for your spots you may continue to do so. If you are on tablets for your acne e.g. minocycline we will ask you to wait until these have worn out of your system before entering you into this study.

Please do not use any medication or medicated soaps on your back, unless we have prescribed it for you. If you are given treatment for your skin e.g. steroids or antibiotics to be taken by mouth by another doctor, please tell us about it as soon as possible. This is so that we can decide if it affects your IPL treatments. We may be able to suggest a suitable alternative for that medication while you are helping in this study.

More detailed information on acne and its treatment is available on the British Skin Foundation's website at: http://www.britishskinfoundation.org.uk/standard.aspx?id=208

What are the possible benefits of taking part?

Your acne may get better. IPL is also used to help sun-damaged skin appear younger and more even-toned, with a smoother texture. Therefore, you might or might not receive some of these benefits. Also, it is hoped that results from this study will help us to better understand the benefits (or otherwise) of this type of treatment. Therefore in the future, you will help doctors and patients to decide if this is a good treatment option for them based on our findings.

You will not be expected to pay for the special investigations required for this study. Your IPL treatments will be provided at no cost to you throughout the period of the study. You will be paid a flat fee to cover the cost of transportation to see us.

You will be given a form to complete for our records. This money will be paid to you by the end of study.

What are the side-effects of any treatment received when taking part?

For IPL, many patients experience little more than warmth, burning or tingling during treatment. Other effects include mild to moderate pain, redness and mild swelling of the area for up to a week. Especially in darker-skinned individuals, temporary darkening of the treated area has been noted. Uncommonly, some have said that their acne worsened and the area became infected.

The biopsy may be painful, get infected or heal with an enlarged scar ('keloid'). However, if the area is taken care of properly, these complications are unusual. If you have a tendency to get keloids, please let us know as it would not be in your best interests to participate in this study.

Contact names and numbers are listed below if you are concerned in any way or have an emergency related to this study.

What if I get pregnant or is there anything else I should know?

If you are a female of child-bearing age, you may be asked to have a pregnancy test before taking part to exclude the possibility of pregnancy. Women who can become pregnant must consistently use effective birth control during the course of their treatment. Please use methods such as condoms or a non-hormonal intrauterine device, rather than hormonal

methods such as the pill or implants beneath the skin, since the hormones in them can affect the results of this study.

It is very unlikely that IPL treatments are harmful to the unborn child, but if you become pregnant while in this study, please inform us as soon as possible using the contact information below. Likewise, it is highly unlikely that this treatment will have any negative effect on a man's sperm causing damage to a foetus.

Due to the non-invasive nature of this treatment, it should not affect your life or private medical insurance. However, you should check with your provider to make sure that assisting us with this study does not affect your coverage.
If we discover a condition of which you were previously unaware, we will inform your GP and re-assess your ability to participate in this study.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment being studied. If this affects the study, your research doctor will tell you about it and discuss with you whether you can/ want to continue in the study. If you decide to continue in the study, you will be asked to sign an updated consent form.

What happens when the research study stops?

Unfortunately, IPL is offered in Europe and the United States for acne, but is not yet available on the NHS in the UK. Therefore it is not available to public patients on the NHS. If you require further help for your acne after the end of the study, please return to your usual GP or dermatologist.

What if something goes wrong?

If you have a complaint about the way you have been dealt with by a member of staff, please inform the investigators listed below. Your complaint will be investigated and dealt with according to standard disciplinary proceedings.

In the event of an emergency or other concerns related to this study, please use the contact details below.

If you are harmed by taking part in this project, there are no special compensation arrangements. If you are harmed by someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated

during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?

All the information that is collected about you during the course of this research will be kept strictly confidential.

Only staff members directly involved in your care will have access to it and these will be secured in a locked room within the department. Your files will be identified only by your study number and gender so that you cannot be recognised from it. Your GP would probably like to know about your participation in this study, but we will inform them only if you give us your permission for this to be done and your GPs contact information.

What about my photographs?
These will be stored on a secure laptop owned by Cardiff University, kept in the Dermatology Department that is accessible only to the members of the study team. A back-up copy will also be held on the University Hospital of Wales' FotoWeb site in a password protected area – this means that only special members of the study team will have a password allowing them to access this site. Of course, since it is your back, it is very unlikely that anyone would be able to identify you from the picture.

What will happen to the results of this study?
The results of this study will be published in reputable medical journals and in an academic thesis. The results will not contain any personally identifiable information about you.
Due to the nature of this study, we may use your photographs to illustrate the outcomes of your treatment in these publications and for teaching purposes. We will not use them without your permission. Therefore, you will be asked to give your consent for this separately.

Who is organising and funding the research?

Cardiff University, through the Department of Dermatology, is sponsoring and funding this study. Your doctor is not being paid for including you in this study.

Who has reviewed the study?

To ensure your safety and that the highest research standards are being met, the Cardiff & Vale Research & Development Committee and the South East Wales Research Ethics Committee have reviewed this study's protocol and information sheet. They are satisfied that they are in accordance with the latest version of the ethical principles for human research.

Contacts for Further Information

If you have any queries, or experience injuries or adverse events related to this study please contact any of the persons below during working hours:

Professor Andrew Finlay (Principal Investigator)
Work: (0) 2920 742615 Email: FinlayAY@cardiff.ac.uk

Dr. Maria Gonzalez (Academic Supervisor)
Work: (0) 2920 744398 Email: gonzalezml@cardiff.ac.uk

Sister Anne Thomas (Research Sister)
Work: (0) 2920 742672 Email: thomasag1@Cardiff.ac.uk

Dr. Marisa Taylor (Project Coordinator)
Work: (0)2920 745876 Mobile: 07722502563 (for emergencies)
Email: taylorm4@cardiff .ac.uk

Dermatology Department

3rd Floor Glamorgan House

University Hospital of Wales

Heath, Cardiff

CF14 4XN

Fax: (0) 2920 744312

Remember, you will get a copy of this information sheet and consent form to keep. Thank you again for agreeing to help with this study.

Consent Form

CONSENT FORM

CANDIDATE NUMBER			PATIENT D.O.B (dd/mmm/yyyy)								
0											

Title of Project: **An analysis of the action of intense pulsed light on inflammatory markers and sebaceous gland function in mild to moderate acne vulgaris lesions**

Name of Researchers: Prof. Andrew Finlay, Dr. Maria Gonzalez, Dr. Rebecca Porter, Dr. Marisa Taylor

Please initial box

1. I confirm that I have read and understand the information sheet dated April 14, 2008, Version 2 for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason. My legal rights and medical care will not be affected. ☐

3. I understand that sections of any of my medical notes may be looked at by those named above or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

5. I agree to my GP or family doctor being informed that I am taking part in this study. ☐

6. I agree for my photographs to be taken and stored as outlined in this patient information sheet ☐

7. I agree for my photographs to be used in medical publications and for teaching purposes. ☐

Name of Patient	Signature	Date
Name of Person taking consent (if different from researcher)	Signature	Date
Researcher	Signature	Date

FURTHER CONSENT FOR ANALYSIS OF SKIN BIOPSIES

Please initial box

1. I consent to a skin biopsy for the purpose of this research study. ☐

2. I consent to the following tests being done on my biopsy samples. I understand that the results will be used mainly for the purpose of the current study, but my doctors may use these results in another study. ☐

   a. Inflammatory markers & cytokines including but not limited to: MMP-1, IL-8, β-defensin 2 ☐

   b. Sebum markers including but not limited to: Scd, cEPB ☐

   c. Inflammatory cells including but not limited to: neutrophils, macrophages, lymphocytes ☐

3. I consent for any remaining skin samples to be stored securely in the Dermatology Laboratory, University Hospital of Wales, Cardiff for future tests as detailed above or mentioned below. ☐

4. Please initial to tests you consent to on your skin from the list below:

   a. I agree to my sample being used in case more unforeseeable tests are required in future for this research, depending on the results of my current biopsy results e.g. additional inflammatory markers ☐

   b. I would like to be contacted before further tests as mentioned in 4a are done on the stored sample for research purposes. ☐

   c. I consent to further tests being done on the stored sample without being contacted. ☐

   d. I agree that the laboratory can anonymise my stored sample for future research and the monitoring of the quality of the results. I understand that these results may not have any direct benefit in the evaluation of my disease. ☐

____________________	____________________	
Name of Patient	Signature	Date
____________________	____________________	
Name of Person taking consent *(if different from researcher)*	Signature	Date
____________________	____________________	
Researcher	Signature	Date

Advertisement

Do you have a problem with spots (also called ‘acne’) on your back? Do you have problems getting your creams onto your back? Or maybe you’re not keen on using creams and tablets for your spots. Would you like to try something different? If so, you may wish to think about taking part in a study which aims to improve our understanding of how a light treatment, called **intense pulsed light**, works to get rid of spots. Intense pulsed light has been used extensively in Europe and the United States to treat acne, but is not currently available through the National Health Service (NHS).

ALL participants’ backs will be treated with this new light treatment, free of cost. This research project will take place at the Department of Dermatology, University Hospital of Wales.

For more information please call or write to:

Dr. Marisa Taylor
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OR

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Email: ThomasAG1@cardiff.ac.uk

Questions in Online Questionnaire

1. Email address
2. Full Name
3. Address and Postcode
4. Do you have acne on your back?
5. Have you read the patient information sheet?
6. Skin type: How easily do you tan?
    a. I burn and do not tan
    b. I burn first and tan after
    c. I tan easily and burn sometimes
    d. I tan easily and burn rarely
    e. I tan and do not burn
7. Have you had IPL treatments before?
    a. If so, when?
8. Do you have eczema?
    a. Where?
9. Do you have any other medical conditions?

Appendix 2

- Summary of Clinical Studies of Visible Incoherent Light in Acne
- Leeds Revised Acne Grading System (back)
- UHW Medical Photography Protocol for Acne Study
- Leeds Assessment Data (Blinded)
- Excel Sheet with Raw Clinical Data

Summary of Clinical Studies of Visible Incoherent Light in Acne

Table 2: The Use of Blue Light in Acne: A Summary

KEY: * - 'Patient features' listed as: average age, skin type (if given), acne severity (if given), site (if given); § - number completed study / number enrolled (if given); *contrlld* – controlled; *F/U* – follow-up; *INF* – inflammatory; *NI* – non-inflammatory; *n.s.* – not significant; *PIH* – post-inflammatory hyperpigmentation; *R* – randomised; *S/B* – single blind; *S/F* – split face; *tx* – treatment; *uncontrlld* – uncontrolled; *wks* – weeks ↑- increase; ↓ - decrease; ↔ - no change; ♀ - female ; ♂ - male

AUTHOR, REFERENCE	PATIENT FEATURES* N = §	STUDY TYPE	LIGHT SOURCE (S)	IRRADIATION VALUES	REGIME	STUDY DURATION	FINDINGS
BLUE LIGHT							
Kawada et al. *J Dermatol Sci* 2002. **30**: 129-135.	N= 26/30 22 yr 27 ♀, 3 ♂ Mild – mod acne Face &/or back	Open	Clearlight ™ 407- 420 nm	90 mW/cm^2, 20 x 20 cm^2	Bi-weekly x 5 wks	9 weeks	**At week 5:** 6/30 no change or worse acne grade INF: (papules + pustules) mean ↓ 71.3% NI: ↓57.8% Total counts: ↓64% **1 month post-tx in 17/30 patients:** Total counts: ↑6%
Ammad et al. *J Cosmet Dermatol* 2008. **7**: 180-188	N= 21 Mild - Mod Face	Single-blind uncontrlld	Clearlight™ 415 - 425 nm, Peak emission 420 nm	70 -90 W/cm^2 20x 20 cm area Time: 14 min	Bi-weekly x 4 wks	4 weeks	**At week 4:** Acne grade: ↓ 1.64 ± 1.19 to 1.35 ± 1.28 (*p= 0.001) **↓17.7%** INF: 47.71±26.97 to 35.33±28.63 (*p=0.001) **↓26.0%** NI: 23.86 ± 23.42 to 19.43 ± 24.15 (p=0.06) **↓18.6%**

AUTHOR, REFERENCE	PATIENT FEATURES* N = §	STUDY TYPE	LIGHT SOURCE (S)	IRRADIATION VALUES	REGIME	STUDY DURATION	FINDINGS
							DLQI : 6.1± 4.17 to 4.0 ± 3.24 (*p=0.001) *P. acnes* colony count change n.s.
Elman et al. *J Cosmet Laser Ther* 2003. 5: 111-117	3 trials: 10, 13 & 23 recruits	1. S/F dose-response study (10) 2. Full-face open trial (13) 3. S/F double - blind contrlld study (23)	Clearlight™ Narrow band 420 nm	8 -15 min	Bi-weekly x 4 wks	12 weeks	**Study 1, week 4:** no difference between 8 min and 12 min irradiation times **Study 2 (full-face open trial):** Week 4 INF: 77% responded, ↓59% Week 8 INF: 92% responded, ↓81% **Study 3 (S/F blinded trial):** Week 4 INF: mean ↓ 60% and ↓30% for control side. Steady ↓ at wks 2 (59%), 4 (61%) and 8 (53%) after tx.
Omi et al. *J Cosmet Laser Ther* 2004. 6: 156-162	N= 28 28.1 y Burton grades 1-5	Open	Clearlight ™ (410 - 420 nm)	200 mW/cm^2 25 - 30 cm distance	Bi-weekly x 4 wks	4 weeks *3 months* (for 9/20)	**At week 4:** Total lesion count: ↓64.7% ($p < 0.01$) *P. acnes* cultures & PCR levels: ↔ ↓Moisture ($p < 0.001$) & ↑sebum ($p < 0.001$) **2 months post- tx:** 6/20 patients had sustained improvement
Tzung et al. *Photoderm Photoimmunol Photomed* 2004. 20: 266-269.	N= 28/31 21 ± 4 y 18 ♀, 10 ♂ Type III - IV	S/B, S/F controlled	F-36 W/Blue V, Waldmann 420 ± 20 nm	15 cm distance 40 J/cm^2	Bi-weekly x 4 wks	4 wks	**At week 4:** Control side had 10% improvement 'Peak' improvement on tx side = 52%. Most improvement in papules & pustules. ($p < 0.001$) 4 patients worsened Significant worsening of nodulocystic lesions weeks 4 and 8 ($p = 0.026$)

AUTHOR, REFERENCE	PATIENT FEATURES* N = §	STUDY TYPE	LIGHT SOURCE (S)	IRRADIATION VALUES	REGIME	STUDY DURATION	FINDINGS
	Mild -severe						*P. acnes* fluorescence unchanged. Only treated side assessed.
Morton et al. *J Dermatolog Treat* 2005. 16: 219-223	N= 30 18 yr 14♀, 16♂ Mild - mod Face		*Omnilux Blue™* (LED) 409 - 419 nm	48 J/cm^2 x 10 - 20 min 40 mW/cm^2 5 - 10 cm distance	Bi-weekly x 4 wks	12 wks	**2 months post-tx:** INF: ↓60% (p = 0.001) NI: ↑2% (no change)
Gold et al. *J Drugs Dermatol* 2005. 4: 64(67)	N= 18/34 31 yr Types II–VI Mild - mod	R S/B pilot	*Blu-U™ 4170 (12) vs. 1% clindamycin solution (13)*		Bi-weekly x 4 wks x 1000 s Clinda 2x daily	8 wks	**Week 4, 1% Clindamycin vs. Blu-U:** INF: ↓ 30% vs. 36% (n.s.) NI: ↓ 14% vs. 21% (n.s.) In 9/34 who continued follow-up to week 8, changes in counts for either group did not reach significance.
Tremblayet al. *J Cosmet Laser Ther* 2006. 8: 31-33	N= 43/45 26 yrs 31 ♀, 14 ♂ Types I -IV Mild - mod	Open	*Omnilux Blue™* 415 nm	48 J/cm^2 x 20 min	Bi-weekly x 4 – 8 wks	12 weeks	No lesion counts recorded **At 8 weeks:** 20% had complete clearing Mean global score: 2.9 (i.e. 51- 75% improvement), an 8% ↓ from week 4. 10% dissatisfied with outcome 50% "very satisfied" with outcome Comments: ↓skin oiliness
Noborio et al. *Photoderm Photoimmunol Photomed* 2007. 23: 32-34	N= 8/10 29.7 years 8 ♀, 2 ♂ III – IV	Open	*MultiClear™* 405 - 420 nm	Spot size : 73 x 23mm Fluence: 4 J/cm^2 per pulse (6 passes per area)	Once or twice weekly x 12.4 tx (average)	Until 'satisfac-tory results'	80% improved after an avg. of 12.4 tx. Severity scores at enrolment; **2** (1), **3** (1), **4** (6), **6** (2). No change for subject at grades 2 or 3 (drop outs). ↓ to grade 2 for everyone else. Allen & Smith facial acne severity scores

Author, Reference	Patient features* N = §	Study type	Light source (s)	Irradiation values	Regime	Study duration	Findings
	Burton 2 - 4						Burton's scale for acne on body
Kumaresan, M. & Srinivas, C. *Indian J Dermatol* 2010. 55: 370-372	N= 10 4 ♀, 6 ♂ Types IV-VI Michelson: 22.4/ 27	Blinded assessor	*IPL (Vcare Medical Sys, India)* 420 nm cut-off	R face: 5 pulses, 6ms delay L face: auto mode, pulse width 12 ms Fluence: 15 – 21J	Once weekly x 4 weeks	4 weeks	Lesion counts not recorded Michelson Acne Severity Index (*MASI*): Mean improvement: 49.2 – 56.7%. Burst pulse more efficacious than single pulse mode. Both caused statistically sig. improvement in acne (*no P values given*).

Table 3: Red Light and Combination Blue & Red Light in Acne: A Summary

KEY: * - '*Patient features*' listed as: average age, skin type (if given), acne severity (if given), site (if given); § - number completed study / number enrolled (if given); *contrlld* – controlled; *F/U* – follow-up; *INF* – inflammatory; *NI* – non-inflammatory; *n.s.* – not significant; *PIH* – post-inflammatory hyperpigmentation; *R* – randomised; *S/B* – single blind; *S/F* – split face; *tx* – treatment; *uncontrlld* – uncontrolled; *wks* – weeks ↑- increase; ↓ - decrease; ↔ - no change;♀ - female ; ♂ - male

AUTHOR, REFERENCE (YR)	PATIENT FEATURES* N = §	STUDY TYPE	LIGHT SOURCE (S)	IRRADIATION VALUES	REGIME	STUDY DURATION	FINDINGS
RED LIGHT							
Na et al. *Dermatol Surg* 2007. 33: 1228-1233	N = 28/30 23.6 yr 23 ♀, 7 ♂ Mild – mod	R S/B S/F	**Hand held** *SoftLaser SL30* 635- 670 nm	Cumulative dose: 604.8 Jcm^{-2} Irradiance: 6 mW	15 min twice daily x 8 weeks	16 weeks	**Week 8, treated vs. untreated sides:** INF: ↓66% vs. ↑74% NI: ↓59% vs.↓3% ($p< 0.005$) Total lesion count: ↓55% 95% (21/22) relapse at 8 weeks post tx.
Zane et al. *Photoderm Photoimmunol Photomed* 2008. 24: 244-248	N = 15 22.4 yr Types I - IV Moderate acne Face and trunk	Open	Waldmann PDT Lamp, 600 – 750 nm	Face: 20 mW/cm^2, 20 J/cm^2 x 8 mins 40 cm distance	Bi-weekly x 4 wks	12 weeks	No lesion counts Global Acne Grading System **(GAGS)** used **At week 4:** Face GAGS: ↓50% ($p < 0.05$), maintained 2 months post-tx Sebum (C+K SM810): ↓ 43.8% ($p <0.05$) Moisture (C+ K TEWL): ↓ 55.2% ($p < 0.05$) pH: no change **At week 12:** ↓ GAGs, sebum and TEWL maintained.
RED & BLUE LIGHT							
Papageorgiou et al. *Br J Dermatol* 2000. 142: 973-978	N = 82/ 107 14 - 50 yrs	S/B contrlld study	**Hand-held** ***Blue light* (27):** 415 nm ***Blue + red light***	4.23 mW/cm^2 blue & 2.67 mW/cm^2 for red light	15 min daily x 12 weeks	12 weeks	Blue – red combo superior to blue alone and BP, however no statistical significance. **At week 12 (Blue + red vs. blue only):**

AUTHOR, REFERENCE (YR)	PATIENT FEATURES* N = §	STUDY TYPE	LIGHT SOURCE (S)	IRRADIATION VALUES	REGIME	STUDY DURATION	FINDINGS
	Mild - mod		(30): 415nm and 660nm ***Cool white light*** *(25)* Benzoyl peroxide *(25)*	Cumulative dose: 320 J/cm^2 & 202 J/cm^2			INF: ↓ 76% vs. ↓63% NI:↓ 58% vs. ↓45% **S/E:** dryness and itch especially in BP group (8 ppl vs. 2 in other tx grps), acne flare even across groups (2 each), headache; rash seen in treatment grps.
Goldberg, D. & Russell, B. *J Cosmet Laser Ther* 2006. 8: 71-75	N= 22/24 Types II - V Mild - severe Facial acne		415 nm blue light LED 633 nm red light LED	Blue light x 20 min, 48 J/cm^2 Red light x 20 min, 96 J/cm^2	Bi-weekly x 4 wks Red light alternating with blue	12 weeks	Microdermabrasion done before each tx. **At week 4:** Mean lesion count: ↓ 46% (p=0.001) **At week 12:** Mean lesion count: ↓ 81% (p=0.001). Comedones did not respond as well as inflammatory lesions Severe acne slightly better response than mild acne.
Lee et al. *Lasers Surg Med* 2007. 39: 180-188	N = 24/27 22.5 yr 20 ♀, 4 ♂ Mild – moderately severe Face		1. *Omnilux blue™* 415 ± 5 nm 2. *Omnilux revive™* 633± 6 nm	1. 40 mWcm^{-2} x 20 min 2. 80mWcm^{-2} x 20 min	Bi-weekly x 4 wks Red light alternating with blue	12 weeks	**8 weeks post-tx:** INF :↓77.9% & NI: -↓34.3% in 87.5% patients Moisture and sebum unchanged. Melanin ↓by red ($p< 0.005$) and ↑ blue light ($p > 0.1$). Improved skin texture. Nodulocystic lesions said to respond.
Sadick et al. *J Drugs Dermatol* 2008. 7: 347-350	N = 19/21 14-21 yr Mild - mod	Open uncontrlld	Omnilux clear-U ™ **Handheld, contact**	LED area: 60 mm x 50 mm Blue x 20 min, 40 mW/cm^2,	Bi-weekly x 4 wks Red light alternating	12 weeks	**8 weeks post-tx:** Mean INF: ↓ 69% ($p > 0.001$)? Mean NI: ↓ 12%

AUTHOR, REFERENCE (YR)	PATIENT FEATURES* N = §	STUDY TYPE	LIGHT SOURCE (S)	IRRADIATION VALUES	REGIME	STUDY DURATION	FINDINGS
	acne Face		Blue 415 nm Red 633 nm	48 J/cm^2 Red x 30 min, 70 mW/cm^2, 126 J/cm^2	with blue		Subjective rating: 68% had 'marked' or 'moderate' improvement. No differences in response with acne severity

Table 4: Yellow and Broad-spectrum Light in Acne: A Summary

KEY: * - '*Patient features'* listed as: average age, skin type (if given), acne severity (if given), site (if given); § – number completed study / number enrolled (if given); *contrlld* – controlled; *F/U* – follow-up; *INF* – inflammatory; *NI* – non-inflammatory; *n.s.* – not significant; *PIH* – post-inflammatory hyperpigmentation; *R* – randomised; *S/B* – single blind; *S/F* – split face; *tx* – treatment; *uncontrlld* – uncontrolled; *wks* – weeks ↑- increase; ↓ - decrease; ↔ - no change;♀ - female ; ♂ - male

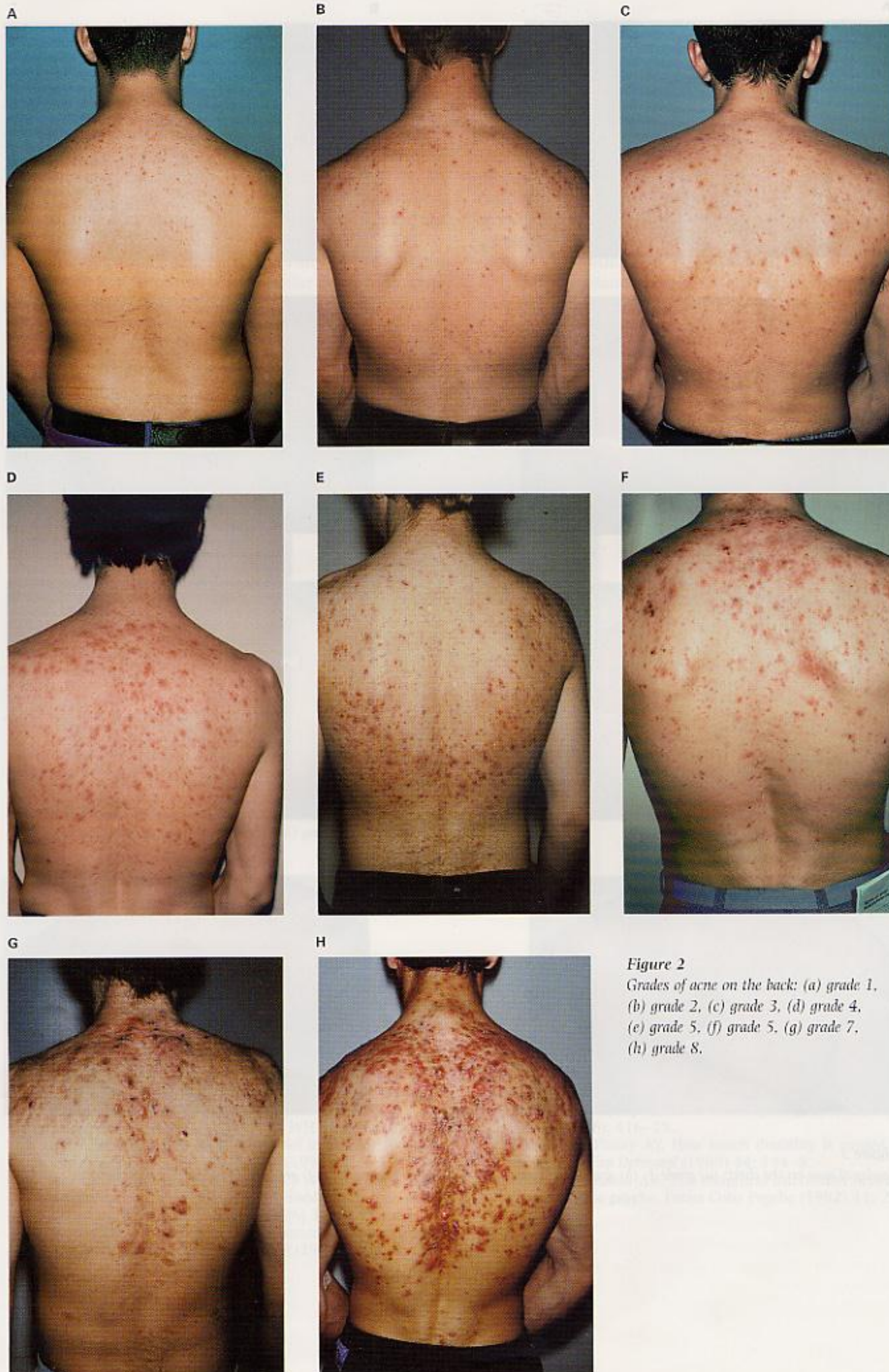
AUTHOR, REFERENCE	PATIENT FEATURES* N = §	STUDY TYPE	LIGHT SOURCE (S)	IRRADIATION VALUES	REGIME	STUDY DURATION	FINDINGS
YELLOW LIGHT							
Chang et al. *Dermatol Surg* 2007. 33: 676-679	N = 30 25.7 yr 30 ♀ Mild-mod acne Type III - IV	R S/B S/F	*Ellipse flex IPL* (530- 750 nm) 2.5ms pulse duration	Skin type II: 8 J/cm^2 Skin type IV: 7.5 J/cm^2	1 every 3 wks x 3 sessions	12 weeks	BP gel used on entire face, a confounder. Korean acne grading system; no comedones included. Lesion counts not reported. **At week 12 (week 3 post-tx):** No difference b/wn IPL + BP and BP alone. (↓ 3.2 vs. 3.1) Skin tone and pigmentation improved on IPL side. S/E: PIH in 3/30, lasted 2 weeks

Author, Reference	Patient features* N = §	Study type	Light source (s)	Irradiation values	Regime	Study duration	Findings
Sami et al. *J Drugs Dermatol* 2008. 7: 627-632	N= 45 29 yr 27♀, 18♂ Types III - IV	R S/F S/B Compara-tive study	1. IPL (*Epi-C/plus*®, Italy) 550 – 1200 nm 2.5 x 4.5 cm spot *(15)* 2. Pulsed dye laser, 595 nm *(15)* 3. Blue-red LED 470 nm & 623 nm *(15)*	1. 22J/cm^2; 30 ms pulse 2. 6- 8 J/cm^2; 0.5 ms pulse; 7mm spot 3. Blue = 10 mW/cm^2 vs. Red = 40 mW/cm^2	1. Weekly (6.0±2.05 sessions) 2. Weekly (4.1±1.39 sessions) 3. Biweekly x 30 min (10.0±3.34 sessions)	Until ≥90% 'clearance' achieved.	Lesion counts for control side of face not reported. Non-inflammatory lesions not counted. **Inflammatory lesion count improvement from baseline at 1 month vs. 1 month post-tx:** IPL: ↓ 41.7% vs. 94.3% ($p \leq 0.05$) PDL: ↓ 73.8% vs. 98.9% ($p \leq 0.05$) Blue-red LED: ↓ 35.6% vs. 91% ($p \leq 0.05$)
Choi et al. *J Eur Acad Dermatol Venereol* 2010. 24: 773-780	N = 20 26 yr 19 ♀, 1♂ Types III - V	R S/F S/B	*Ellipse flex IPL* (530- 750 nm) 2.5ms pulse duration 2. PDL 585 nm (*Cynergy*, Cynosure)	1. 7.5 -8.3 J/cm^2, triple pulse, 9 ms delay, double pass 2. 10mm, 40ms, 8-10 J/cm^2	Once every 2 weeks x 4 weeks	12 weeks Follow up at weeks 1, 4, 8 and 10	Improvement with both modalities, almost a dead heat between IPL and PDL **Reduction in inflammatory lesion counts 1 month post tx & 2 months post-tx:** IPL: ↓ 66.7% & 55% ($p \leq 0.05$) PDL: ↓ 62% vs. 86% ($p \leq 0.05$) **Reduction in non- inflammatory lesion counts 1 month post tx & 2 months post-tx:** IPL: ↓ 33% & 43% ($p \leq 0.05$) PDL: ↓ 47% vs. 59% ($p \leq 0.05$)
BROAD SPECTRUM							
Sigurdsson et al. *Dermatology* 1997. 194: 256-	N = 23/30 23.5 ± 5.2 yrs	S/B, Full face/ Split- back	Phillips HP3136	1. *Full-spectrum + UVA:*	Thrice weekly x 20 min	7 weeks	Assessments at treatments 0, 6, 12 & 20. Those that dropped out, improvement at tx

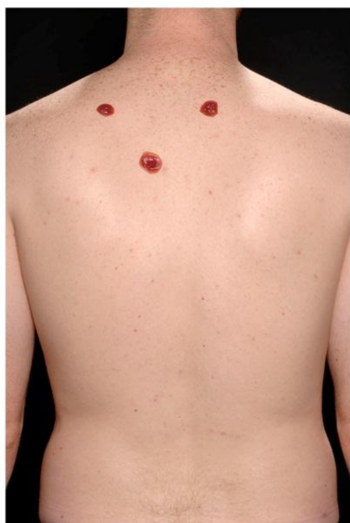
AUTHOR, REFERENCE	PATIENT FEATURES* N = §	STUDY TYPE	LIGHT SOURCE (S)	IRRADIATION VALUES	REGIME	STUDY DURATION	FINDINGS
260.	15♂, 15♀ Mild - mod Face ± back ± chest. Face = 1 unit Chest & back divided into 2 tx areas	or chest	1. Phillips HPA 400W 2. Phillips HPM-10 400W + 390 nm filter 3. Green light + UV & blue light filter *N.B. Peak wavelengths not explicitly stated* Max. skin temp 40 - 41˚ C	(UVA) **5 J/cm^2:** (violet/blue) **16 J/cm^2:** (green) **9 J/cm^2** - 17 fields treated *2. Violet light:* **0.5 J/cm^2: 20 J/cm^2: 5 J/cm^2** - 20 fields treated *3. Green light:* **0 : 0.5 J/cm^2: 50 J/cm^2** - 19 fields treated 40 cm distance	Total 20 sessions		12 comparable to others who completed study. Mean ↓ severity score at week 7: Violet light - 30%(p < 0.02) Green - 22% (p< 0.05) Full spectrum - 14% (n.s.) **After session 20:** Significant reductions only for inflamed lesions Full-spectrum - ↓50% (p <0.01) Violet light - ↓51% (p <0.01) Green light - ↓24% (n.s.) Intra-patient comparisons of both sides of back and/or chest revealed no significant differences. Patient opinion on severity score correlated with physician (no values). Conclude: violet light most effective. Monotherapy with visible light not possible.
Elman, M. & Lask, G. *J Cosmet Laser Ther* 2004. 6: 91-95	N= 19 19 yr 12♂, 7♀ Mild - mod	Open prospective /Full face	Clear Touch™ 430 - 1100 nm Predominantly green/yellow light + heat	3.5 J/cm^2 (per pulse) 35 ms pulse width	Bi-weekly x 4 wk	12 weeks	**At 1 month post-tx:** INF: ↓ 74 ± 20% NI: ↓ 79 ± 22% **At 2 months post-tx:** INF: ↓ 87 ± 17%

AUTHOR, REFERENCE	PATIENT FEATURES* N = §	STUDY TYPE	LIGHT SOURCE (S)	IRRADIATION VALUES	REGIME	STUDY DURATION	FINDINGS
			energy	22 x 55 mm spot			NI:- ↓ 85 ± 25% (no p values) Patient avg. rating of improvement ‘good’ vs. physicians ‘very good’.

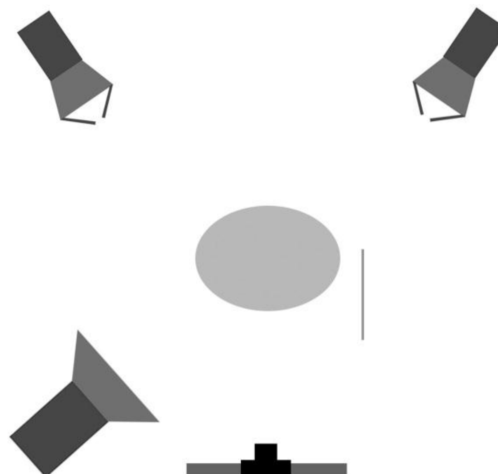
Leeds Revised Acne Grading System (Back)



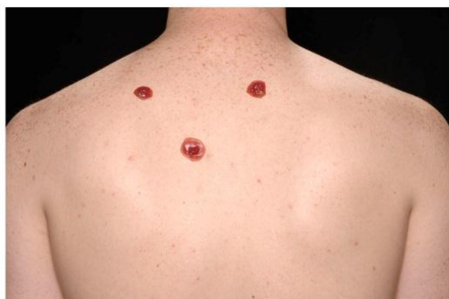
UHW Medical Photography Protocol for Acne Study



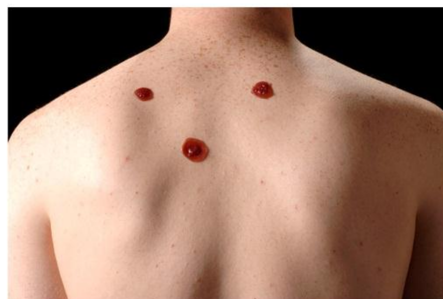
Whole back (portrait) co-axial lighting



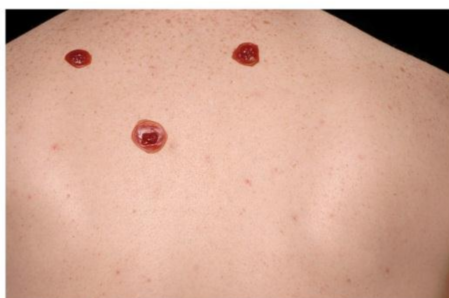
Lighting plan for cross lighting



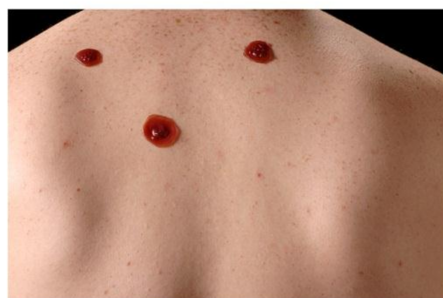
Upper back (landscape) co-axial lighting



Upper back (landscape) cross lighting



Identified area approx A4 co-axial lighting



Identified area approx A4 cross lighting

Leeds Assessments (Blinded)

Leeds Scores from blinded assessors												
CASE	**Chantal1**	**Babar 1**	**Ausama 1**	**Basra 1**	**AVG 1**	**Chantal 2**	**Babar 2**	**Ausama 2**	**Basra 2**	**AVG 2**	**Delta score**	**% change**
6	1	1	1	1	1	1	1	1	1	1	0	0
7	2	3	3.5	1	2.375	2	3	3.5	1	2.375	0	0
9	3	3	3	3	3	2	2	3	2	2.25	-0.75	-25
10	1	1	1	1	1	1	1	1	1	1	0	0
11	1	1	1	1	1	1	1	1	1	1	0	0
12	1	1	1	1	1	1	1	1	1	1	0	0
13	1	1	1	1	1	1	1	1	1	1	0	0
14	1	1	1	1	1	1	1	1	1	1	0	0
15	1	1	1	1	1	2	3	2	2	2.25	1.25	125
16	2	2	2	1	1.75	1	1	2	1	1.25	-0.5	-28.571429
18	1	1	1	1	1	1	1	1	1	1	0	0
20	1	1	1	1	1	1	1	1	1	1	0	0
21	1	1	1	1	1	1	1	1	1	1	0	0
22	2	2	2	1	1.75	2	1	1	1	1.25	-0.5	-28.571429
23	1	1	1	1	1	2	2	2	1	1.75	0.75	75
24	1	1	1	1	1	1	1	1	1	1	0	0
25	3	1.5	2	3	2.375	2	1	1	2	1.5	-0.875	-36.842105
26	1	1	2	2	1.5	2	1	2	2	1.75	0.25	16.666667
27	1	1	1	1	1	1	1	1	1	1	0	0
29	1	1	1	1	1	2	2	1	1	1.5	0.5	50
30	2	2	2	1	1.75	3	2	3.5	2	2.625	0.875	50

Leeds Scores from blinded assessors

AVG Score	1.380952	1.357143	1.452381	1.238095	1.357143	1.47619	1.380952	1.52381	1.238095	1.404762	0.047619048	9.4134145
STD DEV	0.669043	0.654654	0.740013	0.624881	0.593303	0.601585	0.669043	0.858432	0.436436	0.546158	0.499180877	37.604462

Raw Clinical Data

Raw Open Leeds Scores for Full Cohort of Patients (piloted and drop out cases highlighted)

CASE	Back leeds	Back 2nd	δ back	Face grade	Face 2nd	δ Face	Chest grade	Chest 2nd	δ Chest
6	1.5	0	1.5	0	0	0	1		
10	3	2	1	0.5	0.25	0.25	0.5	0.5	0
15	3	2	1	5	0.5	4.5	0	0	0
18	1.5	0.75	0.75	0	0	0	0	0	0
26	2.75	2	0.75	1	1	0	0.5	0.25	0.25
11	1	0.5	0.5	3	1	2	0	0	0
16	2	1.5	0.5	0	0	0	0.5	0	0.5
22	2.5	2	0.5	4	6	-2	1.5	1	0.5
27	1.5	1	0.5	0	0	0	0	0	0
29	2.5	2	0.5	4	1.5	3.5	2.5	2	0.5
21	0.75	0.5	0.25	0.5	0	0.5	0.25	0	0.25
25	2.5	2.25	0.25	0.5	0	0.5	0	0.25	0
7	3.5	3.5	0	0.5	1	-0.5	2	1.5	0.5
9	4	4	0	6	7.5	-1.5	2	2	0
12	1	1	0	0.5	1	-0.5	1.5	0.5	1
14	0.5	0.5	0	0.25	0.5	-0.25	0	0	0
30	4	4	0	0.5	1	-0.5	0.5	0.5	0
20	0.75	1	-0.25	1	1.5	-0.5	0.5	0	0.5
24	1.5	2	-0.5	0.25	0	0.25	0.5	0	0.5
13	1	2	-1	2	2	0	0.5	0	0.5
23	1	2	-1	0	0	0	0	0	0
1	4			0			2		

Raw Open Leeds Scores for Full Cohort of Patients (piloted and drop out cases highlighted)

2	2			2			1		
3	1			0			1		
4	1.5			0.25			1		
5	0.5	1	-0.5	0	0	0	0	0.5	-0.5
8	1.5			0.5			1		
19	1			0			0.5		
AVG 28	1.901785714			1.151786			0.741071429		
SD 28	1.095739404			1.686465			0.731190502		
AVG 21	1.988095238	1.738095238	0.25	1.404762		0.27380952	0.678571429		0.25
SD 21	1.093949812	1.105317235	0.62249498	1.846409		1.46395176	0.771130895		0.29244883
AVG 20	2.0375	1.725	0.3125	1.375		0.2875			0.23684211
SD 20	1.098069478	1.132358972	0.567050309	1.889201		1.50060295			0.29431753

Raw SER Data for Full Cohort of Patients (piloted and drop out cases highlighted)

CASE	Sebum 1	Sebum 2	sebum3	1st Avg	CASE	2nd sebum	2nd sebum2	2nd sebum 3	2nd Avg	δ sebum
6	6	9	5	6.67	**6**	3	9	4	5.33	1.34
10	29	36	25	30	**10**	26	37	28	30.33	-0.33
15	31	41	40	34	**15**	32	9	17	17.33	16.67
18	1	1	1	1	**18**	7	12	7	8.67	7.67
26	12	20	7	13	**26**	31	10	20	20.33	-7.33
11	6	6	4	5.33	**11**	15	48	17	26.67	21.34
16	2	2	0	1.33	**16**	0	5	1	2	-0.67
22	16	20	20	18.67	**22**	9	12	16	12.33	6.34
27	13	19	20	17.33	**27**	9	7	10	8.67	8.66
29	9	5	7	7	**29**	3	3	8	4.67	2.33
21	19	43	27	29.67	**21**	5	5	9	6.33	23.34
25	21	23	17	20.33	**25**	8	13	5	8.67	11.66
7	11	17	11	13	**7**	15	9	11	11.67	1.33
9	13	7	16	12	**9**	24	24	22	23.33	-11.33
12	4	6	5	5	**12**	3	3	6	4	1
14	17	25	17	19.67	**14**	25	21	16	20.67	1
30	7	6	3	5.33	**30**	2	1	0	1	4.33
20	31	45	17	31	**20**	35	34	28	32.33	-1.33
24	9	15	16	13.33	**24**	6	9	4	6.33	7
13	37	62	61	53.3	**13**	24	21	33	26	27.3
23	9	7	5	7	23	11	9	10	10	3
1	8	13	8	9.67	1	3	1	4	2.67	7
2	30	27	46	34.3	2	65	73	80	72.6	-38.3
3	7	15	11	11	3	8	5	14	9	2
4	18	12	11	13.67	4	15	18	17	16.67	-3

	5	30	31	23	28	**5**	52	51	42	48.33	-20.33
	8	15	19	38	24	8					
	19	14	11	12	12.33	19					
AVG 28					17.03321					16.76654	
SD 28					12.23281					16.08255	
AVG 21					16.37905					13.65048	5.872381
SD 21					13.11553					9.720177	9.698687
AVG 20					14.533					13.033	4.801
SD 20					10.28291					9.540773	8.581469

Raw Lesion Count Data for Full Cohort of Patients (piloted and drop out cases highlighted)

inflamm 1	inflamm 2	δ Inflamm	% δ Infl	Resolving 1	Resolving 2	δ resolved	CASE	N-Inf 1	N-Inf 2	δ non-inf
15	18	-3	-20%				**6**	9	19	-10
53	34	19	36%		4		**10**	55	31	24
11	9	2	18%	3	8	-5	**15**	78	38	40
4	4	0	0%	2	4	-2	**18**	17	20	-3
27	10	17	63%	1	4	-3	**26**	55	43	12
17	9	8	47%		1		**11**	18	15	3
12	3	9	75%	4	4	0	**16**	110	85	25
54	31	23	43%	0	1	-1	**22**	77	95	-18
6	7	-1	-17%	0	2	-2	**27**	12	11	1
24	8	16	67%	1	3	-2	**29**	42	19	23
11	2	9	82%	3	1	-2	**21**	11	32	-21
28	8	20	71%	6	0	6	**25**	56	19	37
36	32	4	11%		17		**7**	9	40	-31
100	103	-3	-3%		14		**9**	71	102	-31
30	37	-7	-23%		2		**12**	42	19	23
2	3	-1	-50%	1	0	1	**14**	37	4	33
15	7	8	53%	1	2	-1	**30**	12	14	-2
9	0	9	100%	1	3	-2	**20**	12	8	4
20	12	8	40%	3	4	-1	**24**	56	29	27
20	136	-116	-580%	0	0	0	**13**	28	125	-97
11	12	-1	-9%	1	2	-1	23	5	14	-9
60	43	23	38%				1	6	3	3
24	39	-15	-63%				2	12	103	-91
28	5	23	82%				3	16	20	-4
11	9	2	18%				4	10	1	9

Raw Lesion Count Data for Full Cohort of Patients (piloted and drop out cases highlighted)

6	21	-15	-250%		**5**	3	12	-9
14					8	61		
16				0	19	10		
23.71429			-0.06536			33.21429		-2.38462
21.09565			1.356612			28.53634		33.36235
24.04762	23.09524	0.952381	0.001863			38.66667	37.2381	1.428571
22.41534	34.31604	28.10423	1.389704			29.25121	34.30729	31.19547
24.25	17.45	6.8	0.291956			39.2	32.85	6.35
22.97796	23.13229	8.691193	0.415544			29.90617	28.51828	22.11281

Appendix 3

- TLR2 IHC Image Analysis Data
- TLR2 Semi-qPCR Densitometry Data
- TLDA Data with CSF-1 as the Housekeeping Gene

TLR2 IHC Image Analysis Data

TLR2 Image Analysis Data (Image Pro Plus)			
Case	**B1**	**B2**	**B3**
2a	0.215	0.265	0.204
2b	0.209	0.239	0.221
2c	0.19	0.24	0.221
2d	0.189	0.243	0.213
3a	0.255	0.226	0.228
3b	0.253	0.227	0.217
3c	0.249	0.203	0.198
3d	0.244	0.207	0.208
4a	0.229	0.209	0.226
4b	0.236	0.215	0.245
4c	0.232	0.229	0.233
4d	0.231	0.218	0.224
5a	0.265	0.227	0.225
5b	0.261	0.209	0.218
5c	0.254	0.176	0.245
5d	0.245	0.226	0.26

Mean scores for image analysis			
Case	**AVG B1**	**AVG B2**	**AVG B3**
2	0.2007	0.2469	0.215
3	0.2503	0.2159	0.213
4	0.2321	0.2178	0.232
5	0.2563	0.2097	0.237
6	0.1673	0.2154	0.253
9	0.2457	0.2353	0.156
10	0.2086	0.28	0.203
12	0.2334	0.246	0.04
14	0.1935	0.2827	0.328
18	0.2489	0.2252	0.225
AVG	**0.2237**	**0.2375**	**0.21**
% change	B1 vs B2	B2 vs B3	B3 vs B1
	6.1824	-11.5	-6.032

TLR2 Image Analysis Data (Image Pro Plus)				Mean scores for image analysis
6a	0.186	0.207	0.261	
6b	0.123	0.238	0.235	
6c	0.18	0.236	0.256	
6d	0.181	0.181	0.259	
9a	0.272	0.235	0.216	
9b	0.247	0.246	0.173	
9c	0.247	0.222	0.15	
9d	0.217	0.239	0.085	
10a	0.208	0.312	0.208	
10b	0.208	0.277	0.2	
10c	0.205	0.266	0.206	
10d	0.214	0.264	0.197	
12a	0.228	0.258	0.042	
12b	0.247	0.244	0.065	
12c	0.227	0.243	0.037	
12d	0.232	0.238	0.016	
14a	0.19	0.288	0.343	
14b	0.177	0.318	0.327	
14c	0.202	0.255	0.329	
14d	0.205	0.292	0.314	

TLR2 Image Analysis Data (Image Pro Plus)				**Mean scores for image analysis**
18a	0.246	0.231	0.232	
18b	0.254	0.221	0.218	
18c	0.25	0.216	0.22	
18d	0.245	0.233	0.228	
AVG	**0.224**	**0.238**	**0.21**	

TLR2 Semi-qPCR Densitometry Data

Densitometry Data from Agarose Gel Images (SemiqPCR) using Alpha Imager			
CASE	**TLR2 B1**	**TLR2 B2**	**TLR2 B3**
1	0.068456	1.671763	0.628743
2	1.564153	0.733494	0.672913
3	0.24395	1.668062	0.863118
4	0.242567	0.082767	0.006374
5	1.519897	0.027824	0.044103
6	0.012827	0.086629	1.564121
9	0.677026	0.166702	0.598754
10	1.795397		0.070617
12	2.26179	3.535246	5.729383
14	0.66413	5.29467	1.276844
18	0.946663	0.428601	0.117073
AVG	0.908805091	1.3695758	1.052003909
% change	**B2-B1**	**B3-B1**	**B3-B2**
	50.70071831	15.75682395	-23.18760969

TLDA Data with CSF-1 as HKG

TLDA Data with CSF-1 as the House-keeping Gene							
GAPDH				**IL-10**			
Case	**B1**	**B2**	**B3**	**Case**	**B1**	**B2**	**B3**
2a	-4.762	-4.57	-3.844	**2a**	7.345	4.105	3.801
2b	-4.72	-4.129	-4.214	**2b**	4.8	4.526	4.681
2c	-4.943	-3.821	-3.835	**2c**	6.331	5.212	4.57
14a	-3.289	-3.91	-3.981	**14a**	9.809	3.225	6.63
14b	-4.273	-3.659	-4.071	**14b**	5.108	3.863	5.903
14c	-4.06	-3.276	-4.292	**14c**	2.959	4.031	5.783
22a	-4.709	-3.727	-4.468	**22a**	2.243	5.607	3.44
22b	-4.028	-4.168	-3.844	**22b**	3.005	4.805	2.862
22c	-4.059	-4.047	-5.584	**22c**	3.204	3.065	3.897
24a	-4.584	-3.443	-4.067	**24a**	4.567	8.84	3.376
24b	-4.311	-3.361	-3.721	**24b**	4.077	4.816	3.311
24c	-4.293	-3.783	-4.168	**24c**	4.077	8.869	3.523
25a	-4.597	-4.65	-5.123	**25a**	4.116	3.281	4.712
25b	-4.605	-4.442	-3.923	**25b**	3.273	4.505	4.306
25c	-5.069	-5.276	-5.206	**25c**	4.736	4.825	3.386
26a	-4.296	-4.611	-4.375	**26a**	4.37	4.418	4.322
26b	-4.393	-4.971	-4.461	**26b**	3.999	4.676	2.945
26c	-4.192	-4.825	-4.519	**26c**	5.743	2.977	3.063
30a	-3.967	-4.295	-4.367	**30a**	3.012	2.886	4.136
30b	-3.891	-4.171	-4.14	**30b**	2.948	3.326	2.729
30c	-3.622	-3.845	-4.617	**30c**	4.35	3.387	4.042

TLDA Data with CSF-1 as the House-keeping Gene

TNF				**TNFR**			
Case	**B1**	**B2**	**B3**	**Case**	**B1**	**B2**	**B3**
2a	3.098	2.604	3.615	**2a**	2.598	3.559	4.808
2b	2.831	3.248	4.621	**2b**	3.223	3.589	4.921
2c	4.457	3.627	1.509	**2c**	3.668	3.419	4.446
14a	1.921	2.736	3.856	**14a**	2.973	6.688	5.109
14b	4.433	3.904	3.337	**14b**	4.265	4.004	3.919
14c	2.076	7.096	5.169	**14c**	3.724	7.096	3.464
22a	2.338	3.336	3.613	**22a**	2.455	4.023	7.615
22b	3.514	3.703	3.655	**22b**	3.243	5.412	5.781
22c	3.367	4.242	3.178	**22c**	3.572	10.079	2.513
24a	2.812	4.405	7.9	**24a**	2.307	3.211	3.637
24b	3.31	3.748	5.129	**24b**	2.863	2.619	4.074
24c	3.951	3.479	8.066	**24c**	2.926	5.267	4.605
25a	2.946	3.307	4.666	**25a**	5.215	1.991	3.914
25b	2.104	2.54	3.446	**25b**	3.877	3.289	3.214
25c	3.49	2.75	1.693	**25c**	4.648	3.405	4.725
26a	4.164	3.717	1.757	**26a**	3.078	3.544	5.537
26b	2.433	2.611	4.951	**26b**	2.748	0.841	5.86
26c	3.315	2.063	2.552	**26c**	3.293	3.306	3.689
30a	2.828	1.955	2.226	**30a**	3.243	3.835	3.01
30b	2.584	3.149	4.142	**30b**	4.282	4.799	4.782
30c	2.829	3.085	4.01	**30c**	4.999	7.748	3.188

TLDA Data with CSF-1 as the House-keeping Gene						
IL-8				**STAT-3**		
Case	**B1**	**B2**	**B3**	**B1**	**B2**	**B3**
2a	7.409	6.923	5.283	-1.058	-1.814	-0.823
2b	4.183	5.644	10.026	-0.615	-1.406	-0.905
2c	3.739	7.294	10.187	-0.965	-1.952	-0.708
14a	5.491	3.53	5.714	-1.269	-0.414	-0.522
14b	5.687	3.948	5.483	-0.667	-1.151	-0.468
14c	6.449	7.096	6.784	-1.734	-0.809	-0.119
22a	4.793	4.338	7.615	-0.931	-0.608	-0.6
22b	10.058	6.972	5.444	0.026	0.009	-0.827
22c	9.975	6.827	4.326	-0.149	-0.02	-0.945
24a	-0.82	8.84	-1.808	-1.057	-0.768	-0.924
24b	0.302	2.494	3.167	-0.274	-0.621	-0.524
24c	-0.915	-4.538	3.211	-0.999	-0.443	-0.533
25a	10.191	6.493	7.9	-1.507	-1.414	-1.136
25b	3.637	4.89	9.351	-1.968	-0.775	0.093
25c	3.571	6.607	8.066	-1.956	-1.623	-1.403
26a	8.674	3.571	4.553	-1.04	-1.476	-1.879
26b	8.547	5.426	5.787	-1.02	-1.873	-1.762
26c	8.796	3.526	4.841	-1.095	-1.204	-2.058
30a	3.664	5.872	8.493	-0.753	-1.245	-1.269
30b	2.973	5.528	8.883	-1.57	-1.164	-1.274
30c	4.063	5.927	8.061	-0.826	-1.661	-1.384

Appendix 4

Publications & Presentations

Published Articles

- Taylor M, Gonzalez M, Porter R. **Pathways to inflammation: Acne Pathophysiology.** *Eur J Dermatol* 2011; 21(3): 323 – 333

- Taylor MN & Gonzalez ML. **The practicalities of photodynamic therapy in acne vulgaris.** *Br J Dermatol* 2009; 160(6): 1140-1148.

Published Abstracts

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