Protecting Against Skin Cancer Promotion: A Clinical Study to Assess the Effect of Omega 3 Fatty Acid Supplementation on Photoimmunosuppression

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LIST OF ABBREVIATIONS

∆E	Delta E
ADA	Adrenic Acid
ADCC	Antibody Dependent Cellular Cytotoxicity
Ab	Antibody
Ag	Antigen
ALA	Alpha Linolenic Acid
APC	Antigen Presenting Cell
ATPase	Adenosine Triphosphatease
BCC	Basal Cell Carcinoma
BCG	Bacillus Calmette Guerin
BSA	Bovine Serum Albumin
CD	Clusters of Differentiation
CHS	Contact Hypersensitivity
COX	Cycloxygenase
CPD	Cyclobutane Pyrimidine Dimer
CSF	Colony Stimulating Factor
DC	Dendritic Cell
DGLA	Dihomo-Gamma-Linolenic Acid
DHA	Docosahexanenoic Acid
DLN	Draining Lymph Node
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DNFB	Dinitroflourobenzene
DPA	Docosapentaenoic Acid
DTH	Delayed Type Hypersensitivity
ECM	Extracellular Matrix
EFA	Essential Fatty Acid
EI	Erythema Index
ELISA	Enzyme Linked Immunosorbent Assay
EM	Electron Microscope
EPA	Eicosapentaenoic Acid
EPO	Erythropoietin
FITC	Flourescein Isothiocyanante
GLA	Gamma Linolenic Acid
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor

GSH	Glutathione (Reduced Form)
GSSG	Glutathione Disulfide (Oxidized Glutathione)
GTCC	Glyceryl Tricoprylate Coprate Crodamol
HETE	Hydroxyeicosatetraenoic Acid
HLA	Human Leucocytic Antigen
HPETE	Hydroperoxyeicosatetraenoic Acid
ICDRG	International Contact Dermatitis Research Group
IHC	Immunohistochemistry
IEM	Immuno-Electron Microscopy
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IPF	Immune Protection Factor
JAKs	Janus kinases
kDa	kilo Dalton
kJ	kilo Joule
LA	Linoleic Acid
LB	Lamellar Bodies
LC	Langerhans Cell
LCPUFA	Long Chain Polyunsaturated Fatty Acid
LN	Lymph Node
L-NMMA	NG monomethyl-L-arginine (a nitric oxide synthase inhibitor)
LOX	Lipoxygenase
LTB_4	Leukotriene B_4
MED	Minimum Erythema Dose
MHC	Major Histocompatibility Complex
MM	Malignant Melanoma
n-3	Omega 3
n-6	Omega 6
ng	Nanograms
Ni	Nickel
NK	Natural Killer
Nm	Nanometer
NMSC	Non Melanoma Skin Cancer
NO	Nitric Oxide

NOS	Nitric Oxide Synthase
PAF	Platelet Activating Factor
PBS	Phosphate Buffer Saline
PD	Papillary Dermis
pg	Picogram
PGE_2	Prostaglandin E_2
PI	Photoimmunosuppression
PMNs	Polymorphonuclear Leukocytes
PUFA	Poly unsaturated Fatty Acid
PUVA	Psoralen and UVA treatment
RDI	Recommended Daily Intake
ROR	Retinoid-related Orphan Receptor
rIL-12	Recombinant Interleukin 12
SB	Stratum Basale
SC	Stratum Corneum
SCC	Squamous Cell Carcinoma
SG	Stratum Granulosum
SL	Stratum Lucidum
SPF	The Sun Protection Factor
SS	Stratum Spinosum
SSR	Solar Simulated Radiation
STAT	Signal Transducers and Activators of Transcription
TB	Tuberculosis
TBARS	Thiobarbituric Acid Reactive Substances
T-bet	T box protein expressed in T cells
TcR	T cell Receptor
TGF- β	Transforming Growth Factor - β
Th	T Helper Cell
THPO	Thrombopoietin
TNF	Tumor Necrosis Factor
Treg	T Regulatory Cell
UCA	Urocanic Acid
UVA	Ultraviolet A
UVB	Ultraviolet B
UVR	Ultraviolet Radiation

ABSTRACT
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Protecting Against Skin Cancer Promotion: A Clinical Study to Assess the Effect of Omega 3 Fatty Acid Supplementation on Photoimmunosuppression

Ultraviolet radiation (UVR) is a complete carcinogen, inducing skin cancer via DNA photodamage that can lead to mutagenesis, and promoting it's growth via photoimmunosuppression (PI). The omega-3 polyunsaturated fatty acid (n-3 PUFA) eicosapentaenoic acid was shown in murine studies to protect against PI and UV-induced skin cancer although the mechanism is uncertain. The principal objectives of this thesis were to (i) examine whether n-3 PUFA can protect against a clinical model of PI in healthy humans and (ii) explore whether the underlying mechanism could be abrogation of UV-induced depletion of antigen-presenting Langerhans cells (LC) from the epidermis, and/or impact on immunomodulatory cytokines.

Nickel (Ni) allergic females (n=79) were randomized to 3 months of daily supplementation with 5g n-3 PUFA (70% eicosapentaenoic acid, EPA; 10% docosahexaenoic acid, DHA) or the placebo medium chain triglyceride, GTCC. Local PI was clinically assessed post supplementation using the nickel contact hypersensitivity (Ni CHS) model. In each volunteer, Ni patches were applied to 3 skin sites that were irradiated for 3 consecutive days with UV-doses of 1.89, 3.82 & 7.59J/cm^2 respectively. CHS responses were measured and compared to responses of control patches applied on unirradiated skin using a reflectance erythema meter. In the same subjects, assessments of cellular and biochemical mediators of PI were made pre and post supplementation. At 24hr post irradiation with an erythemal UV-dose (4 minimal erythemal doses) to upper buttock skin, half the subjects (n=39) had skin punch biopsies taken and the other half (n=40) had suction blisters raised on this irradiated skin and on unirradiated skin of the contralateral buttock. Epidermal sheets were prepared from the punch biopsies and immunohistochemically stained to assess UV-induced LC numbers. Levels of immunomodulatory cytokines were analysed in the suction blister fluid using Luminex multiplex assay kits. To evaluate compliance and bioavailability, blood samples were taken from all volunteers, pre and post supplementation and EPA% weight in red blood cell membranes was examined using gas chromatography.

Post supplementation, EPA %wt was significantly higher in the active group compared to control: mean 3.61% ± 0.22% (SEM) vs. 0.93% ± 0.06% (p<0.001). 3 volunteers showed evidence of non-compliance and were excluded from further analysis. Compared to placebo, evidence for protection against local PI of Ni CHS was apparent post n-3 PUFA at all UV doses, reaching statistical significance at the UV-dose of 3.8J/cm^2 (p<0.05). No significant difference in post-UV epidermal LC numbers after supplementation was seen between active and placebo groups, with a % fall following UV of 76.61 ± 3.39% (SEM) in the active group and 73.52 ± 5.24% (SEM) in the control group. When intragroup comparisons were made pre vs. post supplementation, a similar increase in UV-induced LC depletion from the epidermis was seen in both groups, reaching statistical significance following n-3 PUFA (p=0.018). Levels of interleukins IL-10 and IL-8, and of TNF-α, increased post-UVR in both active and control groups pre-supplementation, with no changes occurring following supplementation.

In conclusion, supplemental EPA was bioavailable and evidence of protection against clinical PI of Ni CHS was seen in the actively treated group. However, no evidence was found that this abrogation of PI was mediated through a reduced effect of UV on migration of epidermal LC or the immunomodulatory cytokines examined. This original study gives the first evidence that dietary n-3 PUFA may protect against clinical PI, and potentially skin cancer promotion, in humans. Further research is needed to confirm this finding, and to examine the underlying mechanisms, which could involve other immunoregulatory cells of the skin, such as dermal dendritic cells and T regulatory cells and other mediators of UV-immunosuppression including the prostanoids, which may be modified by n-3 PUFA.

1 INTRODUCTION

Skin cancer has become a public health problem of growing concern. It is one of the commonest cancers and its incidence is on the rise. Epidemiologic studies as well as experiments on laboratory animals indicate that exposure to ultraviolet radiation (UVR) is the primary cause of most skin cancers (Murphy 2009). UVR is a complete carcinogen that initiates DNA damage that can lead to carcinogenesis and allows this highly antigenic, UVR induced skin cancer to grow unopposed via photoimmunosuppression (PI) (Kripke et al. 1996; Berneburg and Krutmann 2000; Beissert et al. 2001; Murphy 2009). UVR-induced immunosuppression in murine studies was undoubtedly shown to be one of the key factors in the promotion of skin cancer (Walker and Young 2007). In addition, an individual's susceptibility to immunosuppression of their contact hypersensitivity responses (CHS) by UVR directly correlates with their risk of developing skin cancer. Individuals with a past medical history of skin cancer were found to be more susceptible to PI than those with no skin cancer history (Yoshikawa et al. 1990). Furthermore, squamous cell carcinoma (SCC) in patients on immunosuppressive medications was found to be more aggressive than that developing in non immuno-compromised individuals (Damian et al. 2001). It is thought that PI acts in similar ways to that which makes immunosuppression in transplant patients increase their risk of developing skin cancer (Oberyszyn 2008; Murphy 2009).

Apart from protection via clothing and avoidance of intense sunlight, the use of topical sunscreens is the current method of skin protection against the damaging effects of solar UVR. These topical creams however, are often inadequately applied to provide sufficient coverage & usually require re-application to provide protection throughout the time of UVR exposure (Diffey 2001).

A new dietary approach, with the potential for a more continuous and even protection, may therefore be considered as a useful adjuvant to topical sunscreens in the protection strategies against photo damage. An example of such a dietary approach is omega-3 polyunsaturated fatty acids (n-3 PUFAs). Experimental models have shown that PI, and consequently, photocarcinogenesis is reduced by dietary intervention with n-3 PUFAs. A clarified model of such protection however is yet to be demonstrated in man (Black and Rhodes 2006). The aim of this study is to examine the protective effects of n-3 PUFAs on UV-induced immunosuppression in humans.

In the following introduction I will consider the structure and the immune system of the skin, followed by an outline of UVR and its acute and chronic cutaneous effects, particularly the suppressive effects of UVR on the skin's immune system. I will then look at the clinical and laboratory methods for examining UVR-induced immunosuppression in humans and how dietary agents can provide systemic protection against it. The final section in this chapter will be the hypothesis and aims of my study.

1.1 Skin Structure

The skin is the largest organ in the human body. Histologically, the skin consists of three distinct layers: the epidermis, dermis and hypodermis (subcutis) (Figure 1.1).

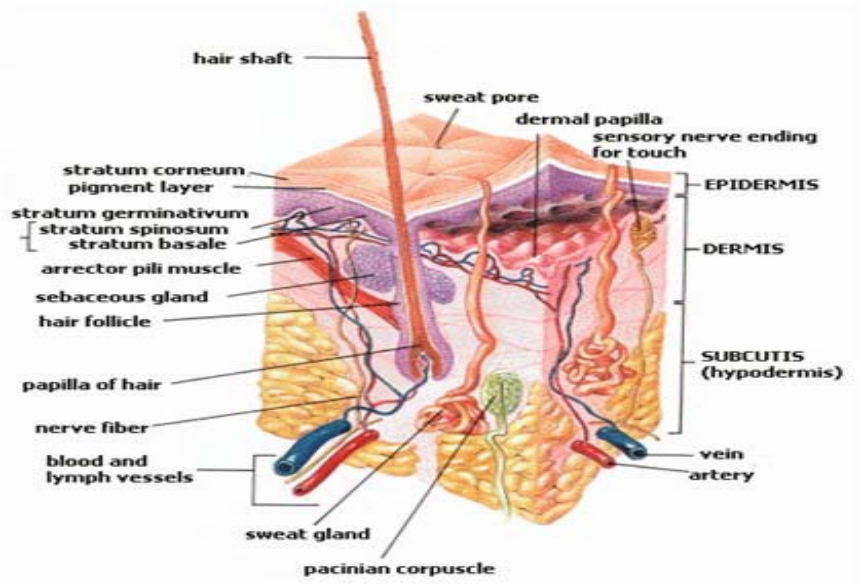


Figure 1.1 - Skin Layers and Appendages

(http://content.answers.com/main/content/wp/en-commons/thumb/3/3d/300px-Skin.jpg)

1.1.1 The Epidermis

The epidermis is the outermost, avascular layer of the skin. It is divided into four layers, from outer to inner: stratum corneum, stratum granulosum, stratum spinosum and stratum basale.

Stratum Corneum (SC)

The stratum corneum is a tight lamellar structure made of corneocytes (terminally differentiated keratinocytes) and secreted contents of the lamellar bodies. It forms a tight barrier that prevents noxious substances from entering the body and protects against water loss (Menon 2002).

Stratum Granulosum (SG)

The stratum granulosum is the highest layer in the epidermis where living cells are found. The most apparent structure in the cells of this layer is the distinct, darkly staining, basophilic keratohyalin granules.

Stratum Spinosum (SS)

Due to desmosome abundance, this layer has spiny appearing cells in histological sections. An increase in cellular keratin filaments in this layer is noticeable compared to the basal cells.

Stratum Basale (SB)

The stratum basale, also known as the stratum germinatum, is a single layer of columnar basal cells, which remain attached to the basement membrane via hemidesmosomes.

1.1.2 The Dermis

The dermis is the bulk connective tissue element of the skin. It is made of collagen, elastin, glycosaminoglycans; collectively named the extracellular matrix (ECM) and fibroblasts that secrete precursors to and help contribute to the structural framework of the ECM.

1.2 Skin Immune System

In addition to the skin's protective role as a physical barrier, it also has a complex immune system to protect against harmful agents. This is achieved by a rapid, more primitive, non-specific first line of defence system known as innate immunity and a higher developed, specific system; adaptive immunity.

Innate immunity is characterized by the lack of an immunological memory. It's immune reactions are less complicated than those of the adaptive immunity and are therefore developed earlier in evolution. Resident skin cells that contribute to the innate immune response include keratinocytes, epidermal Langerhans cells (LC), dermal dendritic cells (DDC) and macrophages (van Beelen et al. 2007).

The adaptive immune response is characterized by specificity due to an accumulative immunological memory. This enables an improved immune response on each successive encounter with a specific antigen. Cells in this system include T & B lymphocytes (Kelley 2001; Schwarz 2003).

The immune system is composed of cellular as well as soluble components that work together to protect the body against noxious factors and foreign antigens.

1.2.1 Cells of the Skin Immune System

The skin immune system is composed of several cell types, some constantly residing in the epidermis or dermis and some that move, mainly to connect the skin with blood or lymphatics.

1.2.1.1 Langerhans cells

Langerhans Cells (LC) are an important part of this study and will be considered here in detail. LCs, first described by P. Langerhans in 1868, are a subset of myeloid dendritic antigen-presenting cells (APC) found abundantly in the epidermis. In conjunction with keratinocytes, LCs form a tight cellular network covering the entire body surface (van Beelen et al. 2007).

LCs respond to foreign antigens by first ingesting and then processing the antigen. This is followed by migration of the stimulated LCs via the afferent lymphatics to the draining lymph nodes (LN) where they present the antigen on their surface to T cell receptors (TcR) found on the surface of naïve T cells (T cells that have never been exposed to antigen before) in a process called T cell priming (Romani et al. 2006). If the antigen on the surface of the LC is carried in conjunction with major histocompatibility complex class I (MHC I) molecules, then cytotoxic T cells (CD8+) who have TcR with affinity to MHC I are generated and activated to track and kill the cells carrying the antigen (e.g. virus infected or cancer cells). If however the antigen is bound to a MHC II complex then naïve CD4+ T helper cells are activated which results in a series of inflammatory/immunoregulatory responses; depending on the cytokines secreted by the APC and on the surrounding micro environment at the time of activation (figure 1.5). Accordingly, dendritic cells (DC) play a pivotal role in deciding between immunity and tolerance based on their expression of variable patterns of T cell co-stimulatory or inhibitory molecules (van Beelen et al. 2007).

During migration, LCs undergo functional and phenotypical maturation where they lose their ability to process antigens and acquire instead characteristics of immunostimulatory DCs. The process of LC maturation and migration mainly occurs under the control of cytokines secreted by the LCs themselves as well as those secreted by surrounding keratinocytes. It has been shown that signals from at least two cytokines are required for the proper maturation and migration of LCs following skin sensitization; interleukin 1beta (IL-1β) and tumor necrosis factor-α (TNF-α) (Griffiths et al. 2005). In animal models, prostaglandin E_2 (PGE_2) has also been implicated in LC migration and maturation (Kabashima et al. 2003).

LCs cannot be specifically identified in routinely fixed and stained histological sections, but only ultra structurally using the electron microscope or by immunohistochemical analysis (Schwarz. 2003) (figure 1.2A). Ultra structurally, LCs are specifically identified by Birbeck granules (rod or tennis racket shaped organelles first described in 1961)(Pena-Cruz et al. 2001; Schwarz. 2003) (figure 1.2b). Histochemically, human LCs can be visualized by staining for the membrane bound enzyme; ATPase (also known as cluster of differentiation no.39; CD39) (Mizumoto and Takashima 2004; Santegoets et al. 2008). Under the influence

of UVA or PUVA however, LCs have been found to lose their ATPase activity early on compared to their visibility under the electron microscopy; 90% of ATPase-stained LCs were undetectable using ATPase enzyme staining after seven PUVA treatments (2 weeks) whereas it took fifteen treatments (5 weeks) for LCs cells to appear reduced using the electron microscope. Antibodies against a number of antigenic markers that are constantly found on the surface of LCs are also used to stain and identify LCs. These markers include the panhematopoietic marker CD45, MHCII (HLA-DR in humans, Ia in mice) (Schwarz 2003; Mizumoto and Takashima 2004), which in healthy epidermis is specific to LCs (Romani et al. 2006), S-100 protein, E-cadherin (a molecule involved in LC adhesion to keratinocytes) (Guironnet et al. 2002), CD1a; the adhesion molecule Ep-CAM (gp40 in mice), the integrin CD11b (Bursch, Wang, Igyarto et al. 2007) and the Birbeck granule-associated C type lectin; langerin (CD207) (Guironnet et al. 2002; Schwarz. 2003; Mizumoto and Takashima 2004; Romani et al. 2006). Amongst those markers, CD1a is a very useful marker for detecting human LCs in the epidermis, since in the epidermis of both normal and inflamed skin, it is found exclusively on LCs (Pena-Cruz et al. 2001; Schwarz. 2003) This does not apply to HLA-DR antigens, which in inflamed skin can also be expressed on keratinocytes (Schwarz. 2003), macrophages and vascular endothelial cells (Romani et al. 2006).

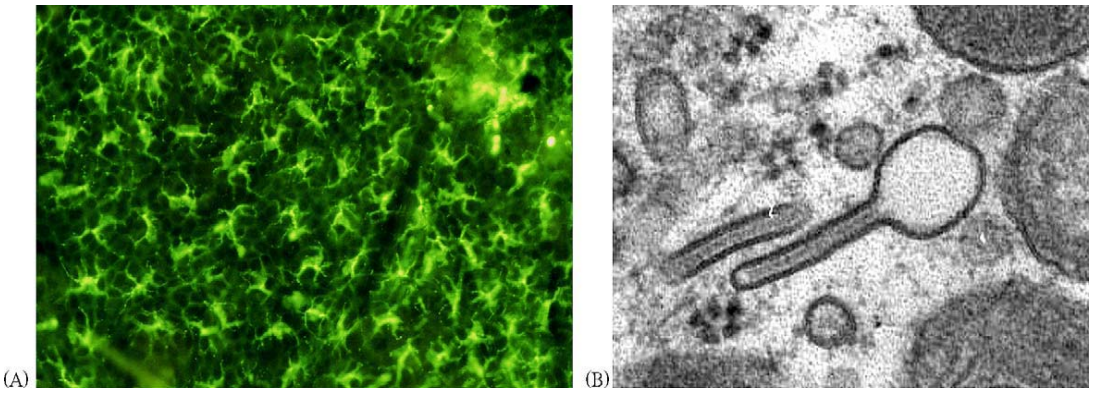


Figure 1.2 - Microscopic images of Langerhans cells

A. Fluorescent microscope image of a network of LCs in murine epidermis

(Monoclonal antibodies against MHC II were used)

B. Typical rod and tennis racket shaped Birbeck granules in a LC

(N. Romani et al. / Immunology Letters 106 (2006) 119–125)

1.2.1.2 Dermal Dendritic Cells (DDC)

Recently, the exact role of LCs in skin immune responses and the identity of the major APC in the skin have both come under question (Ritter et al. 2004; Romani et al. 2006; Henri et al.

2010; Stoitzner 2010). A subset of CD1a+ langerin+ DCs, phenotypically distinct from migrating LCs was found in the dermis (Romani et al. 2006; Poulin et al. 2007; Santegoets et al. 2008). In addition, a third subset of langerin+ DCs was found to permanently reside in the cutaneous draining LNs; lymphoid tissue resident DCs (Henri et al. 2010).

When both epidermal and dermal langerin+ dendritic cells were depleted by treating Lang-DTR EGFP knock-in mice (mice with the fluorescent EGFP protein and the diphtheria receptor inserted into the Langerin loci) with the diphtheria toxin, CHS responses were abrogated. In the absence of only epidermal LCs however, in transgenic hLang-DTA mice that have a normal number of dermal langerin + DCs, CHS responses were unaffected, suggesting that LCs were not essential for CHS responses in this model (Romani et al. 2006; Bursch et al. 2007).

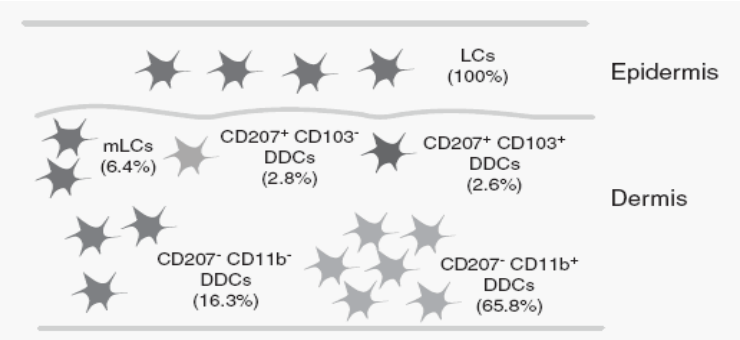


Figure 1.3 - Dendritic Cells in the Skin

Apart from migrating LCs (mLCs), there are two distinct subsets of langerin+ DDCs. In addition, there are two CD207- DDCs that are distinguished from each other using CD11b (Henri et al. 2010)

1.2.1.3 T Lymphocytes

T lymphocytes, derived from hematopoietic stem cells migrating from the bone marrow, acquire and develop their antigen receptors in the thymus. They differentiate into functionally distinct subpopulations which are recognizable by different cluster of differentiation surface molecules (CD). All mature T cells express the surface protein CD3. CD3, along with the T cell receptor (TcR), act as specific markers for T cells.

1.2.1.3.1 T Helper Cells (Th)

Mature Th cells always express the surface protein CD4 (a ligand of MHC II) and are hence also known as CD4+ T cells. T helper cells have no cytotoxic or phagocytic activity; they

cannot kill infected host cells or pathogens and cannot eliminate foreign antigens. Instead, Th cells are involved in activating and directing other immune cells (Schwarz. 2003).

T helper cells are mainly categorized on the basis of the cytokines they produce. Initially, Th cells were essentially divided into two groups; Th1 and Th2. Recently, a third functionally and phenotypically distinct subset of Th cells has been described; Th17 (Bettelli et al. 2007; Chen and O'Shea 2008).

Th1 Cells

Th1 cells participate in cell-mediated immunity to fight intracellular pathogens and eliminate cancer cells (Grewe et al. 1998; Kidd 2003). Th1 cells are produced when APCs form an immunological synapse with naïve CD4+ Th0 cells and secrete factors to direct their differentiation into Th1 cells including IL-12, IL-27, IFN α and IFN β. Differentiation of Th1 cells requires the transcription factors; T box protein expressed in T cells (T-bet), signal transducer and activator of transcription 1 (STAT1) and STAT4. Developed Th1 cells produce high levels of the Th1 signature cytokine; Interferon gamma (IFN-γ) (McGeachy and Cua 2007; Zhou et al. 2007).

In addition to Th1 cells, natural killer (NK) cells (section 1.2.1.3) also respond to the IL-12 secreted by activated APCs and in turn release more IFN-γ. IFN-γ reinforces the production of IL-12 by APCs thus furthering the polarization of more Th0 cells into Th1 in a self reinforcing autocrine loop (Kidd 2003; van Beelen et al. 2007; Zhou et al. 2007).

Th2 Cells

Th2 cells provide help for B cell differentiation, maturation and antibody production to fight extracellular organisms such as multi cellular parasites and are therefore essential for antibody mediated (humoral) immunity. Because Th2 cells can stimulate the activation of mast cells and eosinophils and the production of IgE, they are commonly associated with allergic reactions (figure 1.4) Figure 1.4(Grewe et al. 1998; Kidd 2003).

The differentiation of Th2 cells from naïve T helper cells (Th0) is guided by activated APCs that secrete IL-4 and express the transcription factors STAT6 and GATA3. Th2 cells produce anti inflammatory and immunosuppressive cytokines such as IL-4, IL-5, IL-10 and IL-13 (van Beelen et al. 2007). IL-4 is also secreted by NK cells, mast cells, and eosinophils. As with Th1, an autocrine loop is created when IL-4 stimulates more Th0 differentiation into Th2 (Wang and Mosmann 2001).

Th1 and Th2 cells have been known to antagonize each other's actions, either by blocking mature polarization or by blocking receptor functions of the opposite cell type (Kidd 2003). As

examples, the secretion of IFN-γ by Th1 cells is blocked by IL-10 from Th2. Similarly, the proliferation of Th2 cells is inhibited by the secretion of IFN-γ from Th1 (figure 1.4). Moreover, Th1 cytokines (IFN-γ and IL-12) and Th2 cytokines (IL-4 and IL-13) repress Th17 cell development (Mus et al. 2010).

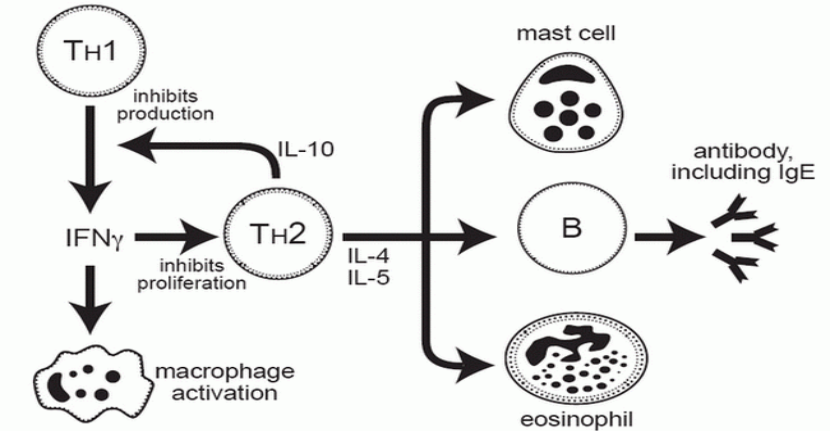


Figure 1.4 - Antagonizing Effects of Th1/Th2 Responses

(Kidd 2003)

Because Th1 cells secrete IL-2, IFN-γ and TNF (Grewe et al. 1998; Kimballs 2008) they are mainly involved in promoting cell-mediated immunity and inflammatory immune responses through the activation of cytotoxic cells and macrophages (Wang and Mosmann 2001) and are therefore regarded as "pro-inflammatory" cells. On the other hand, Th2 cells can be regarded as true helper cells that assist, via the release of IL-4 And IL-5 in the activation of B cells and the induction of humoral immunity, or as anti-inflammatory cells because they secrete IL-10 (Grewe et al. 1998) (Figure 1.4)

Th17 Cells

Th17 cells are so called because they produce IL17; a 32 kDa cytokine. Depending on the presence or absence of IL-6, transforming growth factor beta (TGFβ) can promote the differentiation of Th0 cells into two functionally distinct subsets of effector T cells; proinflammatory Th17 and immunoregulatory/anti-inflammatory Treg (Bettelli et al. 2006; Veldhoen et al. 2006) Figure 1.5(figure 1.5). To regulate tissue inflammation, Th17 and Tregs appear to function in an antagonistic competition similar to that exhibited by Th1 and Th2 (Bettelli et al. 2006; Hatton and Weaver 2009).

Differentiated Th17 cells express activated STAT3 and the Th17 specific transcription factors; RORγt and RORα (McGeachy and Cua 2007; Hatton and Weaver 2009). Although the role of Th17 in mediating tissue inflammation and auto-immunity has been established by a large number of studies, its role in pro/anti tumor immunity remains controversial (Martin-

Orozco and Dong 2009). The expression of IL-17A has been detected in several human tumors including prostate, breast and gastric cancer. Furthermore, in IL-17 knock out mice (IL-17-/-) the growth of B16 melanomas and MB49 bladder carcinomas was reduced. Similarly, mice deficient in the IL-17 receptor showed inhibited tumor growth. Other studies however have shown an anti-tumor role for IL-17: hematopoietic tumor cells over expressing IL-17 showed significant tumor growth inhibition when implanted in syngeneic mice and in IL-17 deficient mice, injecting adenocarcinoma cells resulted in a 5 times or more increase in subcutaneous growth and metastases compared to control mice (Ngiow et al. 2010).

1.2.1.3.2 T Regulatory Cells (Treg)

T regulatory cells (Treg) are a separate lineage of T cells essential for maintaining immunological tolerance to auto antigens. The term Treg comprises several subtypes with different functions, phenotypes and patterns by which they exert their functions. "Natural" Tregs (CD4+ CD25+ FoxP3+) require cell to cell contact and activation of their TcR to exert their suppressive activities in a cytokine and antigen non-specific manner. Another subset of CD4+ Tregs characterized by the production of IL-10 and TGFβ was recently discovered; type 1 regulatory T cells (Tr1) that are "induced" through antigen stimulation. Differentiation of natural Tregs is dependent on the presence of TGFβ while inducing the differentiation of Tr1 cells has been attributed to several factors including the activation by immature dendritic cells and the presence of IL-10 (Ramsdell 2003). UV-induced Tregs (section 1.5.4.3) have recently been characterized as expressing CD4 and CD25. Once activated in an antigen-specific manner, UV-Tregs inhibit immunity in a general manner via the secretion of IL-10 in a phenomenon called "bystander suppression" (Maeda et al. 2008; Schwarz 2008).

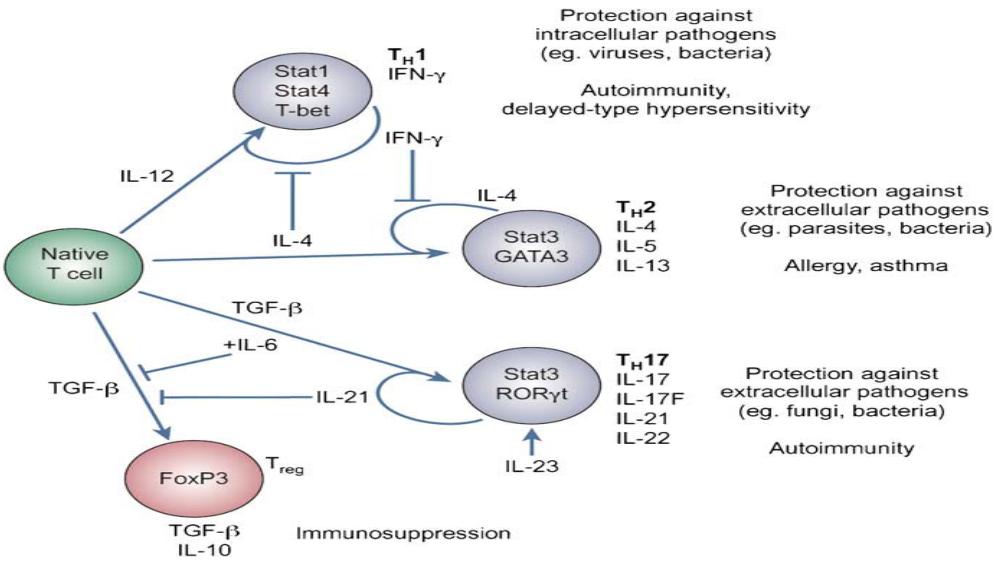


Figure 1.5 - Cytokine directed differentiation of naïve T helper cells (Th0)

(Deenick and Tangye 2007)

1.2.1.3.3 T Cytotoxic Cells (TC)

TCs are essential for immunity against intracellular pathogens and tumor cells. TCs express the CD8 surface glycoprotein and are therefore sometimes referred to as CD8+ T cells. The TcR of TCs can recognize specific antigenic peptides bound to Class I MHC molecules; the CD8 surface protein is attracted to the non-variable portions of the Class I MHC molecule. This affinity between CD8 and the MHC molecule keeps TC and the target cell bound closely together during antigen-specific activation. Tc-related molecules include perforin, granzyme B and FasL (Wang and Mosmann 2001).

1.2.1.3.4 Natural Killer (NK) Cells

NK cells (also called large granular lymphocytes) do not have the specificity and memory properties of T lymphocytes. They are a type of cytotoxic lymphocytes that are an important part of the innate immune system. NK cells were given their name because initially it was thought that they can kill cells without a need for antigen processing or presentation by MHC molecules. The major task of natural killer (NK) cells is to eliminate virus infected or malignant cells (Whiteside and Herberman 1994).

1.2.1.3.5 Monocytes

Monocytes are produced by the bone marrow from hematopoietic stem cell precursors called monoblasts. Monocytes circulate in the bloodstream for about one to three days and then typically move into tissues throughout the body (Ziegler-Heitbrock 2000). After monocytes migrate from the bloodstream to the tissues, they then differentiate into different types of tissue resident macrophages or dendritic cells. Monocytes, in response to inflammatory signals, move quickly (approx. 8-12 hours) to the site of infection/inflammation where they are responsible for the phagocytosis of foreign substances. Monocytes are also capable of killing infected host cells via antibody-mediated cellular cytotoxicity (Schwarz. 2003).

1.2.2 Soluble Factors of the Skin Immune System

Cytokines (proteins) and eicosanoids (lipids) are two important classes of soluble immune response mediators.

1.2.2.1 Cytokines

Cytokines are signalling peptides that consist of water-soluble proteins and glycoproteins that play an important role in intracellular communication. Cytokines are critical to the functioning of both innate and adaptive immune responses and balance between cell-mediated and humoral immunity. Cytokines are secreted by immune cells which have encountered an antigen as a way to activate and recruit more immune cells and increase the system's response to this antigen.

Cytokines demonstrate redundancy where more than one cytokine can perform the same immunological function and pleiotropism in which a single cytokine can perform diverse actions, either on the same cell or different cell types; a fact that does not allow strict categorization. Cytokines have been given various names depending on such things as their cell of origin, function and target of action (Schwarz. 2003). Examples of such names include:

o Lymphokines: a name used to describe cytokines thought to be exclusively made by lymphocytes.

o Interferon (IFN): were given their name because they interfere with viral replication.

o Interleukin (IL): was used to define cytokines that are produced by one leukocyte and target another, but the term is now used to describe newer cytokines with no bearing on their function or target. The vast majority of these interleukins is produced by T helper cells.

o Chemokines: used to describe cytokines that are important in chemoattraction (chemotaxis) between cells and therefore play an important role in leukocyte migration. It is generally accepted that chemokines are important mediators for UVB-induced inflammatory response. Amongst those mediators, IL-8 is one of the most comprehensively studied.

1.2.2.2 Eicosanoids

Eicosanoids come from the Greek word “eicosa” which means twenty. They are twenty carbon compound signalling molecules derived from either n-3 or n-6 PUFAs following metabolism by cycloxygenase (COX) or lipoxygenase (LOX) enzymes. For each eicosanoid, there are two or three separate series (e.g. Prostaglandin E1, 2 & 3), derived either from an n-3 or n-6 polyunsaturated fatty acid (PUFA). The different activities of each series largely explain the health effects of n-3 and n-6 PUFAs. The nature of PUFAs, their metabolism and their products are discussed in detail in section 1.6.3.2.

1.3 Ultraviolet Radiation (UVR)

The sun radiates energy over a broad spectrum of wavelengths (figure 1.6). UVR has a shorter wavelength than visible blue or violet light and is responsible for sunburn and other adverse health effects. The biological consequences of UV exposure result from induced cellular damage mediated by altered signal transduction and gene activation (Vink and Roza 2001). The majority of human UVR exposure is due to sunlight; fortunately the majority of solar UV is shielded by the ozone layer before it reaches the earth's surface. Other sources of

UVR exposure include medical phototherapy lamps, sun beds and arc welding apparatus, unshielded fluorescent lamps and tungsten halogen lamps.

Types of UVR

Depending on wavelength, UVR is divided into three categories; UVA, UVB and UVC:

UVA: 320 – 400 nm

UVB: 290 – 320 nm

UVC: 200 – 290 nm

Although the Commission International d'Eclairage (CIE) defines UVB as ranging between 280-315nm, based on the biological effects of UV and the ozone filtration of wavelengths below 290nm, most photodermatologists define the UVB region as 290-320nm (Noonan and De Fabo 1992; Diffey 2002; Schwarz 2002; Matthews et al. 2010) Furthermore, based on it's photo-biological actions; the UVA region has been further divided into UVA1 (340-400nm) and the more biologically active, UVA2 (320-340nm) (Diffey 2002).

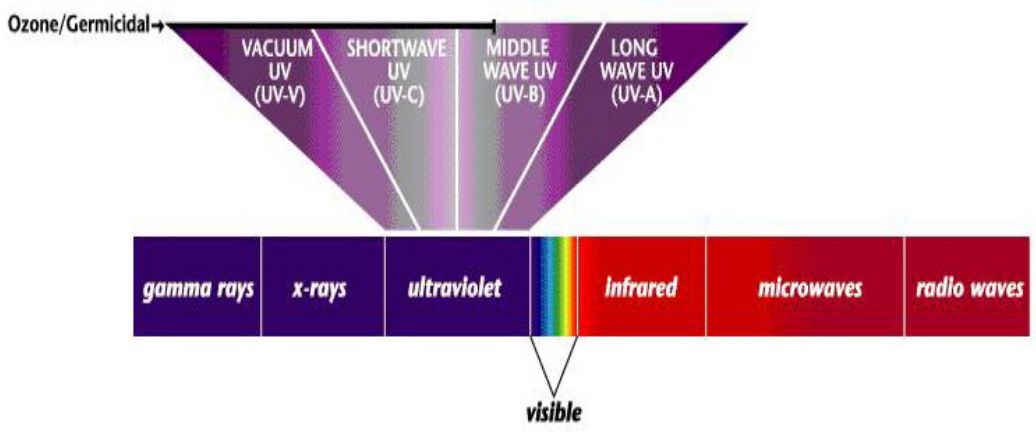


Figure 1.6 The Electromagnetic Spectrum

(http://www.uvabcs.com/uvlight.php)

1.4 Adverse Effects of UVR on Human Skin

Health benefits of the sun include vitamin D synthesis and generation of warmth and light. However, recent advancements in photobiology and photodermatology have identified solar radiation and in particular the UV component as a major environmental health hazard.

1.4.1 Acute Effects

1.4.1.1 Sunburn

Sunburn is the most well recognized acute cutaneous response to UVR and is principally due to ambient UVB (Werth et al. 2003). UVA can also cause skin erythema but approximately

1000 times the dose of UVB is required to produce the same effect (Henry W. Lim 2007). The sunburn response is associated with the classic inflammation signs of redness, warmth, pain and swelling (Nakaguma and Takahashi 1990; Kelley 2001).

Sun Reactive Skin Types

Skin colour is an important factor in determining the ease with which an individual will sunburn. Based on a personal history of sunburn and tanning responses to 45-60 min of exposure to midday summer sun in early June, Fitzpatrick grouped individuals into six sun-reactive skin types (table 1.1) (Pathak et al. 1999).

Table 1.1 - Fitzpatrick's Classification of Sun Reactive Skin Types

(Freedberg et al. 1999)

Skin Type	Skin reactions to solar radiation	Examples
I	Always burns easily & severely; tans little or none	People most often with fair skin, blue eyes, freckles.
II	Usually burns easily and severely; tans minimally or lightly	People most often with fair skin, red or blonde hair, blue, hazel or even brown eyes.
III	Burns moderately and tans about average	White-skinned Caucasian.
IV	Burns minimally, tans easily & above average with each exposure; exhibit IPD reaction (immediate pigment darkening on UV-exposure)	People with olive skin, dark brown hair, dark eyes (e.g. Mediterraneans and Hispanics)
V	Rarely bums, tans easily & substantially; always exhibits IPD reaction	Brown skinned persons (e.g. South Asian)
VI	Never burns and tans profusely; exhibits IPD reaction	People with black skin (e.g. African American Negroes and South Indian Aborigines)

Minimum Erythema Dose (MED)

A MED is defined as the lowest dose of UVR sufficient to produce a just visible erythema on exposed skin after 24 hours or, in a different definition, erythema with sharp margins after 24 hrs (Fourtanier et al. 2005). Since the two definitions will refer to different doses, it is essential to define the method of measurement used. The error in visual interpretation of the just visible erythema was found to be significantly less than that for sharp margins and thus this

definition appears to be preferable (Pathak et al. 1999). MED is commonly tested prior to commencing any UV skin treatment to determine the appropriate starting UV dose and to avoid doses that might cause uncomfortable/harmful erythema (Dawe 2005).

Time Course and Mechanisms

Erythema resulting from UVB-exposure is a delayed erythema response; first becoming evident 3 - 4 hours after exposure, reaching a peak by 8 to 24 hrs and persisting for more than 48 hours (Rhodes et al. 2001). This prolonged nature of UVB-induced erythema suggests that the mediators inducing it are either mediators with a very long half life or that there is a continued synthesis of the mediators throughout the UVB sunburn response. Prostaglandin E2 (PGE_2) and nitric oxide (NO) have both been implicated as mediators of the sunburn erythema response (Strickland et al. 1997; Rhodes et al. 2001); systemically, intradermally or topically applying the cycloxygenase inhibitor, indomethacin or applying the nitric oxide synthase (NOS) inhibitor, L-NMMA led to a reduction in vasodilatation and sunburn erythema (Rhodes et al. 2001).

1.4.1.2 Photodermatoses

The term photodermatoses generally refers to skin disorders caused by an abnormal response to UVR (UVA and/or UVB) or visible light. Photodermatoses are classified into cutaneous diseases caused entirely by solar exposure (including the immunologically mediated, drug/chemical induced and those caused by defective DNA repair) and photoaggravated dermatoses (Table 1.2).

Table 1.2 - Classification of Photodermatoses

(Henry W. Lim and John L.M. Hawk 2007)

Immunologically Mediated	**Defective DNA Repair**
Polymorphous light eruption	Xeroderma pigmentosum
Chronic actinic dermatitis	Cockayne syndrome
Solar urticaria	UV-sensitive syndrome
Actinic prurigo	Trichothiodystrophy
Juvenile spring eruption	Bloom syndrome
Hydroa vacciniforme	Rothmund-Thomson syndrome
	Kindler syndrome
Drug and Chemical-induced	**Photoaggravated**
Exogenous: phototoxicity and photoallergy	Lupus erythematosus
Endogenous: cutaneous porphyrias	Dermatomyositis
	Others

1.4.2 Chronic Effects

1.4.2.1 Photocarcinogenesis

Skin cancer is the most commonly diagnosed malignancy worldwide. Each year, more than 1 million people are diagnosed with skin cancer while the annual incidence of all other cancers combined is 1.3 million. Common skin cancers in humans are BCC and SCC; collectively known as nonmelanoma skin cancer (NMSC) and malignant melanoma (MM). Although MM only accounts for 4% of skin cancers, it is responsible for more than 70% of deaths caused by cutaneous malignancies (Jensen et al. 2007).

Risk factors for photocarcinogenesis

o Ultraviolet Radiation

Although most of our information on UV-induced carcinogenesis comes from investigating the process in animal models (particularly in mice), epidemiological studies also indicate that UVR-exposure is the primary cause of skin cancer in humans (Beissert et al. 2001; Aubin 2003). UVR is a complete carcinogen, initiating DNA damage that can lead to the induction of photocarcinogenesis and allowing this highly antigenic UVR induced skin tumor to escape tumour surveillance and grow unopposed via photoimmunosuppression (PI) (Kripke et al. 1996; Berneburg and Krutmann 2000; Beissert et al. 2001; Murphy 2009).

Analysis of DNA mutations from skin tumors helped make a link between UV-exposure and NMSC (Ortonne 2002). When a cell has too much DNA damage for successful repair, it is instructed to self destruct in a process called apoptosis. One of the pivotal genes for apoptosis is the p53 tumor suppressor gene (Murphy 2009). Approximately 75% of all skin cancer patients were found to have UV-related p53 mutations in non malignant sun exposed skin (Vink and Roza 2001). In addition, more than 90% of SCC and 50% of BCC showed UV-induced p53 mutations (CPD could be localized within the gene) (Beissert et al. 2001).

During the past century, life style changes including recreational activities and clothing styles resulted in increased sun exposure. People who spend the most time outdoors, such as those with outdoor occupations, generally have a higher incidence of SCC than those who work indoors. This suggests the possibility of a dose-related response between sunlight exposure and the incidence of skin cancer.

Skin Pigment is protective against sun burn and also against skin cancer. An example of this is seen in the high incidence and early occurrence of SCC in African albinos, compared with the extremely low incidence in their pigmented counterparts (Pathak et al. 1999).

o Genetic Diseases

In addition to albinism, there are other genetic diseases associated with increased skin cancer incidence on sun-exposed body sites e.g. xeroderma pigmentosum which is characterized by defective DNA repair leading to extreme sun sensitivity and multiple skin cancers on sun-exposed body parts (Kamide 2000).

o Personal/ Family History

Once a person develops NMSC, the risk of developing a second primary cutaneous tumor is greatly increased compared to the risk of a person of comparable skin type. This is probably due to those patients already having UV-damaged skin elsewhere (Trakatelli et al. 2007).

o Chronic Immunosuppression

One factor that has been clearly associated with an increased risk of NMSC is chronic immunosuppression; this implies a person's immune status is an important risk factor for developing skin cancer (Oberyszyn 2008).

Action spectrum for photocarcinogenesis

An action spectrum defines the wavelength dependence for eliciting a specific photo biological effect; it is used as a "weighting factor" to define a given UV spectrum's actual biological effectiveness e.g. photocarcinogenesis and erythema weighting (Figure 1.7). For obvious ethical reasons, direct information on carcinogenicity of different UVR wavelengths can only be obtained from experimental animal models. The peak carcinogenic wavelength of UVR in albino mice was found to be 293nm. After taking into account differences in epidermal UV-transmission between mice and human skin, the peak carcinogenic UV wavelength in humans was estimated to be approximately 300nm (Ortonne 2002).

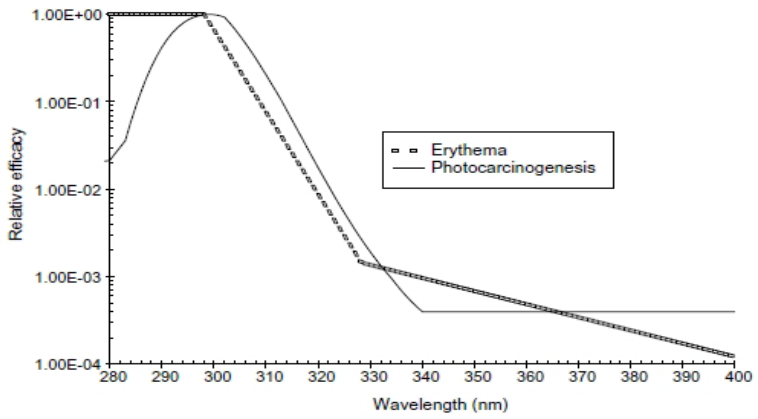


Figure 1.7 - Commission Internationale de l'Eclairage (CIE) (1987) reference action spectrum for erythema (24h after UV-irradiation) & photocarcinogenesis in human skin (Ortonne 2002)

1.4.2.1.1 Malignant Melanoma (MM)

MM, the most serious form of skin cancer is a neoplasm of melanocytes. It is one of the most aggressive cancers seen in adults and has the potential for early metastasis. The incidence and mortality of MM in the United Kingdom has risen dramatically; in the past 20 years, in England and Wales the mortality rate of MM rose by over 100% in males and 50% in females (www.cancerresearchuk.org).

Many dermatologists believe there is a link between intense intermittent UVR exposure, the number of sunburn episodes (particularly during childhood) and the incidence of melanoma &/or BCC later on in life (Tan 2011). Since the number of sunburn episodes in a person's life is related to their skin type, light skinned individuals are more susceptible to MM compared to darker coloured persons.

1.4.2.1.2 Non Melanoma Skin Cancer (NMSC)

NMSCs are generally less serious than melanomas. If detected and treated early, NMSCs can have a cure rate as high as 95% (Madhu A. Pathak et al. 1999). Nevertheless, if left untreated they can spread causing disfigurement and more serious health problems. There are two primary types of NMSC:

1.4.2.1.2.1 Basal Cell Carcinoma (BCC)

Although BCC is the commonest type of skin cancer, it has the lowest mortality of any cutaneous malignancy. BCCs commonly arise on the head and neck but can occur on other skin areas. BCCs grow slowly and rarely metastasize but can penetrate deep; hence the popular term “rodent ulcer” (Schwarz. 2003).

BCCs show a keratin profile that is distinct from the surrounding basal layer of epidermis. When proteins of different hair follicle compartments (cytokeratins) were compared to those of BCC, the closest resemblance was to the outer root sheath. It is hypothesized that stem cells from the bulge region of the outer root sheath give rise to BCCs (Donovan 2009).

Based on growth pattern, BCCs are essentially classified into two subsets; indolent (slow growing) and aggressive-growing. Indolent BCCs can be found on both sun-exposed and sun-protected skin, whereas the aggressive-growing subtypes are most frequent found on sun-exposed skin (Crowson 2006).

1.4.2.1.2.2 Squamous Cell Carcinoma (SCC)

SCC commonly occurs on the sun-exposed skin of elderly individuals. It tends to present as a papule or nodule with or without scaling and ulceration. This cancer can grow into a large mass and unlike BCCs; it can spread to other parts of the body causing significant morbidity

and mortality. Histologically, SCCs are composed of interconnected cords and nests of atypical epidermal keratinocytes that later invade the dermis with partial detachment from the originating epidermal keratinocytes.

Bowen's disease is an *in situ* form of SCC. Because it is confined to the epidermis, there is no risk of metastasis with Bowen's disease. If not treated however, Bowen's disease can progress to invasive SCC with risk of metastasis (Yanofsky et al. 2011).

Keratoacanthomas are a rapid growing SCC variant that usually present as a crateriform (bowl shaped) nodule on sun-exposed skin of elderly, fair-skinned individuals. In addition to UV exposure, other predisposing factors for the development of SCCs include chronic scarring skin conditions such as lichen planus, discoid lupus erythematosus and thermal burns (Rinker et al. 2001)

1.4.2.2 Photoageing

Photodamaged skin is found in sun exposed areas such as the face, neck, forearm and upper chest. Most of the visible skin changes of dermatoheliosis can be attributed to a change in the extracellular dermal components. Clinically, photoaged skin shows a loss of elasticity, increased dryness, a yellowish and irregular pigmentation and coarse, deep rather than fine wrinkling, resulting in a "leathery skin" appearance. In addition, photoaged may show a variety of premalignant lesions such as actinic keratosis (Pathak et al. 1999) .

1.5 Photoimmunosuppression

UVR can suppress the skin's immune system both locally and systemically (Vink et al. 1998; Beissert et al. 2001) (figure 1.8). Local immunosuppression refers to the situation where the antigen is applied to the irradiated skin site while in systemic immunosuppression; the antigen is applied to a skin site distant to that which was irradiated (Chung et al. 1986; Noonan and De Fabo 1992). High doses (5-30 kJ/m^2) of UVB are required to inhibit the immunity at distant non-exposed sites, whereas much lower doses (2 kJ/m^2) are needed to induce local PI (Schwarz et al. 1996; Vink et al. 1998); local PI by UVB is caused even at suberythemal doses (Cooper et al. 1992; Fuchs and Packer 1999; Young 2003). Different mechanisms seem to be involved in systemic and local immunosuppression. The depletion of LCs appears to play a critical role in local while, even though UVR has a limited capacity to penetrate the skin it produces systemic immunosuppression by stimulating the release of soluble mediators and cytokines like PGE_2, cis-UCA, IL-10, IL-4 and IL -13 in both mice and human models (Noonan and De Fabo 1992; Schwarz et al. 1996).

In both local and systemic immunosuppression, UV-irradiation can compromise the immune system in an antigen-specific fashion via the induction of immune tolerance; antigen specific Tregs can be found in the skin's draining LNs and spleen following UV-irradiation (Schwarz 2005; Schwarz 2008). When UV-induced tumor cells were transferred to syngeneic UV-irradiated mice, they were allowed to grow. Cells from chemical induced tumors however, were rejected when transferred into the UV-irradiated mice which indicates that UV-irradiation induces T cells that specifically inhibit immunity against UV-induced tumors (Schwarz 2008).

Examples of the immunosuppressive effects of UV-exposure in humans include diminished CHS responses and re-activation of herpes simplex following sun exposure (Damian et al. 2001; Norval 2006).

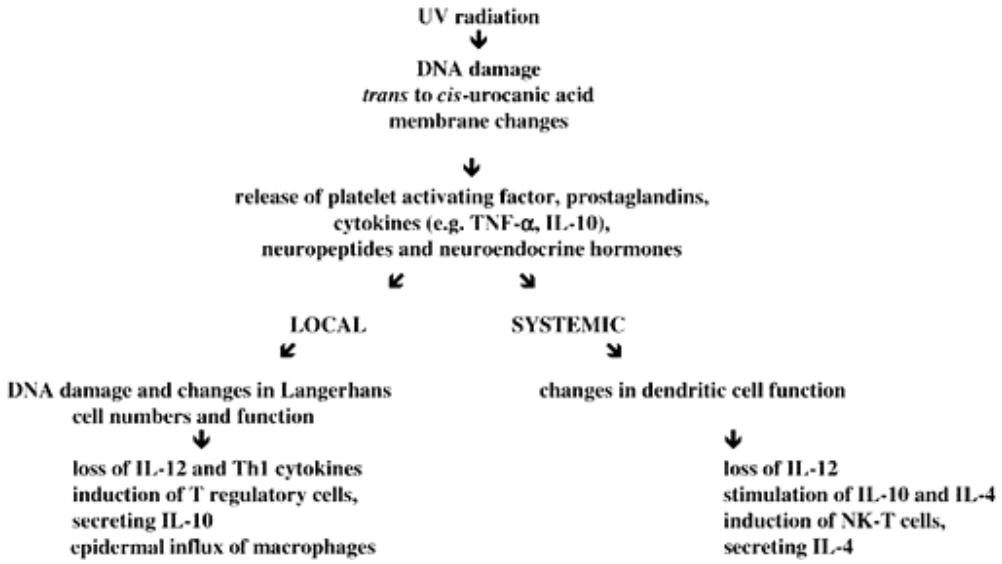


Figure 1.8 - Pathways Leading to Local and Systemic Photoimmunosuppression
(Norval 2006)

1.5.1 Chromophores for Photoimmunosuppression

For UV to initiate PI or any other biological effect, it must first be absorbed by molecules in the skin known as chromophores. Each type of chromophore absorbs a unique combination of wavelengths commonly referred to as the chromophore's absorption spectrum. Following the absorption of UVR, skin chromophores translate the UV-photon energy into biochemical signals and subsequently cellular responses. Each UV-induced biological response requires different wavelengths for it's generation; this is commonly referred to as the response's action spectrum e.g. the action spectrum for photocarcinogenesis and PI (Vink et al. 1996; Vink et al. 1998; McLoone et al. 2005). When a chromophore's absorption spectrum is similar to the action spectrum for a given response, this strongly implicates it as being at least partially

responsible for inducing the response. For example similarity between the action spectra for CPD formation and erythema implicated DNA as being the chromophore responsible for erythema (Young et al. 1998). Several molecules have been proposed as the initiating PI chromophore:

1.5.1.1 Cellular DNA

DNA is a major UVR target. UV absorption by cellular DNA results in various forms of DNA damage with an interruption in cellular transcription and replication (Kripke et al. 1996; Norval 2002). Cellular DNA is especially efficient at absorbing the shorter UVB wavelengths of sunlight (290-320 nm). Peak DNA absorption is at 260nm (Vink and Roza 2001). DNA can be damaged/mutated by UV, both directly through DNA absorption of photons and indirectly through UV-generated reactive oxygen species (ROS) which result in oxidative DNA damage (Swindells and Rhodes 2004). UV-induced DNA damage is very common, with each cell in the skin undergoing as many as 50-100 per second of UV exposure (Goodsell 2001). Most of those lesions are corrected within seconds by a process called nucleotide excision repair (NER) before causing any permanent damage (Berneburg and Krutmann 2000; Goodsell 2001; Vink and Roza 2001). It has been suggested that the repair of CPDs stimulates skin pigmentation which in turn protects against further DNA damage (Bestak and Halliday 1996).

UV-induced DNA damage is a major molecular trigger for PI; the primary molecular event mediating UV-induced immunosuppression is postulated to be the formation of DNA based pyrimidine dimers (Kripke et al. 1992; Vink et al. 1998; Kuchel et al. 2005). The major pyrimidine dimers produced by DNA photodamage are cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (Berneburg and Krutmann 2000; Goodsell 2001; Vink and Roza 2001). UV-induced dimer formation leads to the keratinocyte release of immunosuppressive cytokine such as IL-10 and TNF-α (Rivas and Ullrich 1994); the action spectrum for UV-induced TNF-α release by basal keratinocytes and CPD formation were found to be identical (Walker and Young 2007). Topical treatment of patients with past history of skin cancer with liposomes containing T4 endonuclease significantly reduced UV-induced mRNA upregulation of IL-10 and TNF-α (>90%) (Wolf et al. 2000). In a study by Kripke et al. (1992), introducing a dimer specific repair enzyme into mice epidermis following UVR exposure prevented the suppression of both DTH and CHS responses and inhibited the formation of LN suppressor cells. Furthermore, XPA knockout mice (XPA protein participates in the initial step of NER) showed extreme sensitivity to PI of CHS (Vink et al. 1998).

1.5.1.2 Urocanic acid (UCA)

UCA is an isomer of deaminated histidine that is synthesized by keratinocytes during the process of keratinisation (Beissert et al. 2001). UCA is a major stratum corneum chromophore; compared to wild type mice, aqueous extracts from the stratum corneum of histidinaemic mice (mice with histidase gene mutations causing diminished UCA levels) showed a significant reduction in UVB absorption (Barresi et al. 2011).

UV-irradiation results in the photoisomerization of trans-UCA (the major skin UCA isomer) to cis-UCA, which was found to have immunosuppressive properties (Noonan and De Fabo 1992; Beissert et al. 2001; McLoone et al. 2005). In mice, both topical and systemic cis but not trans-UCA, induces immunomodulatory effects that closely resemble PI (Gibbs et al. 2008). Applying cis-UCA 24 hours prior to the DNCB sensitization of Balb/c mice significantly reduced ear swelling responses to DNCB and induced a prolonged (13 weeks) state of immune tolerance (Wille et al. 1999). Intradermal injection of cis-UCA in mice altered the morphology of LCs and impaired the induction of CHS (Kurimoto and Streilein 1992). Furthermore, cis-UCA found in the serum of UV-irradiated mice inhibited tumor antigen presentation by LCs and injecting mice with an antibody to cis-UCA led to approximately a 60% reduction in PI and significantly reduced photocarcinogenesis (Hart et al. 1997). Moreover, feeding mice a histidine-rich diet increased epidermal UCA and enhanced PI while histidinaemic mice showed resistance to PI (Norval and El-Ghorr 2002). In addition, treating human keratinocytes with cis-UCA led to the upregulation of immunomodulatory mediators such as PGE_2, TNF-α and IL-6 (Kaneko et al. 2009).

A keratinocyte receptor specific to cis-UCA is yet to be identified. It was postulated that cis-UCA may act through the 5-hydroxytryptamine (5HT2A) serotonin receptor or the platelet activating factor (PAF) receptor. However, treating primary human keratinocytes with antagonists to both receptors did not prevent COX-2 gene expression and PGE_2 production or TNF-α and IL-6 secretion in response to cis-UCA; this suggests that keratinocyte stimulation by cis-UCA is done outside those receptors (Kaneko et al. 2009).

The role of UCA in PI still needs to be further explored. Although UCA has recently been shown to protect against UV-induced CPD formation (Barresi et al. 2011), it was also found to induce oxidative DNA damage and lead to the expression of UV-induced 6,4 photoproducts in vivo and CPD in vitro. Despite this however, no direct relationship was found between UCA and photosensitivity; implying that UCA can be acting in a multi-factorial manner involving more than one UV-induced pathway. To further investigate the role of UCA in UV-induced carcinogenesis, a study comparing photocarcinogenesis in wild-type vs. histidinaemic mice might perhaps now be warranted (Gibbs and Norval 2011).

1.5.1.3 Membrane Lipids

The absorption of UV by membrane lipids can induce signalling pathways directly via lipid peroxidation or indirectly through the induction of oxidative DNA damage (Vink et al. 1998; Murphy 2009). UV-irradiation alters the redox equilibrium of a cell which, upon exceeding the cell's capacity to counterbalance leads to free radical formation, oxidative stress and subsequent oxidation of lipids, proteins and DNA. Lipid peroxidation at the plasma membrane leads to enzyme activation with subsequent expression of several mediators such as platelet activating factor (section 1.5.3.1) as well as phosphorylation and activation of important transcription factors such as nuclear factor-kappa β (NF-kβ) (Norval 2006; Zhang et al. 2008).

1.5.2 Protein Mediators of Photoimmunosuppression

Cutaneous UV-irradiation stimulates the release of several protein mediators (cytokines) that are involved in the mechanisms of PI (Figure 1.8).

1.5.2.1 Interleukin-4 (IL-4)

The major signal for Th2 differentiation is IL-4. UV-irradiation induces serum IL-4 in a dose dependent manner which favours a shift towards Th2 responses while inhibiting those of Th1; compared to unirradiated control mice, T cells from UV-irradiated mice secrete less IL-2 and IFN-γ but more IL-4 (Shreedhar et al. 1998) (for more details, please see section 1.5.3.2).

IL-4 was found to have immunosuppressive properties; injecting UV-irradiated mice with anti IL-4 blocks immune suppression. Moreover, transferring immune suppression through the adoptive transfer of T cells from UV-irradiated mice to normal recipient mice was prevented by injecting the recipient mice with anti IL-4 beforehand (Ullrich 1996).

There is no evidence however to support the secretion of IL-4 by UV-irradiated keratinocytes. Instead, it has been postulated that prostaglandins released by UV-irradiated keratinocytes are responsible for the induction of serum IL-4 and subsequently IL-10. Injecting normal mice with PGE_2 induced serum IL-4 and IL-10. Furthermore, treating UV-irradiated mice with a COX-2 inhibitor led to blockage of IL-4 production and treating them with anti-IL-4 suppressed serum IL-10 (Shreedhar et al. 1998).

1.5.2.2 Interleukin-10 (IL-10)

IL-10 was found to be essential for UV-induced systemic immunosuppression and photocarcinogenesis. The expression of IL-10 can be seen in invasive BCC and secretion of IL-10 by MM denotes poor prognosis (Rivas and Ullrich 1994; Enk et al. 1995). Furthermore,

treating UV-irradiated mice with anti-IL-10 blocked UV-induced immunosuppression of established immune responses (Nghiem et al. 2002).

In a study by Loser et al. (Loser et al. 2007), chronic UVB irradiation of IL-10 knockout mice (IL-10-/-) failed to develop skin cancer, dysplasia or even mitosis (mice were put under observation for a year). Irradiation of IL-10+/+ or IL-10+/- mice on the other hand, showed comparable levels of UV-induced skin tumors; mainly poorly differentiated SCCs. Moreover, IL-10-/- but not IL-10+/+ mice were immune to the development of UV-induced hapten-specific tolerance. Similar to IL-4, IL-10 also showed an antagonizing role to Th1 responses. When supernatants from stimulated spleen and LN cells taken from IL-10+/+ and IL-10-/- mice were assayed for cytokine presence; T cells from IL-10-/- mice had significantly higher levels of the Th1 cytokine, IFN-γ and lower levels of the Th2 cytokine, IL-4 (Loser et al. 2007).

IL-10 is not only implicated in PI, it is also heavily involved in UV-induced hapten specific tolerance mediated by UV-induced Tregs. UV-induced Tregs (CD4+ CD25+ CTLA-4+) secrete large amounts of IL-10 and ultimately mediate UV-induced hapten-specific tolerance. Treating naïve recipient mice with anti-IL-10 antibodies (Ab) prior to the intravenous injection of spleen and regional LN cells from syngeneic, UV-tolerized mice blocked the transfer of tolerance. In addition, culturing Tregs from the LN and spleen of UV-tolerized mice with bone marrow derived DCs and the DNBS hapten (water soluble DNFB) for 11 days led to a significant increase in IL-10 secretion by Tregs. This increase was blocked by adding CTLA-4 Ab; confirming that CTLA-4+ Tregs were the source of IL-10 (Schwarz et al. 2000).

IL-12, a pivotal Th1 cytokine with antitumor/anti immunosuppressive and the ability to reverse UV-induced tolerance (Schwarz et al. 1996; Schwarz and Schwarz 2002) was found to directly suppress IL-10 secretion from irradiated keratinocytes cells (Beissert et al. 2001).

1.5.2.3 Tumor necrosis factor alpha (TNF-α)

TNF-α secretion by skin keratinocytes and fibroblasts is normally elevated following UVB but not UVA exposure and has been shown to play a significant role in PI (Werth et al. 2003). Systemic administration of anti TNF-α antibody abolishes PI and mice genetically deficient in TNF-receptor 2 did not show an inhibition of their CHS responses to DNFB following irradiation with UVB (Kurimoto and Streilein 1992). In addition, TNF-α has been shown to produce morphological changes in LCs identical to those induced by irradiation with UVB (Kurimoto and Streilein 1992).

TNF-α together with IL-1β were found essential for the migration of LCs. Intradermally injecting IL-1β in human skin stimulated the migration of LCs; a significant reductions in LCs

was noted at both 2 and 4 hours. Suction blister fluid analysis showed significant levels of TNF-α. Topical treatment with human recombinant lactoferrin inhibited IL-1β production and blocked LC migration. Taken together, these findings demonstrate the association of IL-1β with epidermal LC migration and local TNF-α production (Cumberbatch et al. 2003).

1.5.2.4 Interleukin-8: The neutrophil-activating chemokine

IL-8, a potent chemokine and inflammatory mediator, is constitutively expressed in the epidermis. It is up regulated in human keratinocytes following UVB-irradiation in both in vitro and in vivo studies, which would explain the accumulation of neutrophils at the site of irradiation (Baggiolini and Clark-Lewis 1992; Storey et al. 2007). Neutrophils attracted into the dermis by IL-8 mediate inflammation and cause tissue damage secondary to the release of reactive oxygen species (Storey et al. 2007).

In a study by Strickland et al (Strickland et al. 1997) several components of UV-induced skin inflammation were examined over a 24 hour period. In addition to PGE_2 and NO, the increased mRNA expression of the chemokines IL-8 and TNF-α and upregulation of the endothelial adhesion molecule, E-selectin appeared to play an important role in UV-induced skin inflammation in particular, post-UV neutrophillic infiltration that peaked 24 hours post-UV.

In addition to inflammation, IL-8 is also a mediator of angiogenesis and is being associated with tumor growth including the development of malignant melanoma (Storey et al. 2007). Furthermore, IL-8 acts as a stimulus for the keratinocyte synthesis of the platelet activating factor (PAF) with resulting PAF-induced gene transcription for two important mediators of systemic PI, COX-2 and IL-10 (for more details on the immunosuppressive properties of PAF, please see section 1.5.3.1) (Konger et al. 2008).

1.5.3 Lipid Mediators of Photoimmunosuppression

In addition to the secretion of immunosuppressive cytokines, cutaneous UV-irradiation induces lipid peroxidation and release of lipid mediators such as platelet activating factor (PAF) and PGE_2 (Halliday 2005).

1.5.3.1 Platelet Activating Factor (PAF)

As previously outlined in section 1.5.1.3; the absorption of UV by membrane lipids leads to lipid peroxidation and subsequent enzyme activation. Synthesis of PAF is mediated through the UV-activation of a two-enzyme pathway in the presence of an arachidonate-containing phospholipid. Initially, phospholipase A_2 (PLA_2) releases an arachidonic acid side chain from the plasma cell membrane constituent, phosphatidylcholine to generate lyso-

phosphatidylcholine. An acetyl residue from acetyl CoA (a co-enzyme containing acetyl transferase) is then transferred to lyso-phosphatidylcholine to form PAF. The released arachidonic acid side chain is either re-acylated during the formation of new phosphatidylcholine or is used as a substrate for eicosanoid synthesis (Barr et al. 1993; Walterscheid et al. 2002; Konger et al. 2008; Travers et al. 2010).

The platelet activating factor receptor (PAF-R) is found on a variety of cells including monocytes, mast cells and keratinocytes (Dy et al. 1999; Kaneko et al. 2009). Although PAF is produced enzymatically, UV can also produce agonists of the PAF-R non-enzymatically through the oxidation of phospholipids with PAF-like activity (Travers et al. 2010) . The major PAF-metabolizing enzyme is PAF-acetylhydrolase (Pei et al. 1998). Oxidative stress is reported to inactivate PAF-acetylhydrolase (Dy et al. 1999).

Upon binding to its receptor, similar to UV-irradiation, PAF induces gene transcription for two important mediators of systemic PI, COX-2 and IL-10. In agreement with this, and implying PAF-dependency, in a dose dependant manner UVB-induced systemic immunosuppression of DTH was blocked in wild type mice upon pre-UVB intraperitoneal injection of the PAF-receptor antagonist, PCA-4248. To exclude possible specificity to PCA-4248, the same experiment was repeated with similar results achieved using two unrelated PAF-R antagonists. Furthermore, both PAF and UVB-induced suppression of DTH were blocked using a selective COX-2 inhibitor or IL-10 neutralising antibody. Since cutaneous synthesis of PAF leads to upregulation of COX-2 gene transcription and arachidonic acid release (a substrate for COX-2) this consequently results in PGE_2 production. Taken together, these findings suggest that PAF-induces systemic immunosuppression through a cytokine cascade involving PGE_2 and IL-10 (Walterscheid et al. 2002).

In addition, PAF was also shown to increase the production of another UV-induced immunosuppressive mediator; TNF-α. Treatment of PAF-R +ve human epidermal KB cell lines with the carbamoyl PAF agonist (cPAF) or 400J/m^2 UVB led to a significant increase in TNF-α mRNA and TNF-α protein. The same could not be observed in PAF-R –ve KB cell lines. Furthermore, prior incubation of PAF-R +ve KB cell lines with 10 μm of PAF-R antagonists (WEB 2086 or A-85783) inhibited both the UVB and cPAF-induced TNF-α production (Dy et al. 1999).

1.5.3.2 Cycloxygenase Metabolites of n-6 PUFA

PUFA metabolism by COX is the first step in a cascade of reactions that give rise to a variety of molecules with prostanoic acid as their central structure; prostanoids. Prostanoids are a subclass of eicosanoids that includes prostaglandins, prostacyclins and thromboxanes.

The effects of prostanoids are exerted by G protein-coupled receptors on plasma membranes. The relative activity of those receptors plays a role in deciding a prostanoid's biological actions. In addition, the expression and distribution of enzymes that are involved in a prostanoid's synthesis also contribute to it's final action in a given cell. Furthermore, factors like cell type and nature of the stimulatory response can cause a prostanoid to have opposing outcomes (Gualde and Harizi 2004). An example of this are the actions of the major cutaneous prostanoid, PGE_2 which can be pro or anti-inflammatory, based on interactions with one of it's four E-receptors (EP1 to EP4) found in different cell types. When the expression patterns and mRNA levels of the various EP receptors were examined in murine and human NMSCs, a significant difference was noted between the cancer subtypes as well as between the sun-exposed and non-exposed skin which suggest that altered EP receptor expression may play a differential role in the development of UV-induced SCC and BCC in murine and human skin (Lee et al. 2005). Using selective antagonists to EP4, UVB-induced immunosuppression in mice models was blocked. In accordance with this, administering an EP4 agonist managed to restore UV-induced immunosuppression that was previously inhibited by the COX-inhibitor, indomethacin. Mice deficient in other EP receptors did not show similar resistance to PI (Soontrapa et al. 2011).

Based on their precursor PUFAs, eicosanoids are divided into n-3 and n-6 derived (Bagga et al. 2003). The n-6 membrane PUFAs, in particular AA, are considered precursors for a number of key UV-induced mediators of inflammation and immunosuppressive. EPA, an n-3 membrane phospholipid component competes with n-6 PUFAs as a substrate to COX. The action of COX on EPA results in the less potent PGE3 (Reeve et al. 1996; Moison and Beijersbergen Van Henegouwen 2001) (for details on the opposing actions of n-3 and n-6 PUFAs, please see section 1.6.3.2.6).

1.5.3.2.1 Prostaglandin E_2 (PGE_2)

Prostaglandins (PG) are bioactive lipids derived from the metabolism of membrane PUFAs. Prostaglandins play an important role in a number of biological processes including wound healing, inflammatory and immune responses. All prostaglandin contains 20 carbon atoms, including a 5-carbon ring.

UVR is a potent inducer of PGE_2. As previously outlined, UVR leads to the release of AA from the phospholipid domain of cell membranes following cutaneous UV-irradiation (Flower and Perretti 2005). Also, COX-2 (the type 2 isoform of COX) is rapidly induced by UVB-irradiation. Subsequently, this leads to AA metabolism by COX and formation of PGE_2. Furthermore, in

another non-enzymatic pathway, the UV-induced release of ROS leads to the oxidation of AA and further PGE_2 metabolism (Wiswedel et al. 2007).

PGE_2 is the principal eicosanoid understood to play a major role in skin immunity; it is a potent mediator of skin inflammation and PI (Fogh and Kragballe 2000; Gualde and Harizi 2004) and is implicated in tumorigenesis; increased levels of PGE_2 have been found in both BCC and SCC (Lee et al. 2005). Mice that were given SC pellets that slowly release indomethacin at 1.25μg/day over a 20 day period (a dose sufficient to block prostaglandin synthesis) showed resistance to the UV-induced suppression of both systemic and local CHS responses to DNFB (Chung et al. 1986). Moreover, while UV-irradiation upregulates serum levels of the immunosuppressive cytokine, IL-4 (section 1.5.2.1); this upregulation was suppressed by injecting UV-irradiated mice with the selective COX-2 inhibitor, SC236. This inhibition was dependent on the dose of SC236 injected; a complete inhibition was seen when 0.2μg of the COX-2 inhibitor were injected per mouse (Shreedhar et al. 1998). Furthermore, another murine study looking into the effects of topical naproxen (a non-specific COX inhibitor) on acute and chronic cutaneous UV-irradiation revealed a decrease in PGE_2 with resulting reduction in chronic UV-induced skin tumor incidence (Gonzalez Maglio et al. 2010). In addition, topical celecoxib treatment of human subjects following UVB-irradiation inhibited the cutaneous production of PGE_2 with the resulting suppression of skin inflammation and oxidative damage. Also, long term studies of topical celecoxib were found to be effective in decreasing malignant and pre-malignant skin lesions with a reduction in UVB-induced carcinomas and papillomas (Wilgus et al. 2003) and PI of CHS responses were abrogated by treatment with inhibitors of PG synthesis. The function of Tregs; a key component of PI was additionally found to be PGE_2-dependent (Black and Rhodes 2006).

1.5.3.2.2 Lipoxygenase Metabolites of n-6 PUFA

In addition to being metabolized by COX, AA released from membrane phospholipids following UVR exposure is also used as a substrate for another enzyme; lipoxygenase (LOX). The initial product of AA metabolism by LOX is hydroxyeicosatetraenoic acid. When 5, 8, 12 or 15-LOX introduce an oxygen molecule into the respective AA backbone position; this gives rise to the unstable HPETEs. HPETEs then undergo reduction to their respective HETEs and under certain circumstances maybe further oxidize to diHETEs (Figure 1.9). An example of a diHETE is the biologically active leukotriene (LT) (produced by 5-LOX) (Fogh et al. 1992).

1.5.3.2.3 15-Hydroxyeicosatetraenoic & 15-Hydroperoxyeicosatetraenoic Acid

15- Hydroxyeicosatetraenoic (HETE) is produced in fibroblasts and keratinocytes by the actions of 5-LOX on AA (Figure 1.9). Unlike other LOX metabolites of AA, 15-HETE has no pro-inflammatory effects in the skin; 15-HETE can inhibit the synthesis of 5-LOX and 12-LOX

products that possess inflammatory and proliferative capacities (Fogh et al. 1992). Based on actions like the inhibition of IL-2-induced blastogenesis in CT-6 T cell lines, induction of Tregs and the suppression of IFN-production in lymphoma cells of murine experimental models, both 15-HETE and 15-HPETE are considered immunosuppressive (Vanderhoek 1988).

Peroxisome-proliferator activating receptor-gamma (PPARγ) is a nuclear hormone receptor that is involved in many biological processes including carcinogenesis and immune cell function. 15-HETE has shown the ability to activate PPARγ following UV-irradiation and this ability was found to be blocked by antioxidants. This indicated the photo-oxidation dependency of PPARγ activation and highlighted the oxidative stressor properties of 15-HETE. This finding was further supported using NO-dependent oxidative inflammatory reactions. COX-2 was found to be a target for PPARγ activation and this activation has been implicated in UV-induced carcinogenesis as well as local and systemic immunosuppression. Blocking the PPARγ receptor using selective, specific PPARγ antagonists led to the inhibition of COX-2 expression and subsequent decrease in production of immunosuppressive PGE_2 in KB cells. Taken together, these findings support an indirect, pro-oxidator dependent immunosuppressive role for 15-HETE (Gordon et al. 1990; Konger et al. 2008).

1.5.3.2.4 Leukotriene B_4 (LTB_4)

LTs are produced from AA by the actions of the 5-LOX enzyme (Figure 1.9) (Flower and Perretti 2005). Following reduction of HPETE to HETE and after the oxidation of HETE to LTA_4, the Leukotriene A4 hydrolase enzyme (LTA4H) then catalyzes LTA_4 to LTB_4.

LTB_4 is a potent mediator of cutaneous inflammation with polymorphonuclear leukocytes (PMN) chemoattraction, inflammatory cell activation and epidermal proliferation properties. In murine experimental models, LTB_4 levels show remarkable elevation following UVB-irradiation and this elevation is significantly suppressed following application of LOX enzyme inhibitors (Nakaguma and Takahashi 1990; Fogh et al. 1992).

Elevated LTB_4 levels are frequently found in skin cancer and the LTA4H enzyme is overly expressed in, and has long been associated with, chronic inflammation-associated carcinomas (Black and Rhodes 2006). In agreement with this, the LTA4H inhibitor, Bestatin was found to suppress tumorigenesis in animal models. Studies are now looking at LTA4H inhibitors or LTB_4 receptor antagonists as cancer preventative agents; alone or combined with other agents such as COX- 2 inhibitors (Chen et al. 2004; Fegn and Wang 2009).

In addition to chronic inflammation-associated carcinogenesis, in a similar manner to 15-HETE, LTB_4 contributes to cutaneous tumor-promotion via the induction of oxidative stress,

PPARγ activation and subsequent induction of COX-2 expression and the augmentation of PGE_2 release (Hedi and Norbert 2004; Konger et al. 2008) .

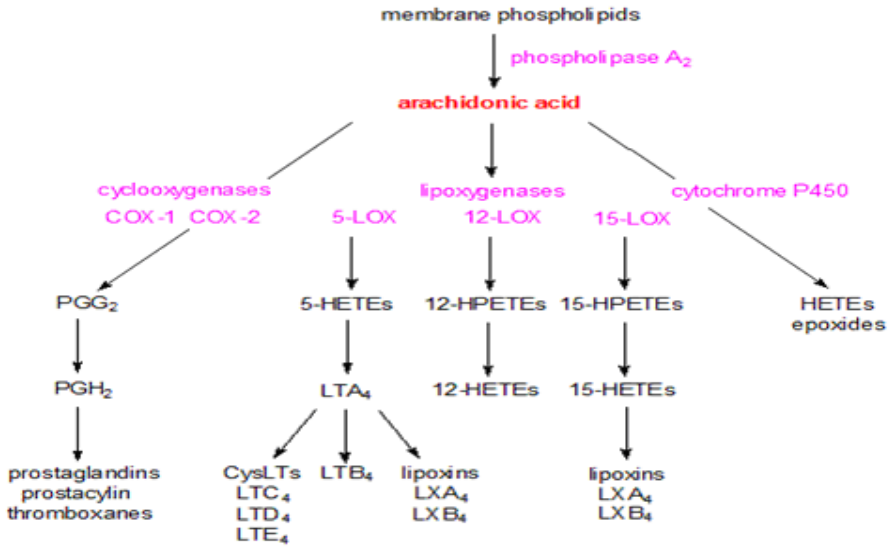


Figure 1.9 - n-6 PUFA Eicosanoid Biosynthesis Pathways

(http://lipidlibrary.aocs.org/lipids/eicintro/index.htm)

1.5.4 Cellular Responses in Photoimmunosuppression

1.5.4.1 Langerhans Cell Depletion

UV-irradiation of the skin leads to a significant drop in number, change in morphology and alteration in antigen presentation by LCs (Schwarz et al. 1996; Vink et al. 1998). Those changes are largely implicated as the main cause for local PI and the induction of UV-Tregs (section 1.5.3.3.). Although it is well documented, the mechanism of UV-induced LC depletion remains unknown (Burnham et al. 1993; Kolgen et al. 2002). Depletion of LCs may be partially explained by the loss of specific LC surface markers (e.g. Ia and ATPase) following UV-induced cell damage (Friedmann et al. 1983; Obata and Tagami 1985).

The two potential causes mostly commonly studied for LC depletion are migration of LCs from the epidermis to draining LNs and apoptosis. Kolgen et al. (Kolgen et al. 2002), used human models to examine this at several time points post UV-irradiation with x6 MED of TL 12 UVR. Punch biopsies were taken to assess apoptotic cells in the epidermis and suction blisters were raised to study LC migration. Apoptotic cells (detected using TUNEL and caspase3 staining) were found in conjunction with epidermal defects in the epidermis of punch biopsies and the roofs of suction blisters. When stained for the LC specific marker,

CD1a; no cells in the epidermis of punch biopsies and less than 3% of the apoptotic cells in suction blister roofs were CD1a+. In addition, most CD1a+ cells were found lower in the epidermis whereas the apoptotic, caspase 3+ cells were detected higher up; just below the stratum corneum. Taken together, these findings suggest that few LCs in skin biopsies or suction blister roofs undergo apoptosis after UVB-exposure; the majority of apoptotic cells found were most probably keratinocytes. On the other hand, when testing for DNA damage (thymidine dimers) was done using H3 staining; a large majority of cells in the epidermis of punch biopsies and all the cells in suction blister roofs were found to be H3+. To test whether the H3+ cells were LCs; double staining of the suction blister roofs and fluid with CD1a and H3 was performed. All suction blister roof cells and 27% of blister fluid cells double stained CD1a+ and H3+. This finding suggested that, rather than undergoing apoptosis; LCs remain viable but with DNA damage following UVB-irradiation. The fact that the number of DNA-damaged LCs in suction blister roofs did not correlate with those of suction blister fluid is partially explained by factors like the time it took to raise the suction blister, LC adherence to dermal blister floor and the blister cross section examined (Kolgen et al. 2002).

UV-induced DNA damage was found to be an important trigger for post-UV LC migration with altered LC antigen presentation and subsequent generation of UV-induced antigen specific Tregs (Vink et al. 1997; Vink et al. 1998; Kolgen et al. 2003; Schwarz et al. 2005). IL-12 was found to protect against PI via NER (Schwarz et al. 2005). Injection of IL-12 after UV exposure prevented the depletion of LC. The same could not be observed in NER-deficient, XPA knockout mice which imply that DNA damage plays an important role in post-UV depletion of LCs (Kolgen et al. 2003).

Repairing the DNA photodamage of LCs significantly reduces their UV-induced depletion, restores their Ag presenting capabilities which in turn abolishes Treg cells from draining LN cells and instead leads to the stimulation of Ag-primed IFN-γ producing T lymphocytes (Vink et al. 1997; Schwarz et al. 2005).

By examining cell surface markers of LCs in the draining LNs of UVB-irradiated skin, the upregulation of CD1d was noted. CD1d is a class I MHC molecule involved in antigen presentation to natural killer T-cells (NKT). While subcutaneously injecting the CD1d+ LCs into syngeneic wild type mice led to a dose dependent suppression of CHS, the sameCD1d+ LCs failed to induce immunosuppression in NKT-deficient mice. Furthermore, the NKT-deficient mice were resistant to immunosuppression following UVB-irradiation. When IL-4 secretion by the NKT cells isolated from LNs of UV-irradiated mice was measured; it was found to be significantly higher than that of non-irradiated NKTs. Taken together these findings indicate another photoimmunosuppressive role for skin residing LCs in which UV-

exposure activates mature, non-damaged LCs to migrate to the draining LNs and stimulate IL-4 secreting NKT cells (Fukunaga et al. 2010).

Injecting the diphtheria toxin (DT) into langerin-DTR mice (mice with the diphtheria toxin receptor inserted into the langerin locus) resulted in the complete depletion of LCs and langerin+ DDCs. UV-irradiating the langerin depleted mice for four consecutive days and then sensitizing them through the irradiated skin did not result in PI of CHS responses upon Ag challenge. In addition, injecting spleen and LN cells from those mice into syngeneic, sensitized mice did not lead to the transmission of tolerance and resulted in a normal CHS response. When the same experiment was repeated in the absence of LCs but not langerin+ DDCs; the mice continued to show resistance to UVR-induced inhibition of CHS and Tregs in both spleen and LN could not be detected. This showed that even though LC is not required for the induction of CHS, it is essential for the induction of PI and of UV- Tregs (Schwarz et al. 2010).

1.5.4.2 Th1/Th2 Balance

One potential mechanism that may explain the suppressive effects of UVR on immune reactions like DTH and tumor rejection may be a shift in the activation of T cells from a Th1 to a Th2 type response.

Th1 cells are critical for cell-mediated immunity such as CHS, DTH and tumor antigen responses (Muller et al. 1994; Schwarz et al. 1996; Beissert et al. 2001) (but not the initiation of irritant contact dermatitis or the induction of tolerance) (Muller et al. 1994). Th1 responses are characterized by the production of IFN-γ and were found to play an important role in inhibiting tumor incidence and growth. The finding of T cells that produce high levels of IFN-γ in LNs that drain a tumor site correlates with it's rejection (Kacha et al. 2000).
While normal LCs can present antigen (Ag) to both Th1 and Th2 cells, direct UV-irradiation of LCs blocked their ability to present Ag to Th1 but not Th2 (Simon et al. 1990). In addition, the Ag presentation by cells extracted from the spleen of UV-irradiated mice was suppressed to Th1 while enhanced to Th2 cells (Ullrich 1996). Compared to normal controls, T cells from UV-irradiated mice secrete less IL-2 and IFN-γ and more IL-4; indicating a shift toward a Th2 like reaction. IL-4 not only promotes naïve CD4+ T cells to differentiate into Th2 but also inhibits low dose IL-12 to induce the development of Th1 cell (Shreedhar et al. 1998).

1.5.4.3 T regulatory Cells

Ultraviolet (UV) radiation was found to induce immune tolerance in an antigen specific fashion through the generation of UV-induced Tregs (UV-Tregs) (Schwarz 2008). UV-Tregs express CD4, CD25, CTLA-4 (CD152), GITR (Glucocorticoid-induced TNF family-related receptor)

and bind the lectin dectin-2 (Maeda et al. 2008). Unlike natural Tregs that suppress immune responses through direct cell-to-cell contact, UV-Tregs exert their suppressive activities through the secretion of IL-10 upon hapten-specific stimulation/activation. Injecting UV-Tregs from spleen/LN cell extracts of UV-tolerized mice into IL-10-deficient mice successfully transferred tolerance which indicates that the IL-10 required for suppression is derived from UV-Tregs and not from the host cells (Maeda et al. 2008). In a TGFβ-dependant manner, both Foxp3+ and Foxp3- CD4+ T cells can be induced to express IL-10 (Maynard et al. 2007) and it remains unclear whether UV-Tregs express Foxp3; further UV-Treg characterization is required. The type of Tregs induced may vary depending on several factors including UV dose, mouse strain and type of hapten applied post-UV (Schwarz 2008).

UV-Treg induction is an active process requiring migration and subsequent Ag presentation by viable LCs with UV-induced DNA damage (Schwarz 2005; Schwarz et al. 2010). Accordingly, since IL-12 reduces UV-mediated DNA damage, it also prevents UV-Treg induction (Schwarz et al. 2005).

1.5.5 Action Spectrum of Photoimmunosuppression

Few data exists on the action spectrum of UVR-induced immunosuppression in humans (Matthews et al. 2010). Having a clear action spectrum for PI would help identify it's initiating chromophore. In addition, it would enable a more accurate hazard assessment and spectral weighting which would in turn allow for a better protection strategy.

The immune effects of different UV-wavebands must be interpreted in the context of their relative amounts in sunlight. Since the majority of ambient UVR is UVA (95%), multiplying the effectiveness of this waveband by it's abundance in the solar spectrum revealed it's relatively higher significance in inducing immunosuppression compared to UVB (Damian et al. 2011). Similarly, the abundance of ground level UVB (5% of ambient UVR) varies substantially at different wavelengths and this accordingly affects each wavelength's relative significance in inducing PI; UVB at 310nm is 40,000 times more abundant than at 290 nm and approximately 15 times more than at 300nm (Matthews et al. 2010).

Accordingly, when the immunosuppressive properties of ambient UVR were tested in humans using the CHS response to Ni, two peaks were found; a UVB peak at 300nm and a UVA peak at 370nm (Damian et al. 2011). In addition to highlighting the importance of broad spectrum sunscreens that protect against UVA and UVB, this finding suggested a different chromophore might be involved in UVA-induced immunosuppression.

UVA and Photoimmunosuppression

The majority of studies looking into the immunomodulating effects of UVR have focused on the UVB waveband which is 1000 times more erythemogenic than UVA and was seen as more immunosuppressive. UVA however, is more immunosuppressive than it is erythemogenic (Walker and Young 2007). Via ROS and NO, UVA induces oxidative stress that can subsequently cause damage to DNA, proteins and lipids and plays an important role in the immunomodulating effects of solar UVR (Halliday 2005).

UVA can cause both local and systemic PI and can induce UV-tolerance (Bestak and Halliday 1996; Damian et al. 1999; Byrne et al. 2002; Norval 2002; Baron et al. 2003; Murphy 2009). Human melanocytes and keratinocytes exposed to UVA or UVB in vivo showed comparable levels of thymine dimmers (Young et al. 1998) and skin exposed to UVA-tanning lamps showed p53 mutations induced by oxidative damage and alterations of the p53 protein similar to those of UV-exposed experimental animals (Woollons et al. 1997). In addition to the indirect DNA damage induced by oxidative stress, UVA was also found to induce direct CPD formation in supercoiled pUC18 plasmid DNA in vitro (Jiang et al. 2009)

Unlike the linear dose response seen with UVB, where increasing doses of UVR cause greater levels of immunosuppression, UVA was found to be immunosuppressive only over a particular dose range with higher UVA doses failing to induce immunosuppression. Although the cause of this bell-shaped UVA dose-response curve is not fully understood, several mechanisms have been postulated; higher doses of UVA may lead to the destruction of an unknown UVA chromophore or may switch on UV-protective mechanisms (Byrne et al. 2002; Halliday and Rana 2008). In agreement with the latter, UVA radiation was found to counteract the immunosuppressive effects of UVB; a single suberythemal UVA-dose blocked the immunosuppressive effects of UVB in mice (Reeve 2002) and UVA-induced tan protected against further cutaneous UVA damage (Margolis et al. 1989). This UVA immunoprotection was found to be related to secretion of IFN-γ and the upregulation of the antioxidant stress enzyme, heme oxygenase (de Gruijl 2000; Reeve and Domanski 2002).

1.5.6 Assessment of Photoimmunosuppression

1.5.6.1 Clinical Models to Assess Photoimmunosuppression

UVR can suppress the activation of both primary immunity, where UV-exposure is prior to the initial contact with an antigen and secondary (memory/recall) immunity where UV-exposure is after the primary antigen exposure (sensitization) but before antigen re-exposure.

Use of secondary rather than primary immune responses to assess PI allows for the use of multiple test sites on each volunteer. In addition, each volunteer can act as his/her own unirradiated control (provided that the UVR dose is kept below that of systemic PI). On the other hand, with UV-induced suppression of primary immune reactions, human volunteers can only be tested once; as UV-irradiation needs to be prior to the first antigen exposure. It is also ethically unacceptable to immunize a person to an antigen he/she have never been exposed to before. Taken together, these factors limit the use of primary immune responses as an experimental model for the assessment of PI in humans. In addition, since immune reactions and the development of memory cells against a tumor most probably continue throughout the time of the tumor's development, UV-induced inhibition of secondary immunity is likely to play an important role in photocarcinogenesis (Damian and Halliday 2002).

Delayed type hypersensitivity (DTH) and contact hypersensitivity (CHS) are two examples of secondary immune responses that are frequently used to asses PI in humans (Figure 1.10) (Vink et al. 1998; Damian and Halliday 2002; Narbutt et al. 2005). Like the immune responses targeting tumor cells, CHS and DTH responses are Th1 driven immune reactions (Matthews et al. 2010). For this reason, they can be used as ethically acceptable substitutes for *in vivo* evaluation of UV-induced immunosuppression of tumor immunity (Damian et al. 2001).

These two types of reactions however, are initiated at different depths in the skin and by different antigen-presenting cells; epidermal LCs in CHS and dermal dendritic cells (DDCs) in DTH, thus response differences have been seen although both can be suppressed by UVR (Damian and Halliday 2002). It remains unclear whether LCs or DDCs are the main APCs in tumor immunity (Noordegraaf et al. 2010). Recent findings however suggest that LCs are essential for PI (Schwarz et al. 2010) which might indicate that CHS is a better model for it's assessment. In addition, CHS provides a less invasive clinical model for testing PI; CHS requires superficial antigen application whereas DTH involves intradermal antigen injection (Damian and Halliday 2002; Fourtanier et al. 2005).

1.5.6.1.1 Delayed Type Hypersensitivity (DTH)

Another secondary immune response used to test modulation of memory T cell responses following UVR-irradiation is DTH response to microbial antigens (Damian and Halliday 2002; Norval 2002). Unlike CHS, the antigen in DTH is injected deep into the skin (subcutaneously or intradermally), and is thus captured and processed by different APCs: LCs in CHS and DDC in DTH. Mantoux-positive volunteers are commonly used as a model for DTH (Figure 1.10). Mantoux testing is usually performed on the forearm, using a purified protein derivative (PPD) to the microbial tuberculosis (TB) antigen. Immunity to TB following a successful BCG vaccination (Bacille Calmette-Guerin) manifests with moderate DTH to the Mantoux test. The

reaction to a DTH response is assessed by measuring the diameter of induration “wheel” (not the surrounding skin erythema) 48-72 hours after the test is performed. Similarly, antigen specific responses of healthy contacts of leprosy patients (who become lepromin-positive) is used to examine for PI by injecting lepromin (the heat killed suspension of Mycobacterium leprae) into UV-exposed and non-exposed skin. Following UV-irradiation, the granuloma resulting from the DTH response, which is characterized by tissue necrosis and fibrosis, is reduced in size with a decreased number of infiltrating CD4+ cells (Norval 2001).

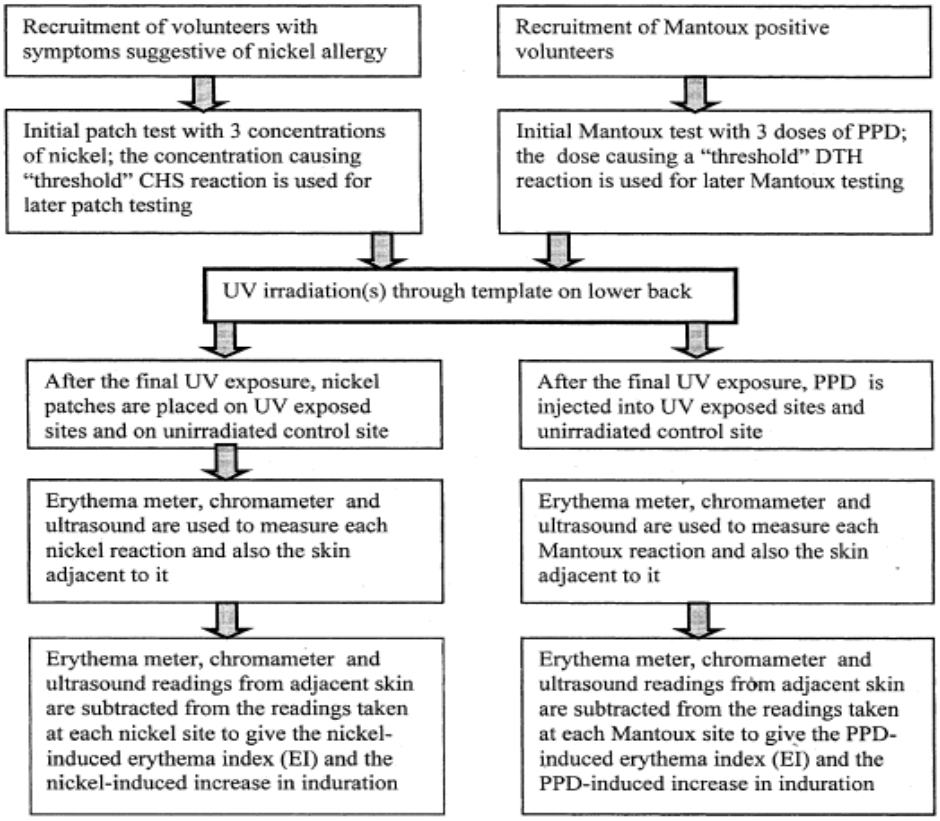


Figure 1.10 - Assessment of Photoimmunosuppression Using CHS and DTH Responses

(Damian and Halliday 2002)

1.5.6.1.2 Contact Hypersensitivity (CHS)

CHS is a special form of DTH (Schwarz 2002) that occurs when an antigen is topically (epicutaneously) applied to the skin's surface (Figure 1.11). One of the most widely used models of CHS is nickel (Ni) allergy. Ni allergy is common in the general population, affecting approximately 10-15% of women and 1-5% of men; women are more exposed to Ni due to their higher prevalence of jewellery use (Fourtanier et al. 2005; Matthews et al. 2010). When Ni patch tests were used to examine PI in human models; both total and partial body UVR exposure suppressed the Ni CHS response (Fuchs and Packer 1999).

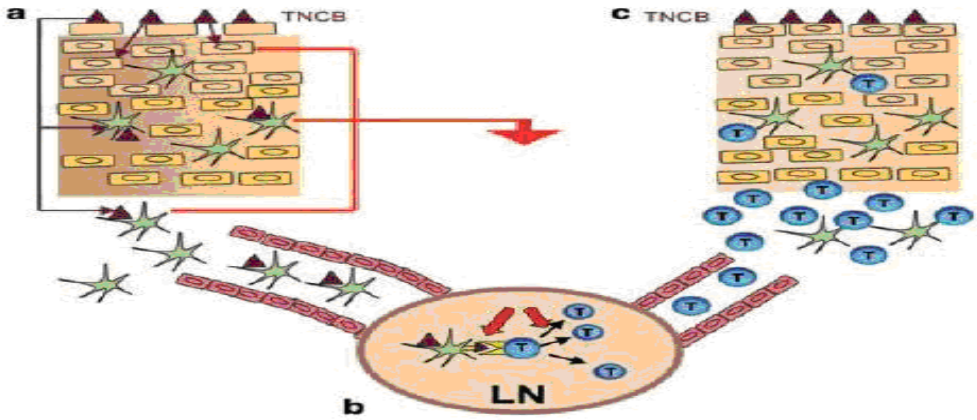


Figure 1.11 - Contact Hypersensitivity Response to trinitrochlorobenzene (TNCB)

a) Sensitization after detection of TNCB by APCs **b)** T cell priming by APCs following their migration to the draining LNs with subsequent expansion of specific T cells. After cutaneous re-exposure **c)** primed T cells are recruited from the LNs into the dermis and epidermis where they evoke a CHS response

(Yazdi et al. 2007)

Post-UV Ni Patch Testing as a Model for Clinical Photoimmunosuppression

Over a number of consecutive days, the back of Ni allergic subjects is irradiated with different UV doses. This is followed by applying Ni patches to the irradiated skin sites as well as adjacent non-irradiated skin (used as a control to compare Ni reaction with and without UV).

o Selection of Ni concentrations

Prior to UV-irradiation, to confirm Ni allergy and determine it's severity (and hence the Ni concentration that will be used post-UV), a Ni patch test is performed using multiple Ni concentrations. The Ni patch tests are removed after 48h and to ensure a settled CHS response with clearly defined readings, they are assessed 24hrs later (i.e. 72hrs after the Ni patches were applied). The Ni concentration selected for post-UV assessment of PI is one that gives a threshold CHS reaction with mild induration and erythema and no vesiculation. This ensures the Ni patch test reactions can be suppressed by suberythemal doses of UV and prevents them from overlapping with adjacent Ni patches (Damian and Halliday 2002).

o Assessment of Ni CHS Responses

Ni induced erythema is assessed clinically (using the grading scale of the International Contact Dermatitis Research Group (table 3.3) as well as by readings from a reflectance spectrometer. Ni induced induration can also be measured using ultrasonography. Readings from the Ni patches of surrounding, unirradiated skin of the same volunteer are used as a

control. The surrounding skin of each patch test, which was neither exposed to Ni nor UVR can be used to calculate the "background corrected" reading of each Ni reaction. All readings are measured in triplicates and an average is taken.

o Potential adverse effect of patch testing

Depending on the initial intensity of the Ni response, clinical resolution of the patch test sites usually occurs in no more than two weeks. The main potential adverse effect of a patch test is the development of a severe, unexpected allergic reaction with vesiculation, ulceration and rarely; an angry back syndrome with a strong widespread reaction causing adjacent patch test sites to become hyper-responsive. This can be further complicated, especially in ulcerated reactions, with superinfection and scarring. To prevent this, severe reactions are usually managed with topical steroids before they exacerbate (Damian and Halliday 2002).

UV-irradiation in clinical models of photoimmunosuppression

o Light Source

To model human solar UV-exposure, a solar simulator is used. Solar simulators have an arc lamp with two dichroic mirrors that only reflect wavelengths in the 280 to 400nm range; this effectively blocks out visible and infrared radiation. In addition, an atmospheric attenuation filter in the solar simulator removes UVC and modifies UVB to closely resembles natural, ambient sunlight (Figure 1.12) (Damian and Halliday 2002).

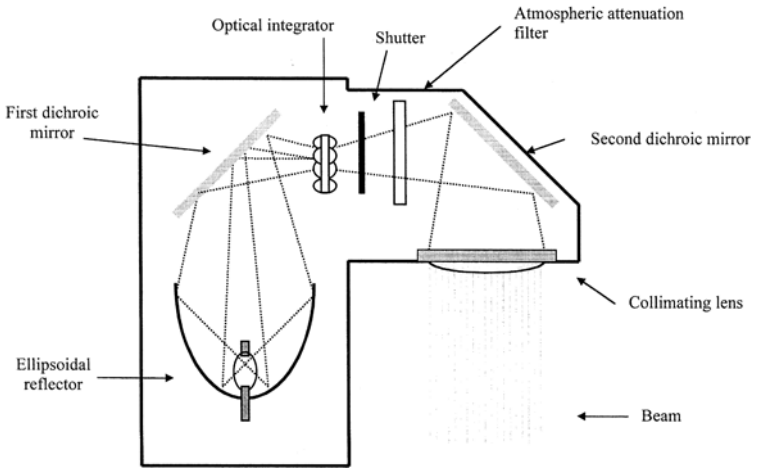


Figure 1.12 - Components of a Solar Simulator

(Damian and Halliday 2002)

o Light Doses

To study biological agents that can potentially protect against PI, such as sunscreens or n-3 PUFAs, a UV-dose response study is usually performed. This helps determine each agent's

protective abilities and limits. The maximum of any UV-dose series used is usually determined in relation to the MED of the subject or group of subjects participating in the study; although skin type is not directly related to PI (Damian et al. 2001), the use of a suberythemal UV-dose usually aids in the proper visual as well as reflectometer assessment of cutaneous post-UV immune reactions (Fuchs and Packer 1999). In addition, suberythemal UV doses more closely mimic day to day solar exposure (Narbutt et al. 2005).

1.5.6.2 Assessing Cellular Responses in Photoimmunosuppression

Depletion of epidermal LCs following UV-irradiation can be immunohistochemically (IHC) assessed by comparing LC density in epidermal sheets from punch biopsies (figure 1.2A) or the roofs of suction blisters, before and after UV-irradiation (Friedmann et al. 1983; Kolgen et al. 2002) (for more details on using epidermal sheets from punch biopsies to examine LC trafficking post-UV, see section 2.15.1). A number of antigenic markers constantly found on the surface of LCs can be used for this purpose. These markers include MHCII, E-cadherin, CD1a, CD11b and CD207 (langerin) (Guironnet et al. 2002; Mizumoto and Takashima 2004; Romani et al. 2006). Amongst these markers, CD1a is very useful marker for detecting LCs in human epidermis (Pena-Cruz et al. 2001; Schwarz. 2003) (for more details on LC-specific markers please see section 1.2.1.1). When LC count from suction blister roofs was compared to that of epidermal sheets from punch biopsies, a 30% reduction in CD1a+ LCs was noted in the blister roofs and this number did not decrease further by the injection of the stimulating signal for LC migration, IL-1β. Taken together, these findings suggest that the mechanical trauma from the raising of suction blisters had already stimulated the responsive LCs (30%) to migrate prior to the injection of IL-1β (Dearman et al. 2004). In addition to the detection and counting of LCs, IHC staining can also be used to detect and examine cutaneous cytokine levels in punch biopsies (Ulfgren et al. 2000; Shahbakhti et al. 2004).

The antibodies used in IHC can be polyclonal or monoclonal. Monoclonal antibodies are generally considered more specific while the diverse nature of polyclonal antibodies allows them to recognize several epitopes (the part of an antigen to which an antibody attaches in order to elicit an immune response) (Mashhood 2008).

1.5.6.3 Multiplex Analysis of Cytokines Involved in Photoimmunosuppression

In response to UVB-irradiation, keratinocytes release a variety of cytokines including TNF-α, IL-4, IL-8, IL-6, and PGE_2 (Pupe et al. 2002). Assays can be used to measure cytokine levels in sera, other body fluids or tissues e.g. levels of cutaneous photoimmunosuppressive

cytokines can be assessed in suction blister fluid (Rhodes et al. 1994). The most widely used cytokine immunoassay is the enzyme linked immunosorbent assay (ELISA).

Multiplex assays are a recent development of ELISA assays, used for the simultaneous measurement of multiple cytokines in a single sample. Several different formats of multiplex assays are available; flow cytometric multiplex bead-based assays, chemiluminescence or electro-chemiluminescence based assays. Bead based multiplex assays are currently one of the most widely used multiplex assays (Leng et al. 2008) (for more details on the multiplex bead-based immunoassay see section 2.15.2). Compared to ELISA, multiplex arrays are more time and cost efficient; multiple cytokines can be examined in a single biological sample with less sample volume needed. This enables the ability to study analytes in context of each other and allows for repeated measures to be performed on the same cytokine panels, in the same subjects and under the same experimental assay conditions. In addition, the broader dynamic range allows for the ability to detect proteins over larger concentration limits. Despite these potential advantages, experience with multiplex techniques is relatively limited and it is yet to become a standard form for measuring inflammatory mediators (Leng et al. 2008).

1.6 Photoprotection

Since UVR causes a range of adverse effects on the skin including PI and skin cancer, methods to minimize this damage are sought after:

1.6.1 Physical Protection

o Clothing

Provided consideration is given to good skin coverage and UVR transmittance, clothing is an effective method of UVR-protection. Examples of such clothing include long-sleeved shirt, a wide-brimmed hat and sunglasses.

o Seeking Shade & Avoiding Peak UVR Levels

Under clear skies in the summer, the highest UVB-levels are at and around noon. By avoiding direct sunlight exposure during this period (11am to 3pm), a substantial reduction in personal UV-dose can be achieved.

o Protective Glass & Window Films

While glass efficiently absorbs UVB, it allows the transmission of both UVA and visible light. With recent developments, many types of glass have very good UV-protection with coated, UV-blocking glass keeping out >98% of UV up to 380 nm. Glass coating that absorb UV wavelengths up to 400 nm is available but with high production costs (Bernstein et al. 2006).

1.6.2 Topical Sunscreens

Topical sunscreens act by reflecting, scattering or absorbing UV radiation. The protection achieved by a sunscreen depends on several factors including: the type of sunscreen, the application thickness and technique and the resistance to water and friction as well as how often the sunscreen is re-applied.

The Sun Protection Factor (SPF)

The SPF is defined as the ratio of the amount of UV energy required to produce a minimal erythema on sunscreen-protected skin to the amount of energy required to produce the same erythema on unprotected skin. In the past decade the maximum SPF value of sunscreens has gradually risen with average SPFs ranging from 15 to 30 and some having SPFs of 50 or higher. It should be noted however that the SPF advertised on a sunscreen is usually not achieved as people rarely apply the sunscreen at the recommended density of 2 mg per cm^2 (Fourtanier et al. 2005). In addition, SPF is used to define the sunscreen's ability to absorb, reflect or scatter solar UVB rays (which are the main cause of skin inflammation and redness upon which the SPF is based). There is however no universal standard for measuring UVA protection, although the UVA star rating system is used with more stars indicating higher protection against UVA (Diffey 1994).

The Immune Protection Factor (IPF)

Recently, an index of protection of sunscreens to UVR induced immunosuppression has been advised; immune protection factor (IPF). The IPF was evaluated in human skin in vivo by examining the sunscreens ability to inhibit UV induced local immunosuppression of CHS or DTH responses (Fourtanier et al. 2005). Significantly, IPF is shown to be different to the SPF; reflecting the contribution to PI attributed to UVA (please see section 1.5.5 for more details).

1.6.3 Dietary Agents

Current strategies for cutaneous photoprotection rely on shielding the skin from UVR. Such methods are often inadequate as they usually do not provide sufficient coverage, are not applied in the required quantities or are not re-applied often enough. New dietary approaches to photoprotection may therefore potentially provide a safe systemic adjuvant to topical remedies (Black and Rhodes 2006).

1.6.3.1 Antioxidants

Because UV-induced skin damage is partially induced via the induction of oxidative stress, antioxidants have been widely studied as photoprotective agents. UV-irradiation induces the release of substantial amounts of reactive oxygen (ROS) &/or nitrogen (NOS) species with

the resulting reduction in antioxidants levels and subsequent inflammation, DNA damage and destruction of tissue cells. The intracellular ratio of glutathione (GSH) to oxidized glutathione (GSSG) is a reliable indicator of a cell's antioxidant status (Peterson et al. 1998). Depletion of GSH from APCs *in vivo* leads to a shift towards the immunomodulatory Th2 pathway, while restoration of GSH levels leads to the opposite effect. In addition, the Th2 cytokine IL-4 leads to reduction in intracellular GSH levels while IFN-γ induces it's increase (Kidd 2003).

Antioxidants can be administered orally or topically. Topical antioxidants are poor UV absorbers and are therefore commonly combined with other UV filters in sunscreen products. Antioxidants exert their protective effects through a range of properties including modulation of inflammatory pathways and gene expression.

High dose oral supplementation of 12 Ni allergic volunteers with skin type II, with a combination of two antioxidants; Vitamin E (RRR-α-tocopherol; 2gm/day) and Vitamin C (L-ascorbic acid; 3gm/day) for 50 days, managed to protect against PI of their Ni CHS responses following irradiation with a low dose solar-simulated protocol (x10 unexposed back skin areas; each 4cm^2 were irradiated for 5 consecutive days with a suberythemal UV-dose of 70 mJ/cm^2). The 12 volunteers in the placebo group of this study, who were irradiated with the same solar-simulated protocol after receiving 50 days of supplementation with identical looking lactose capsules, continued to show significant UV-induced suppression of their CHS responses. These findings indicate a potential use for oral antioxidants as a dietary means for protection against PI and prevention of skin cancer (Fuchs and Packer 1999).

In accordance, when β-carotene (a Vitamin A precursor) was examined for it's ability to protect against UV-induced immunosuppression, using an alternative clinical model; the post-UV DTH response, similar result were achieved. Using 24 male volunteers between the age of 19 – 39 years old, equally divided into active and control groups, DTH tests to the tetanus and diphtheria toxoid, inactivated Streptococcus, Candida, filtrate of Proteus mirabilis, Trichophyton Mentagrophytes as well as old tuberculin, were performed on back skin that was not exposed to the UV-light source and assessed 48hrs later, led a significant reduction in diameter of the response post-UV in the placebo group (who received supplement capsules containing 70% sterile glycerine solution). Volunteers in the active group on the other hand, all put on a course of 30mg of β-carotene/day for 28 days; showed no significant change in DTH responses post UV-irradiation (x12 exposures over 16 days to a UV light source containing 90% UVA and 10% UVB). Furthermore, the β-carotene plasma levels of the control group subjects inversely related to their degree of PI (Fuller et al. 1992).

When the same research group performed a similar study; also testing the β-carotene protective properties against PI, this time in older individuals with a mean age of 65.5 years,

very similar results to those found in the younger groups were observed. Using the same suberythemal UV-irradiation protocol, clinical DTH model for PI (with the same multi-antigen kits) and the same β-carotene doses resulted in significant decline in DTH responses in the placebo group in response to UV-irradiation. Although the active, β-carotene group did exhibit a slight decline in their DTH response this time, the extent of PI was only significant in the placebo group. This mild dampening effect of UV-irradiation on the DTH responses of the active group might be explained by the reduced increase in the β-carotene plasma levels in the older active groups compared to the younger one. Also, the older active group showed significantly higher Vitamin E levels than those previously found in the younger group (38µl vs. 23µl) which might have a yet unexplained effect (Herraiz et al. 1998).

1.6.3.2 Omega 3 Polyunsaturated Fatty Acids (PUFA)

The concept that specific fat components may be necessary for the proper growth and development of animals and possibly humans was introduced over 70 years ago (Levy et al. 1992; Flower and Perretti 2005). Studies conducted in the 1930s by George and Mildred Burr demonstrated that certain fatty acids were essential to the health of mammals. Further discoveries in the 1960s demonstrated that in addition to being an important component of cell structures, those essential fatty acids (EFA) also play a key role in the synthesis of prostaglandins by acting as a substrate for COX enzymes. This led to the realization that EFAs are important precursors for potent hormones with considerable effects on immune and cardiovascular systems. In addition, it was later found that these lipids also act as precursors to other important mediators (eicosanoids) such as leukotrienes and thromboxanes.

Two fatty acid series are classified as essential and hence must be supplied in human diet; omega-3 and omega-6 polyunsaturated fatty acids. Reports highlighting the association between a high-intake n-3 PUFA diet and the low incidence of ischemic heart disease and inflammatory symptoms in general, led to an increased interest in the health benefits of those macronutrients (Fischer and Black 1991; Black and Rhodes 2006).

1.6.3.2.1 Structure of n-3 PUFAs

Fatty acids are straight chain hydrocarbons with a carboxylate (COOH) group at one end and a methyl (CH3) group at the other end. The term omega-3 (n-3 or ω-3) signifies that the first double bond (first point of unsaturation) begins at the third carbon atom from the methyl end (n) of the carbon chain (Figure 1.13) (Kelley 2001; Black and Rhodes 2006) (Figure 1.13). The n-3 PUFAs that are important in human nutrition are (Lands 1992):

- Alpha linolenic acid (ALA): 18:3n-3 (major dietary source n-3 eicosanoids)
- Eicosapentaenoic acid (EPA): 20:5n-3 (immediate tissue precursor of n-3 eicosanoids)

- Docosahexaenoic acid (DHA): 22:6n-3 (competitive antagonist of n-6 eicosanoid formation)

These three PUFAs have 3, 5 or 6 double bonds in a carbon chain of 18, 20 or 22 carbon atoms, respectively.

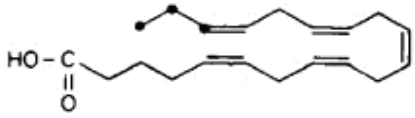


Figure 1.13 - The chemical structure of EPA

The chemical structure of EPA is 20:5n-3; a chain of 20 carbon atoms with 5 double bonds the first starting at the 3rd carbon atom from the methyl end

(Black and Rhodes 2006)

1.6.3.2.2 Sources of n-3 PUFAs

Essential fatty acids including AA (n-6) and EPA (n-3) are given this name because they cannot be produced *de novo* by mammals. Mammals cannot introduce double bonds in the fatty acid structure closer to the omega end than n-9. Only specific plants, algae and fungi can synthesize short chained n-3 PUFAs. Although fish are a rich dietary source of n-3 PUFAs, they also cannot synthesize them *de novo*. Marine animals ingest short chain n-3 PUFAs from algae and phytoplankton found in their diet and through a series of elongations and desaturations; convert them to long chain PUFAs that then accumulate in their fat (figure1.16) (Aronson et al. 2001; Black and Rhodes 2006).

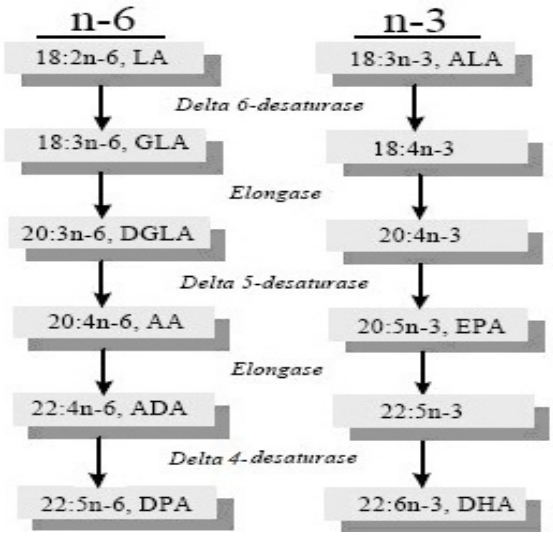


Figure 1.14 - Classic n-6 and n-3 Long Chain PUFA Synthesis Pathways

LA: linoleic acid, GLA: gamma-linolenic acid, DGLA: dihomo-gamma-linolenic acid, AA: arachidonic acid, ADA: adrenic acid, DPA: docosapentaenoic acid, ALA: alpha-linolenic acid, EPA: eicosapentaenoic acid, DHA: docosahexanenoic acid

(Sardesai 1992)

While marine animals are the essential dietary source of long chain n-3 PUFAs, short chain n-3 PUFAs can be obtained from the ingestion of some plant oils and green leafy vegetables (Simopoulos 2002; Black and Rhodes 2006) (table 1.3). Similar to marine animals, human tissue can then synthesize long chain PUFAs from their short chain precursors. Due to enzyme-limitations however, most probably in Δ-6 desaturase (Simopoulos et al. 1999), this conversion is at a much lower efficacy. Using radioisotope-labelled ALA and while being fed a high-saturated fat diet, the total body conversion of ALA to EPA and to DHA was found to be approximately 6% and 3.8% respectively (Gerster 1998). For that reason; direct ingestion of long chain n-3 PUFAs is considered a more efficient method of intake (Brenna 2002). Moreover, due to the lack of inter-conversion between n-3 and n-6 PUFAs, a competitive interaction exists between the two; n-3 PUFAs suppress the metabolism of n-6 PUFAs and vice versa. The accumulation of long-chain n-3 PUFAs in tissues is thus more effective when they are obtained directly from food or when the competing amounts of n-6 analogues do not greatly exceed those of n-3 PUFAs (Lands 1992; Gerster 1998; Black and Rhodes 2006). Recently, food fortification with n-3 PUFAs has become a trend with products like n-3 PUFA fortified bread, orange juice and infant formula being released (Simopoulos et al. 1999).

- Fish

The most widely available source of long chain EPA and DHA is cold water oily fish such as salmon, herring, mackerel, anchovies and sardines. Oils from these fish have a profile with around seven times as much n-3 as n-6 PUFAs. Other oily fish such as tuna also contain n-3 in somewhat lesser amounts (Aronson et al. 2001).

- Vegetable Oils

The primary source of ALA in human diet is PUFA-rich vegetable oils such as soybean and canola oils. Flax seed is also used to manufacture a very rich source of n-6 and n-3 PUFAs; linseed oil (table 1.3) (Gerster 1998; James et al. 2000).

- Algae

The microalgae Crypthecodinium cohnii and Schizochytrium are rich sources of DHA (22:6 n-3). Oil from brown algae (kelp) is also a good source of EPA (Conquer and Holub 1996; DeFilippis and Sperling 2006).

- Walnuts & Butternuts

Walnuts and butternuts are amongst the few nuts that contain appreciable levels of n-3 PUFAs (table 1.3) (Simopoulos 2002).

Table 1.3 - ALA Content in Select n-3 PUFA-Rich Botanical Dietary Sources

(Simopoulos 2002)

Source	ALA (per100gm)
Linseed oil	55.3gm
Canola oil	8.6gm
Soybean oil	7.6gm
Walnut	6.8gm
Butternut	8.7gm

1.6.3.2.3 The n-6: n-3 ratio

Since n-3 and n-6 PUFAs compete for the same metabolic enzymes (LOX and COX), the n-6: n-3 ratio will significantly influence the relative amounts of resulting metabolites e.g. prostaglandins and leukotrienes and this in turn will lead to alterations in the body's metabolic functions (Black et al. 1992; James et al. 2000). Several products of these enzymatic pathways influence immune responses and tumor biology. Examples of this are the tumor promoting properties of PGE_2 (for more details please see section 1.5.3.1).

Aside from modulating the amount and type of eicosanoids, other biological effects of PUFAs are induced in an eicosanoid-independent manner; including alterations in intracellular signalling, gene expression and transcription factor activity (please see section 1.6.3.2.6 for more details) (Simopoulos 2002).

Accordingly, this will necessitate the consumption of n-6 and n-3 PUFAs in a balanced proportion; a healthy ratios of n-6:n-3 ranges from 1:1 to 4:1 (Simopoulos 2002). Evolutionary human diets, rich in seafood and other sources of n-3 PUFAs may have provided such a ratio. Today's typical Western diet however is dramatically shifted toward n-6 PUFAs with ratios between 10-20:1 (Simopoulos 2002; Flower and Perretti 2005).

1.6.3.2.4 Recommended Daily Intake of n-3 PUFAs

As outlined in section 1.6.3.2.5, modernized Western diets are heavily shifted toward the intake of n-6 PUFAs with increased consumption of n-6 PUFA rich dietary plant oils such as corn, safflower, and soybean oils. In addition, modernized agricultural techniques rely on feeding livestock n-6 rich grains with resulting meat and vegetable produce containing higher levels of n-6 PUFAs. This shift along with it's subsequent adverse effects can be avoided through increased intake of ALA together with EPA and DHA, along with the reduction of vegetable oils with high n-6 PUFA content. Not enough data exists to determine a recommended daily intake reference for n-3 and n-6 PUFAs. There is enough data however

to make recommendations for the adequate daily intake of polyunsaturated as well as saturated dietary fats (table 1.4) (Simopoulos et al. 1999).

Table 1.4 - Adequate Intake Recommendations for Saturated and Unsaturated Fats
(Simopoulos et al. 1999)

Fatty Acid	Grams/day (2000 kcal diet)	% Energy
LA LA Upper Limit	4.44 6.67	2.0 3.0
ALA	2.22	1.0
DHA+EPA DHA to be at least EPA to be at least	0.65 0.22 0.22	0.3 0.1 0.1
TRANS-FA Upper Limit	2.00	1.0
SAT Upper Limit	---	<8.0
MONOs	---	---

1.6.3.2.5 Bioavailability of n-3 PUFAs

By definition, essential fatty acids cannot be synthesized in human tissue and must be obtained from the diet. Following intestinal absorption, dietary PUFAs are transported in chylomicrons to the liver where they are processed into triglycerides. Very low density lipoproteins then circulate the fatty acids; to keep them soluble during passage down the bloodstream. Via passive diffusion or transport by fatty acid transport proteins (FTP) the fatty acids pass the plasma cell membrane to reach their target destination. In the skin, PUFAs are essentially maintained in the membrane lipids of the stratum corneum (Schmuth et al. 2005).

Both animal and human studies have been used to examine the bioavailability of n-3 long chain PUFA. Endpoint measures to assess n-3 PUFA bioavailability include; amount or concentration of EPA/DHA in total plasma lipids, serum, platelets and red blood cells. Also, lipid fractions in phospholipids, cholesterol esters and free fatty acids have been used (Bryhn et al. 2006; Dyerberg et al. 2010).

Few studies, with conflicting results have been done on the bioavailability of EPA and DHA. In an attempt to further clarify this, Dyerberg et al. (Dyerberg et al. 2010) recruited 76 subjects in a double blinded placebo (corn oil) controlled study to compare the bioavailability of different chemical preparations of n-3 PUFA. An apparent "bioavailability index" of 100% was given to the increase in EPA and DHA seen with natural fish oil (mixture of fish body/cod

liver oil which is mainly composed of natural-triacylglycerol) and this was used to assess the relative EPA and DHA increase seen with re-esterified triacylglycerol, ethyl esters and free fatty acid forms; all commonly used in DHA/EPA supplements. All active n-3 PUFA groups showed a significant increase in both EPA and DHA, compared to the placebo groups. The DHA/EPA bioavailability index from re-esterified triacylglycerol was found significantly higher compared to the ethyl esters form; 124% vs.73%. An intermediate index of 91 % was found with the free fatty acid form. In addition, despite keeping the daily EPA/DHA doses constant (3.3gm/day of EPA/DHA mixture for 2 weeks), the re-esterified triacylglycerol group showed a 62% greater increase in serum phospholipids, compared to the ethyl ester group.

In addition to chemical structure, the matrices of n-3 PUFA-fortified foods were also found to significantly affect n-3 PUFA bioavailability. In a single meal study recruiting 12 volunteers, Schram et al. (Schram et al. 2007) examined the bioavailability of n-3 PUFAs incorporated into two different food types; a yoghurt drink or a fitness bar and compared the results with the bioavailability of regular fish oil capsules. Bioavailability was analyzed using the fatty acid composition of chylomicrons. The fish oil incorporated into food products were absorbed differently from those administered as capsules; yoghurt drink was the best matrix for providing fast absorption of omega-3 PUFA. This might be due to the pre-formed emulsions.

Results of these bioavailability studies can help consumers and physicians evaluate the cost/benefit of various n-3 PUFA supplements and can also aid in choosing supplements for clinical trials on n-3 PUFA.

1.6.3.2.6 Mechanisms of Photoprotection by n-3 PUFAs

Competition with n-6 PUFA

Several studies have pointed to the pivotal role of n-6 PUFA intake in the induction of photocarcinogenesis. Studies using hairless mice showed an association between high n-6 PUFA levels and the increased risk of photocarcinogenesis. Murine studies examining the effects of corn or soybean oils (rich n-6 PUFA sources with approximately 50% linoleic acid) on photocarcinogenesis revealed an approximated linear relationship; the lowest fat levels led to the longest tumor latency periods and lowest tumor multiplicity (Black and Rhodes 2006).

By feeding groups of hairless mice a diet containing 20% fat by weight; with increasing sunflower (n-6 PUFA rich) to cottonseed oil (hydrogenated, saturated fat) proportions, Reeves et al. (Reeve et al. 1996) managed to demonstrate an increasing UV-induced tumor response with increasing PUFA-levels in the mixed diets. The UV-induced tumors were initiated in the mice by applying 10 weeks of chronic UV-irradiation and the photocarcinogenic responses were assessed as follows; tumor incidence, tumor multiplicity, progression of

benign tumors to SCC and reduced survival. In addition, 6 months after completing the 10 week chronic UV-irradiation treatment (most of the irradiated mice had developed UV-induced tumors by then), the immunosuppression of CHS responses in the groups were analyzed. Mice fed the diet with the lower concentrations of PUFA (0, 5% or 10%) and were hence bearing the smallest tumor load, showed the lowest PI of CHS. Moreover, mice in the 20% saturated fat diet group showed a near-complete protection against UV-induced suppression of their CHS responses. Mice in the group fed 20% PUFA on the other hand, showed a 57% suppression of their CHS responses. When the animals on the hydrogenated fat were switched to a normal mixed fat diet, a large number of skin tumors rapidly developed. This suggested that the PUFA deficiency provided by the hydrogenated fat diet did not prevent the UVR-initiation stage of carcinogenesis but only inhibited it's promotion stage and once PUFA levels were restored; photocarcinogenesis resumed. Taken together, these findings suggest; enhancement of photocarcinogenesis by dietary PUFAs and mediation of this enhancement by the induction of PI (as seen from the inhibition of CHS in the 20% PUFA but not the hydrogenated fat group). Furthermore, it appears that n-6 PUFAs support the promotion rather than the initiation stage of photocarcinogenesis.

In comparison, n-3 PUFAs appeared to have the opposite effects. Black et al. (Black et al. 1992) placed six groups of hairless mice on isocaloric diets containing either 12% or 0.75% corn oil (n-6 PUFA rich) or 12% menhaden oil (rich in n-3 PUFA) for 2 weeks after which, all animals were treated with 11 weeks of chronic UV-irradiation using fluorescent sunlamps; UV-induced tumors started appearing after 14 weeks. Mice on the high-corn oil diets (12%) showed significant photocarcinogenesis (measured by tumor latency period and tumor multiplicity) in comparison to those on the lower 0.75% corn oil group. Comparing the two groups that were on equal concentrations of PUFA; 12% n-3 menhaden and 12% n-6 corn oil, revealed a significant inhibition in photocarcinogenesis in favor of the menhaden-oil group. When the high-corn diet was replaced with a low corn oil diet after UV-induced tumor initiation (i.e. in the promotion stage) this led to inhibition of the enhancing effects of high-corn oil. This confirmed that n-6 PUFA enhancement of photocarcinogenesis occurs during the promotion stage. Suppression of photocarcinogenesis in the menhaden oil group on the other hand remained uninhibited when different concentrations of the oil were given. This confirmed protection against photocarcinogenesis using the n-3 PUFA-rich diet.

The major immediate n-3 and n-6 eicosanoid precursors maintained in tissue (EPA and AA respectively) compete for metabolism by LOX and COX to produce the biologically active eicosanoids. Many eicosanoids act as immunomodulators; particularly prostaglandins (Kelley 2001; Gualde and Harizi 2004). As previously outlined, following it's UV-induced release from

membrane phospholipids, AA is oxidized by COX-2 to give rise to the "2" series of prostaglandins, such as PGE_2 and PGF_2. EPA on the other hand, in addition to not being a very good substrate to COX; produces PGE_3 which is noticeably less potent than PGE_2 in producing various biological effects including immunosuppression (Flower and Perretti 2005; Black and Rhodes 2006) (for more details on the immunosuppressive effects of PGE_2, please see section 1.5.3.1). Alternatively, if AA is oxidized by LOX, it gives rise to another inflammatory series of eicosanoids; the "4" series of leukotrienes, such as LTB_4 (Figure 1.9) (Kelley 2001; Black and Rhodes 2006) (for details on LTB_4, please see section 1.5.3.3.2).

Alteration in Signal Transduction

In the human body, a dose-related replacement of membrane PUFAs occurs after ingesting up to 1.6 gm EPA/day. This effect is more pronounced if the amount of AA in the diet is simultaneously restricted (Flower and Perretti 2005). Incorporation of n-3 PUFAs into membrane lipids alters membrane composition leading to an increase in viscosity with negligible alterations in osmotic cell fragility (Fischer and Black 1991). This in turn results in increased membrane unsaturation and fluidity with re-localization of membrane signalling proteins with subsequent modification in signal transduction and alteration in intracellular signaling pathways (Stulnig et al. 2001; Simopoulos 2002).

It has long been understood that signal transduction is initiated by complex protein interactions. Recently, lipid microenvironments on a cell's surface; lipid rafts and caveolae, have been recognized to play an important role in this process. In response to intra or extracellular signals, membrane lipids that incorporate specific protein sets can change their size and composition. This in turn leads to specific protein interactions and the induction of further signalling cascades (Zeyda et al. 2002).

An example of this cell membrane lipid-protein communication can be seen in T lymphocytes. Fyn is a Src family kinase signalling protein that is highly concentrated in cell membrane lipid rafts. Enriched in saturated fatty acyl-containing lipids, Fyn acts through TcR in a dual (myristate and palmitate) fatty acylation dependant manner. Treatment of T-lymphocytes with EPA inhibited palmitoylation of Fyn kinase which led to it's displacement from lipid rafts and subsequently inhibited signalling through TcR (Webb et al. 2000).

In similar studies examining the molecular mechanism by which n-3 PUFAs inhibit T cell signalling, the 2-day incubation of T-cells in EPA altered their membrane fatty acyl content by successfully incorporating EPA and it's elongated derivative DHA in lipid rafts. Subsequently, this interfered with the transduction of T-cell signals by blocking tyrosine phosphorylation of the linker for activation of T cells (LAT) and displacing the Src family kinase, Lck by n-3

PUFAs. The same results could not be observed when the T-cells were incubated with stearic acid; an 18-carbon saturated fatty acid (Stulnig et al. 2001; Zeyda et al. 2002).
The ability of n-3 PUFAs to block T-cell signal transduction can partly explain their anti-inflammatory properties and warrants their clinical application as an adjuvant treatment of inflammatory disorders. In addition, n-3 PUFAs have also shown anti-carcinogenic properties mediated via the inhibition of cancer cell growth, in part by modifying cell signalling. The epidermal growth factor receptor (EGFR) signalling pathway is pivotal for cellular development, growth and proliferation. Mutations causing an increased expression or activation of EGFR have been associated with cancer development; many therapeutic modalities currently target EGFR. DHA supplementation was found to exclude epidermal growth factor from caveolae with modification of EGFR phosphorylation, localization, and subsequent signalling. Furthermore, disrupting membrane localization of EGFR interfered with Ras signalling. Since sustained signalling/distorted activation of Ras is found in 20–30% of all human malignancies this further supports the anti-carcinogenic potentials of DHA. Moreover, when DHA was combined with the EGFR inhibitor, PD153035s it was found to enhance it's actions; making the use of DHA to augment anti-EGRF therapies a possibility (Rogers et al. 2010).

Alteration of Cytokine Secretions

An imbalance of n-6 and n-3 PUFAs in the peripheral blood causes an overproduction of proinflammatory cytokines. Since IL-1 and TNF are principal mediators of inflammation, effects of fish oil supplementation on the production of the monocyte cytokines, IL-1β and TNF-α were examined using nine healthy volunteers taking 16gm/day of fish oil concentrate. Six weeks after staring supplementation, the *in vivo* production of IL-1β, IL- α and TNF-α were measured using 24h peripheral blood mononuclear cell cultures followed by cell thawing, lysis and cytokine-specific radioimmunoassays; decrease in all three cytokines was noted. This decrease continued 10 weeks after stopping the fish oil concentrate but 20 weeks after discontinuation, cytokine levels returned to baseline. This might explain another key step in the anti-inflammatory-immunosuppressive mechanisms of n-3 PUFAs (Endres 1993).

Moreover, the long chain n-3 PUFAs, EPA and DHA managed to reduce the baseline secretion of another pro-inflammatory, pro-immunosuppressive mediator, IL-8 (section 1.5.2.3) from keratinocyte cell lines by 66% and 63% respectively. They also managed to decrease the post-UV release of IL-8 by 54% and 42% respectively. A similar result was also seen from fibroblast cell lines. In addition, in another experiment using keratinocyte and fibroblast cell lines; EPA and DHA reduced the expected TNF-α stimulation of IL-8 secretion:

from 11,669 +/- 1692 pg/ng protein to 5405 +/- 594 pg/ng protein using EPA and from 6740 +/- 1049 pg/ng protein using DHA (Storey et al. 2005).

One of the primary mechanisms for the anti-inflammatory actions of n-3 PUFAs is the effect on T-cell proliferation and the secretion of Th1 cytokines. Spleen cells from mice fed menhaden fish oil or maize oil for 6 weeks were examined for the secretion of IFN-γ, TNF-α, IL-4 and IL-10. Splenocytes extracted from the menhaden oil group were found to secrete significantly less IFN-γ and TNF-α and significantly more IL-4 than those of the maize oil group. IL-10 secretion in both groups was found to be similar. Taken together, these findings show that dietary fish oil directs splenocytes cytokine secretion towards an anti-inflammatory, Th2 phenotype (Petursdottir and Hardardottir 2009).

Alteration of Transcription Activation and Gene Expression

n-3 PUFAs were found to modulate the expression of a range of genes and influence the activation of many transcription factors. A key transcription factor influenced by changes in a cell's PUFA content is NFkB (Nuclear Factor kappa-light-chain-enhancer of activated B cells). Upregulation of NFkB is seen in response to stress and inflammation and has been known to transcribe many genes involved in carcinogenesis; NFkB induces COX-2 gene expression and is the transcription factor that induces expression of immunosuppressive cytokines like IL-6 and TNF-α (Hardman 2002). Omega-3 fatty acids were shown to inhibit NFkB; it is thought that the 15-LOX products of EPA are the ones specifically responsible for interfering with the activation of NFkB and hence prevent the activation of inflammatory genes (Flower and Perretti 2005). Inhibition of arachidonate oxidative metabolism was shown to prevent NFkB activation whereas EPA did not appear to exert any significant effect. In accordance, PGE_2 (an AA metabolite) was found to activate NFkB. Seeing that n-6 metabolism activates NFkB while EPA has minimal effects on it's induction, this may point to a possible balancing role for a n-3:n-6 ration in controlling NFkb expression (Camandola et al. 1996).

Another gene-related molecule that is affected by n-3: n-6 ratio is the peroxisome proliferator activated receptors (PPAR). PPARs are nuclear receptors that modulate gene expression. Three PPAR isomers exist; PPAR-α, -β/δ, and -γ; all are expressed in the epidermis and are involved in keratinocyte proliferation and differentiation. As outlined in section 1.5.3.3.1 and 1.5.3.3.2, metabolites of n-6 PUFA can activate PPAR-γ which will in turn induce the expression of COX-2. In human keratinocytes, the n-3 PUFA, EPA can also upregulate COX-2 expression via the PPAR-γ receptor which suggests that gene expression may be regulated by the proportion of n-6/ n-3 in the diet (Chene et al. 2007).

The activator protein-1 (AP-1) transcription factor is one of the earliest nuclear responses to mitogenic stimuli, promoting transcription of genes involved in cell proliferation, malignant transformation and metastasis. In human and murine skin, AP-1 is required for the malignant progression of keratinocytes. AP-1 transcriptional activity is stimulated by the tumor promoting phorbol ester; 12-O-tetradecanoyl-phorbol-13-acetate (TPA). EPA and DHA were found to inhibit TPA-induced transformation and thus prevent the transcriptional activation of AP-1 (Liu et al. 2001).

As previously outlined, Ras signalling is a key element in as much as 30% of human malignancies. Long chain n-3 PUFAs were found to decrease the expression of the Ras oncogenes. This in turn inhibits the proliferation of cancer cells and encourages apoptosis (Collett et al. 2001).

Lipid Peroxidation and the Induction of Apoptosis

Due to their highly unsaturated structure, long chain n-3 PUFAs are susceptible to oxidation by free radicals with the formation of cell membrane-damaging lipid peroxides. This lipid peroxidation has been suggested as a mechanism for n-3 PUFA-induced apoptosis and inhibition of tumour cell growth (Albino et al. 2000); apoptosis induced by n-3 PUFAs was found to be inhibited by the antioxidant, Vitamin E (Begin et al. 1988).

In humans, fish oil supplementation was found to increase UV-induced production of the lipid peroxidation index, thiobarbituric acid reactive substances (TBARS). This increase in TBARS was accompanied by a decrease in susceptibility to UV-induced erythema. Since UV-induced erythema is in part mediated by the generation of ROS and the subsequent damage to vital structures including cell membranes and DNA; it has been suggested that n-3 PUFAs may act as free radical buffers that protects more vital cellular structures against oxidative damage (Rhodes et al. 1994) (for more details please see section 1.6.3.2.7.1).

In addition to lipid peroxidation, n-3 PUFA-induced apoptosis and cancer cell death may be mediated through modulation of apoptotic mediators such as the downregulation of the apoptotic suppressor and caspase inhibitor, bcl-2 (Chiu and Wan 1999). Furthermore, in various cancer cell lines, EPA and DHA were found to induce apoptotic cell death through the induction of caspase 3 and inhibition of COX-2 expression (Shirota et al. 2005; Mund et al. 2007). Similar n-3 PUFA-induced apoptosis was found in human vascular endothelial cells which suggests that n-3 PUFA-induced apoptosis might play a role in the inhibition of tumor angiogenesis (Artwohl et al. 2004). Moreover, in melanoma cells, DHA was found to stimulate cell cycle arrest and apoptosis in association with decreased phosphorylation of the tumour suppressor protein, retinoblastoma (Albino et al. 2000).

1.6.3.2.7 Evidence of Photoprotection by n-3 PUFAs

Several studies have been performed to examine the photoprotective properties on n-3 PUFAs against inflammation in humans. In contrast, the only studies examining n-3 PUFA protection against PI have been in murine models.

1.6.3.2.7.1 Evidence of n-3 PUFA Protection against UV-Induced Inflammation in Humans

To examine the effects of oral EPA supplementation on cutaneous mediators of the sunburn response, Shakhbati et al. (Shahbakhti et al. 2004) gave 28 healthy volunteers, 4gm EPA/day for three months. This was followed by the application of a UVB-dose of 3x MED (TL 12 Philips lamp) to unexposed buttocks skin and 16h later; raising suction blisters and collecting their fluid for the measurement of PGE_2 levels, along with cutaneous levels of other inflammatory mediators; IL-6, IL-8, IL-1β and TNF-α. The median MED of the subjects in this study was erythemally weighted at 28mJ/cm^2 with a range of 20-36mJ/cm^2. The placebo supplement given was the n-9 PUFA, oleic acid (OA); using identical capsules and doses (4gm/day for three months) in both active and control groups. Even though results from the study showed no significant effect on the UV-B induced generation of proinflammatory cytokines TNF-α, IL-6 or IL-8 following three months of EPA supplements, EPA managed to abolish the UV-induced secretion of PGE_2 in the blister fluids collected. In addition, the MED of the subjects in the active EPA group was significantly increased compared to the control OA group. Despite indications that EPA at the given (bioavailability-confirmed) dose has no effect on the examined cytokines, is still confirmed the photoprotective properties of EPA against the UV-induced induction of the proinflammatory and immunosuppressive PGE_2.

In a study by Rhodes et al. (Rhodes et al. 1994), the protective properties of n-3 PUFA-rich fish oil supplements against UVB-induced erythema and epidermal lipid peroxidation were examined. During the winter months, 15 Caucasian subjects (13 females and 3 males, median age of 42 yrs, age range 16-78 yrs; 12 suffering from PLE and 3 from psoriasis) were given 10gm/day of fish oil (x10 1gram capsules in two divided doses with each capsule containing 18% EPA and 12% DHA with the remaining capsule weight comprised of saturated and monounsaturated fatty acids) for 3 or 6 months. In the preceding 6 months, none of the patients had history of systemic treatment, phototherapy/photochemotherapy or suntan and were all instructed to continue with their normal diets during the trial period. At the start of the trial and at intervals of 1,2,3 and 6 months, UVB-MED tests were performed (using a 20W TL 12 lamp) and again, 2.5 months after stopping treatment, in those that continued to take the fish oil capsules for 6 months (n=10). 6 volunteers (3 PLE and 3 psoriasis) had paired split-skin biopsies taken from both irradiated and control (unirradiated) skin at baseline (pre-supplementation) and following 3 months of supplementation. Using gas

chromatography, the epidermal fatty acid composition of the biopsies was analyzed and following tissue homogenization, and the measurement of TBARS, an index of free-radical mediated lipid peroxidation was calculated. A rise in MED was seen after 1 month, with a significant increase in mean MED following 6 months of fish oil: 18.9 ± 13.9 mJ/cm^2 at baseline to 41.1 ± 16.6 mJ/cm^2 at 6 months; $p < 0.01$. 10 weeks after stopping the fish oil, MED fell to 23.1 ± 4.9 mJ/cm^2; $p < 0.05$. After 3 month of supplementation, total epidermal n-3 PUFA rose from 1.8 ± 0.4% of the total fatty acid content to 24.2 ± 3.9%; $p < 0.01$. This was accompanied by a rise in TBARS in irradiated skin from 6 ± 0.3 to 18.5 ± 2.6 A532/gm skin; $p < 0.01$ which indicates increased risk of skin lipid peroxidation. This finding can be explained by the highly unsaturated structure of n-3 PUFAs which makes them more susceptible to oxidation but at the same time could provide an extra protective mechanism by which n-3 PUFAs may act as a free radical buffers, to guard more vital structures against oxidative damage. Sensitivity to UVB-induced erythema was markedly reduced (as assessed by the significant increase in MED) which denotes inflammatory protection by n-3 PUFAs.

In a similar study by the same research group (Rhodes et al. 1995), 13 patients (11 females and 2 males, median age of 45yrs, age range 21-81yrs) with moderate polymorphic light eruption (PLE) were given dietary fish oil supplements, rich in n-3 PUFAs (the same fish oil supplement and dose of the preceding study were used) for three months and then examined for UVA-induced papule provocation threshold (PPT) using a graded challenge dose, UVB-induced MED and cutaneous PGE_2 levels (measured in suction blister fluids from forearm skin that was irradiated with a UVB-dose of 4x MED, 24h prior to blister raising and in blister fluid from contra lateral baseline non-irradiated forearm skin). Three months of fish oil supplements managed to significantly increase both UVB-MED (from a mean of 19.8 ± 2.6 to 33.8 ± 3. 7 mJ/cm^2; $p < 0.01$) and PPT (PPT was positive in 10 patients prior to the intake of the supplement; in 9 of those patients, a significant reduction was noted with $p < 0.001$) as well as significantly reduce the baseline (unirradiated skin) and post-UV cutaneous PGE_2 levels (before fish oil; mean baseline PGE_2 level was 8.6 ± 2.1ng/ml and were decreased to 4.1 ± 1ng/ml post fish oil. The mean UVB-induced increase in PGE_2 levels was 18.6 ± 8.9 ng/ml pre-supplementation and became 5.5 ± 1.4ng/ml post-supplements). Taken together, the results of this study show that fish oil supplements can reduce post-UV skin inflammation (as seen by the significant decrease in MED post supplementation) and that this reduction is at least in part due to the reduction in UV-induced PGE_2 levels. In addition, the observed reduction in UVA-induced PPT suggests a potential clinical application for fish oil in the management of PLE.

1.6.3.2.7.2 Evidence of n-3 PUFA Protection against Photoimmunosuppression in Mice

Experimental murine models demonstrate that PI and consequently photocarcinogenesis are reduced by dietary intervention with n-3 PUFAs. Such n-3 PUFA protection however, is yet to be shown in man (Black and Rhodes 2006).

As previously outlined, in section 1.6.3.2.6, n-3 PUFAs were inhibited photocarcinogenesis in murine models; a significant increase in tumor latency period and decrease in tumor multiplicity were seen following a 2 week-diet of 12% menhaden oil (Black et al. 1992). Furthermore, when a diet rich in n-3 PUFA was compared to one rich in n-6 PUFA; secretion of the immunosuppressive and proinflammatory mediator, PGE_2 was markedly reduced by n-3 PUFAs with the subsequent reduction in UV-induced cutaneous inflammatory responses (approximately 30% less) and an enhanced DTH immune response to DNCB (approximately 4.5 times more) compared to mice in the n-6 PUFA group (Fischer and Black 1991).

In a murine study by Moison et al. (Moison and Beijersbergen Van Henegouwen 2001), the influence of a high fat diet (25% by weight) enriched with EPA, DHA or EPA & DHA (MaxEPA) on UVB-induced systemic immunosuppression in 8-10 week old male BALB/c mice was examined using the CHS response to TNCB. Two control diets were used in this study; an oleic acid-rich diet and a standard commercial rodent diet containing a total of 7% fat. Since linoleic acid is known to affect immune responses, care was taken to ensure that all diets used had equal amounts of this n-6 PUFA; approximately 3.2/100 gm. In addition, because n-3 PUFAs were found to increase lipid peroxidation, the antioxidant state of the mice was also examined using levels of Vitamin C, E and glutathione. Mice were irradiated on their shaved back for 30 min using a TL 12 lamp. Because BALB/c mice are known to be relatively resistant to UVB radiation, a high dose of UVB was given at 15kJ/m^2; a dose expected to result in 50–70% immunosuppression. 4 days post-irradiation, the mice were sensitized to TNCB through their shaved abdominal wall. 5 days later, the animals were ear-challenged to TNCB and 24 hours later the CHS responses were assessed using ear thickness. UVB-induced immunosuppression was only 4% in the group of mice fed the high fat EPA diet. Mice that were on EPA and DHA (MaxEPA) showed a considerably higher level of PI at 24%. Immunosuppression in the control, oleic acid group was 53% and finally, in the DHA group the maximum level of immunosuppression was seen at 69%. For all diets, exposure to UVB radiation increased lipid peroxidation but it did not influence vitamin E levels. Vitamin E levels were only decreased in the mice fed DHA (alone or in combination with EPA) even without being exposed to UV-irradiation. Furthermore, levels of vitamin C and glutathione were only diminished in the mice given fish oil. The augmented lipid peroxidation observed in the mice fed DHA can be explained by the highly unsaturated nature of this long chain n-3 PUFA which makes it more prone to lipid peroxidation. In addition, this can account

for the high levels of PI seen in the mice. In conclusion, this study showed that dietary EPA, but not DHA can protect against UVB-induced immunosuppression and this protection is related to the epidermal antioxidant status post-irradiation.

In another murine study performed by the same group (Moison et al. 2001), the protective properties of topically applied EPA were tested against local PI of the CHS responses to DNFB. In 8-10 week old male BALB/c mice, fed a standard laboratory diet (with 7.2% fat of which 37.5% was linoleic acid) local immunosuppression to DNFB was induced in three different ways: UVB irradiation (1.5J/cm^2 using a TL 12 lamp), topical application of cis-UCA (150nmol/cm^2) and topical application of the pTpT thymidine dinucleotide (5nmol/cm^2). All three methods caused 70% immunosuppression. The influence of EPA on epidermal lipid peroxidation and antioxidant status was again measured. For the control medium, 10mmol of topical oleic acid in acetone (10ml/cm^2) was used and for the topical EPA; 3 different doses of 1, 10 or 100mmol were applied. Following topical treatment with EPA or OLA on shaved back skin, one of the aforementioned immunosuppressive techniques was carried out. At varying concentrations, topical EPA managed to partially protect against local immunosuppression; 10mol/cm^2 for UVB-irradiation, 100nmol/cm^2 for cis-UCA and 1000 nmol/cm2 for pTpT. As with systemic EPA treatment, topical application of EPA caused higher UVB-induced lipid peroxidation and lower vitamin C levels in a dose dependant manner. Glutathione was only decreased with the highest EPA dose whereas vitamin E was not decreased after UVB irradiation. In conclusion, provided that an adequate antioxidant defence is kept, topically applied EPA can protect against local induced immunosuppression.

It should be emphasized that all the studies examining the protective effects of n-3 PUFA against PI have been conducted in murine models. The aim of my study was to examine the protective effects of n-3 PUFAs against UV-induced immunosuppression in humans.

1.7 Hypothesis and Aims

My hypothesis is that dietary omega-3 PUFA will reduce immunosuppression caused by UVR in humans. Since UV-induced immunosuppression is a key step in photocarcinogenesis, this hypothesis will indicate the possible application of dietary n-3 PUFA as a nutritional approach to protect against skin cancer.

In a randomized, double blind randomized controlled study of 79 healthy female volunteers with known Ni allergy, the effects of 3 months of daily n-3 PUFA supplementation (4gm EPA/day) on PI was examined:

- Clinically – by comparing PI of Ni CHS responses in active (n-3 PUFA) and control (placebo) groups after 3 months of supplementation. To assess volunteer compliance and n-3 PUFA bioavailability, n3-PUFA levels in RBC membranes were examined (chapter 4).

  Prior to this, to establish methodology and setup a clinical model for performing and assessing PI of Ni CHS responses, a pilot study was performed to determine an appropriate Ni concentration, light source and dose series to use and decide on a suitable endpoint assessment scale for Ni CHS responses (chapter 3).

- Immunohistochemically and biochemically – by comparing UV-induced LC trafficking (using epidermal sheets from punch biopsies) and changes in cytokine levels (using suction blister fluids) pre vs. post supplementation, within and between groups (chapter 5).

2 MATERIALS & METHODS

2.1 Funding

Funding of this study was generously provided by The Association for International Cancer Research (AICR). Croda Oleochemicals (Hull, UK) provided the fish oil supplement capsules (Incromega E7010) free of charge. Central Pharma (Bedford, UK) packed the supplements & GP Solutions (Manchester, UK) labeled them; both at very low rates to support a cancer charity.

2.2 Ethics and Regulatory Approval

Full ethical approval was obtained from North Manchester Research Ethics committee (Ref No. 08/H1006/30) and from the University of Manchester. The project has full Research and Development, Salford Royal Foundation Trust (SRFT) Hospital approval received on the 24th June 2008 (Research and Development Ref No 2008derm06). The study was also registered with the UKCRN portfolio.

2.3 Study Design

A double-blinded, randomized placebo controlled study was conducted on 79 healthy female volunteers with known Ni allergy. Randomization of the participants was conducted by Andy Vail; senior statistician at the biostatistics department of SRFT Hospital, using blocks of computer generated numbers (mixed blocks of four to six). All study allocations were randomized before any of the research group members had access to them. The randomization schedule was sent to GP Solutions (Manchester, UK) who labelled the capsule boxes accordingly (the boxes were packed prior to the labelling by Central Pharma, Bedford UK).

An outline of the study is shown in figure 2.1:

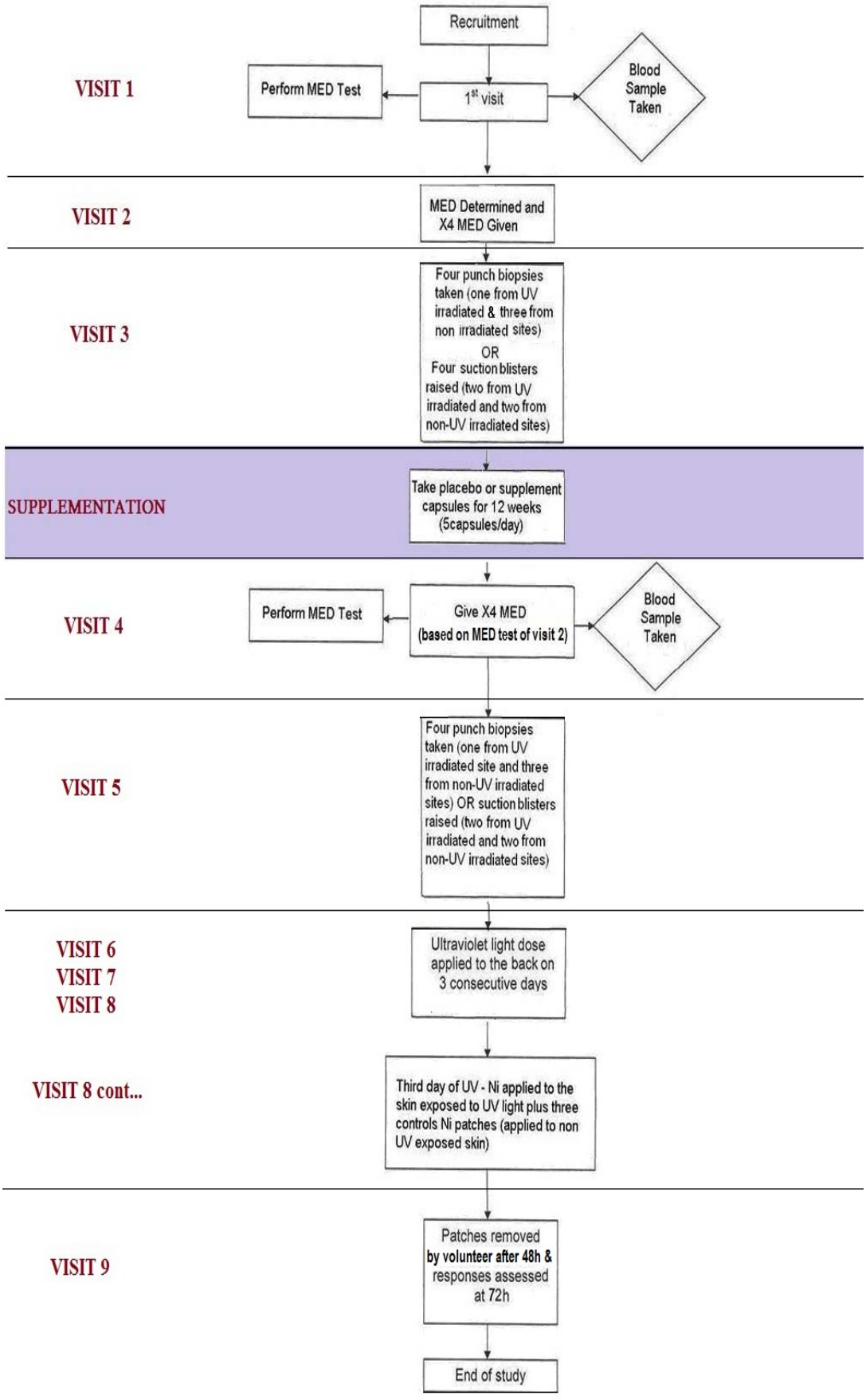


Figure 2.1 - Study Outline

2.4 Study Participants

2.4.1 Inclusion/Exclusion Criteria

Allowing for a potential dropout rate of 25%, a total of 79 healthy female volunteers were recruited for the study using the criteria listed in table 2.1:

Table 2.1 - Inclusion/Exclusion Criteria

Inclusion	Exclusion
Female between18-60 years old Fitzpatrick skin type I or II Known nickel allergy ("allergy to jewellery")	History of atopy History of skin cancer History of photosensitivity disorders On photosensitizing medication Sunbathing/sun bed use in the previous 3 months or any sunny holiday planned in the next 3 months Allergy to fish oil or gelatin Eating more that three oily fish meals per week or taking fish oil supplements.

2.4.2 Recruitment

Recruitment of volunteers was via:

- Study advertisement posters placed around the hospital (for local hospital staff, patients and visitors) and at the contact dermatitis clinic of Salford Royal Hospital.
- Direct approach to Ni allergic patients at the contact dermatitis investigation unit.
- Press release in the local newspaper and posting on the University of Manchester intranet.

Once initial contact with the volunteers was made, with their permission; further screening was performed via a series of short questions; inclusion/exclusion criteria to confirm their eligibility to participate in the study. If found eligible, the volunteer was sent an information sheet fully explaining the study. Those who still expressed a willingness to take part were then asked to attend the Photobiology Unit at SRFT Hospital. At the hospital, any further questions/enquiries were addressed and the volunteer was asked to read and sign a

consent form. A Ni patch test was then performed to confirm the volunteer's sensitivity, upon which her participation in the study could begin.

2.5 Oil Supplements

2.5.1 Fish Oil Capsules

Quotes were obtained from several companies for the packaging, labelling and randomisation of the oil capsules. After consideration, it was decided that Central Pharma (Bedford, UK) would carry out the packaging and GP Solutions (Manchester, UK) the labelling (according to the randomization scheme provided by Andy Vail from the Biostatistics Department, SRFT Hospital). Both the packaging and the labelling were done at very low rates in order to assist a cancer charity. Croda Oleochemicals (Hull, UK) provided the oils free of charge (table 2.2).

Table 2.2 - Contents of the fish oil capsule (Incromega E7010 – 1gm)

(approx.70% EPA, 10% DHA)

Component	Content
Omega-3 Fatty Acids	926.2 mg
Of which EPA	724.1 mg
Of which DHA	117.6 mg
Anti-oxidant /unsaponifiable content	3.6 mg
Free fatty acid	1 mg
Omega-6	40 mg
Saturated fatty acids	3 mg
Mono Unsaturated fatty acids	4 mg
Other poly unsaturated fatty acids	22.2 mg

4gm EPA/day (5 capsules) were given. The exact same dose and formulation (also supplied by Croda Oleochemicals) was found to be effective in previous photoprotection studies (Rhodes et al. 2003).

2.5.2 Selection of the placebo oil

Two options, frequently used in human nutritional studies, were appropriate for use. One was a placebo capsule containing 80% palm oil and 20% soybean oil; this closely resembles the lipid intake of an average UK diet. Another option was the medium chain caprylic capric triglyceride, GTCC (Glyceryl Tricoprylate Coprate Crodamol) containing 55% caprylic acid and 42% capric acid. This had the benefit of not being taken up by cell

membranes and had few biological properties. When the three oils (palm, soybean and GTCC) were filtered and their UV-absorption properties then examined using a spectrophotometer; the results favoured the use of the GTCC oil (Figure 2.2) which showed lower UV-absorption and hence less likelihood to interact with UVR:

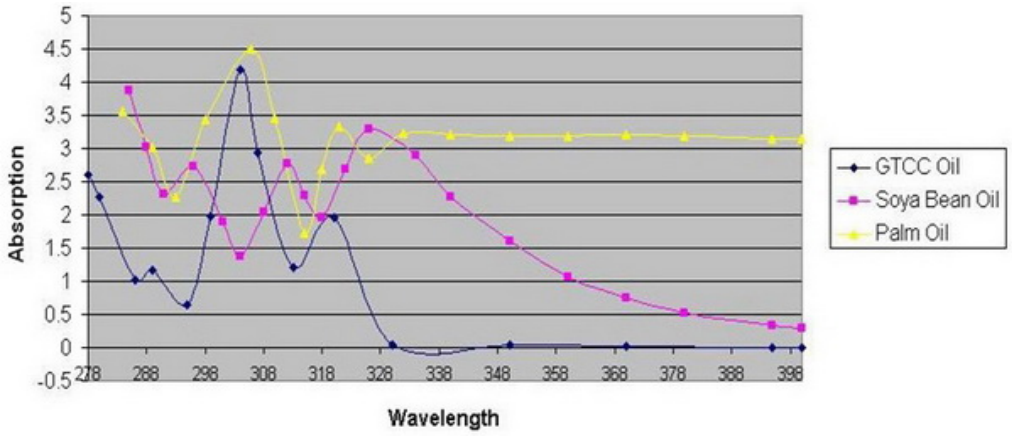


Figure 2.2 - UV-absorption of Tested Placebo Oils

2.6 Lamps

Three UVR lamps were used in this study. Initially, a TL 12 MED tester (section 2.6.1) was used as the UVB rich source for MED measuring and applying 4x MED before the post-UV biopsies and blisters. When later, an unexpected inconsistency was observed while routinely measuring the irradiance output of the TL 12 lamp before every use; it was replaced by another TL 12 source; the Waldman UV 800 (section 2.6.3). For the clinical assessment of PI, using the post-UV Ni CHS responses, a third UVR source with a predominant UVA-output was used; the solar simulator (section 2.6.2). During establishment of methodology for the clinical PI model (the pilot study) a fourth UVB-rich light source was compared to the solar simulator; the Waldmann UV6. Following comparison, the use of this lamp was discontinued (for more details on the comparison between the solar simulator and the Waldmann UV6, please see section 3.4).

2.6.1 The TL 12 MED Tester

The UV source initially used to measure MED and apply 4x MED UV-doses (section 2.2) was a TL 12 lamp (Philips TL 20W/12 RS SLV; spectrum: 280-370 nm, peak: 305nm, 56% UVB & 43% UVA) housed in a black plastic tube with five apertures, each 1cm wide: the TL 12 MED tester. Four of the MED tester apertures were attenuated with mesh grids of variable densities to give variable irradiance outputs (Figure 2.3).

Figure 2.3 - The TL 12 MED Tester

The irradiance of the lamp was measured through it's open aperture and the results recorded prior to every use in order to detect any change in output. This was done using two meters: the IL 1400A & the TL12 lamp sensor (both meters are described in detail in section 2.7.3). Measurements with the two radiometers agreed to within 2%. The spectral emission of the TL12 lamp is shown in figure 2.4.

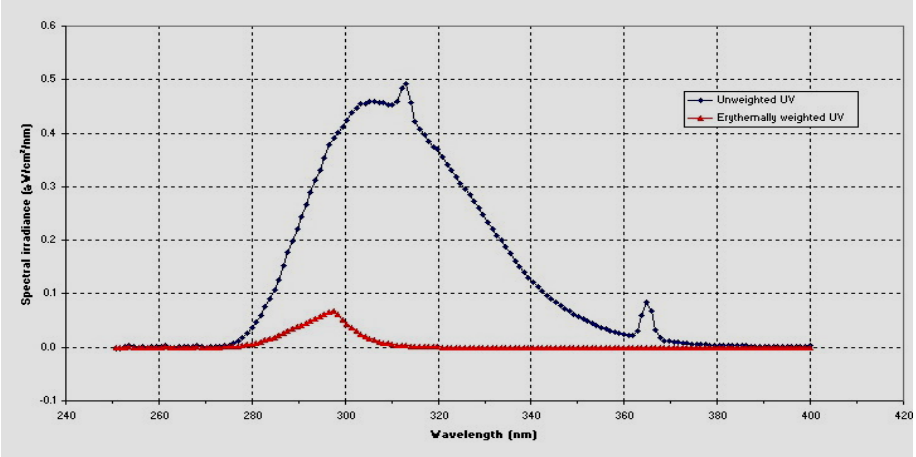


Figure 2.4 - Example of the UVB TL 12 Lamp Spectral Irradiance
(Source: Medical Physics Department, Christie NHS Foundation Trust Hospital)

2.6.2 The Solar Simulator

The solar simulator (figure 2.5) was used for the clinical PI model; the post-UV Ni patch test. The specifications of the solar simulator used were as follows: 1000 Watt Newport model no.91293-1000 fitted with a xenon short arc lamp and a two dichroic mirror, which only reflect wavelengths in the 280 to 400 nm range and minimize both visible and infrared radiation. In addition, an 81017 atmospheric attenuation filter (mimicking the atmospheric absorption of UV) was installed to remove UVC and modify the UVB spectrum. The beam size of the simulator was 6 x 6 inches with a ±3° collimation. The UV-spectral fraction of the solar simulator irradiance was approximately 3.35% UVB & 96.68% UVA (using a non-CIE UVB spectrum of 290-320nm). With these specifications; the solar simulator is the light source that best mimics ambient solar UV (for a comparison between the spectral irradiance of the solar simulator and ambient solar UV, please see figures 2.6 & 2.7).

Figure 2.5 - The Solar Simulator

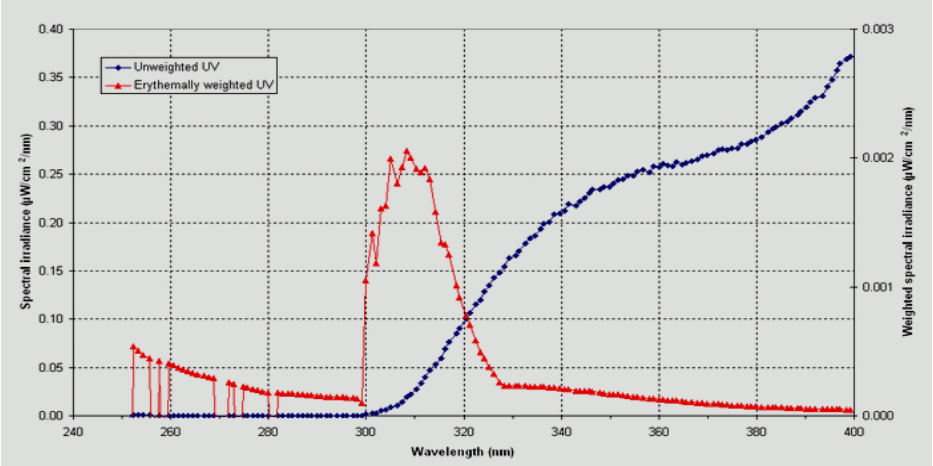


Figure 2.6 - Example of Solar Simulator Spectral Irradiance

(Source: Medical Physics Department, Christie NHS Foundation Trust Hospital)

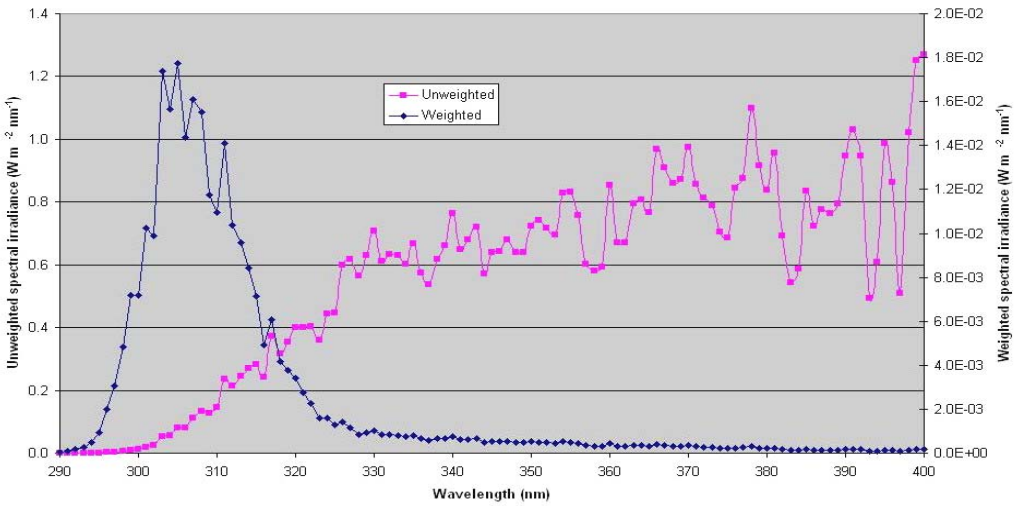


Figure 2.7 - Sample Terrestrial Solar Spectrum

(From Health Physics 73, 456-464, 1997)

When applying solar simulator radiation (SSR) to the volunteer's back, for the clinical assessment of post-UV immunosuppression, the UV-doses given were erythemally weighted. Erythemal weighting defines relative erythemal-effectiveness. If a lamp has relatively more short wavelengths (UVB) in it's spectral irradiance, it's erythemal effectiveness will be higher; shorter wavelengths of UV have higher energy (mW/m^2) and can hence cause more erythema. An erythemal action spectrum has been proposed by McKinlay & Diffey (1987) and adopted by the International Commission on Illumination (CIE) (Figure 1.7) to "weigh" relative erythemal-effectiveness over given wavelengths in a UV-spectrum. Using this CIE action spectrum and the lamp's spectral irradiance, a conversion factor can be calculated to switch between erythemally weighted and total UV. A conversion table showing the erythemally weighted and total UV-doses used in this study can be found in section 2.11 (table 2.3).

2.6.3 The Waldman UV 800

After irradiance output inconsistencies were seen with the TL 12 lamp, it was replaced with another UVB- rich light source for MED testing and applying 4x MED; the Waldmann UV 800 (Waldmann, Villingen-Schwenningen, Germany) (Figure 2.8). The Waldmann UV 800 was fitted with x10 identical bulbs to the one found in the TL 12 (broadband UVB Philips TL 20W/12 RS SLV) giving it an identical spectral irradiance (please see figure 2.4). The expected increase in irradiance output of the Waldmann UV 800 was accounted for by decreasing the lamp's application time.

Figure 2.8 - The Waldmann UV 800

2.6.4 The UV6 Lamp

The UV6 lamp (figure 2.9) was only used for the pilot study; while deciding on the most appropriate light source to use for the clinical model of PI. The irradiance specs of this lamp are as follows; irradiance spectrum: 290 - 400 nm, emission peak 315 nm, 33% UVB, 67% UVA.

Figure 2.9 - The UV6 Lamp

2.7 UVR Meters

All the meters used in this study were tested and calibrated by the Medical Physics Department at Salford Royal Hospital, using standards ultimately traceable to the UK National Physical Laboratory, prior to the start of the study and regularly during it's course. All meters used to measure lamp irradiance in this study were calibrated for the specific lamp emission that they measured.

2.7.1 The Waldmann UV Meter

For measuring the irradiance of the solar simulator and UV6 lamp, a Waldmann UV meter was used; UV6 meter model number 585200000 (Herbert Waldmann GmbH & Co., Villingen-Schwenningen, Germany) (Figure 2.10). The meter was placed 10 cm from the centre of the lamp and the UVA /UV6 button on the side was then pressed for measuring the irradiance of the solar simulator/UV6 lamp respectively. Once the meter display stabilized; the reading was taken. It should be noted that the Waldmann is used to perform point of use check to verify consistent day to day lamp performance. It measures unobstructed beam rather than true skin exposure (prior calibration had established a fixed, precise relationship between these two intensities).

Figure 2.10 - The Waldmann UV Meter

2.7.2 IL-1400A Meter and TL 12 Lamp Sensor

For measuring the irradiance of the TL 12 MED tester, two meters were used to ensure the consistency of the lamp's output; the IL-1400A radiometer photometer & the TL 12 Lamp Sensor. The IL-1400A meter (International Light Inc. Newburyport, USA) was fitted with a SEL 240 detector with a SCS 280 filter and a Telfon Disc (TD) diffuser (figure 2.12a). The TL 12 Lamp Sensor is a UV radiometer designed by Bryan Diffey for the Regional Medical Physics Department, Dryburn, UK (serial no.MJL59). The meter was calibrated for measuring TL 12 lamp irradiance and is fitted with a custom made detector specially designed for use with the TL 12 MED tester (figure 2.12b).

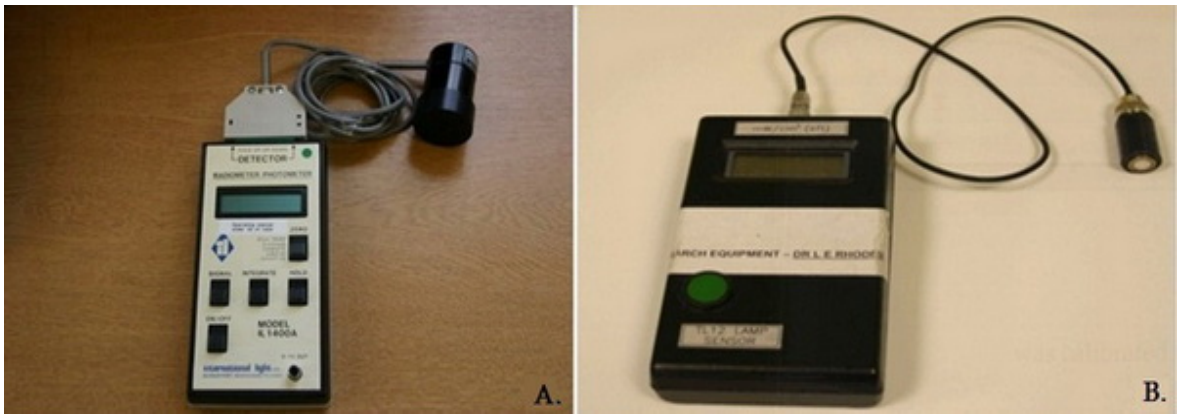


Figure 2.11 - The IL-1400A Meter (A) and TL 12 Lamp Sensor (B)

2.8 MED Testing Protocols

Four different MED testing protocols were used in this study: pilot study MED tests were done using the UV6 lamp and/or the solar simulator, both applied through a 10 aperture MED template (supplied by the Medical Physics Department, Salford Royal Hospital; Manchester) (Figure 2.12). In addition, two MED testing protocols were used in the main study: a TL 12 MED protocol and later on, a UV 800 lamp and MED template protocol.

2.8.1 MED test using the UV6 lamp and MED template

The UV6 lamp was switched on and allowed to warm up for 2 minutes (to allow the lamp irradiance to stabilize). After warm up and shortly before applying the lamp, irradiance was measured using the Waldmann UV meter (section 2.7.2). The Waldmann UV meter readings were between 1.32 and 1.42mW/cm^2. Any unusual readings were reported to the Medical Physics Department. With volunteers lying flat on their stomach, the 10 aperture MED template (Figure 2.12) was secured to the upper back with a 3M Micropore^{TM} hypoallergenic tape (reference no.1530-1). All other areas of the volunteers' skin as well as their eyes were protected from UV-exposure using a UV-opaque cloth and UV goggles respectively. The couch height was then adjusted to keep the lamp centered over the MED template and 10cm away from it (using a pre-cut 10cm candle). Exposure time on the lamp was set to 8 minutes and the lamp switched on to give, through the MED template, the following UV-dose series (mJ/cm^2):

63	50	40	33	25
6.2	7.7	11	13	17

At the end of exposure, the template was removed and the volunteer's skin was quickly marked before impressions of the MED template faded. The volunteer was then asked to come back the next day (at preferably the same time) to read the MED test results, visually and using the Diastron erythema meter (section 2.7.1).

Figure 2.12 - 10 Aperture MED template

2.8.2 MED test using the solar simulator and MED template

MED testing using the solar simulator was only employed during the pilot study to compare MED testing using a UVB-rich lamp, the Waldmann UV6 with testing using a UVA/UVB light source, the solar simulator. As with the other two canopy lamps (the UV6 and UV 800), the 10 aperture MED template (Figure 2.12) was used with the solar simulator to perform the MED test. Before beginning the test, the solar simulator was switched on and allowed to warm up for 10 minutes. During warm up, the MED template was secured to the volunteer's back using 3M Micropore^{TM} hypoallergic tape and their back was then covered using UV-opaque cloth. At the end of the warm period, the solar simulator was positioned above the volunteer and the couch height adjusted to keep the centre of the simulator's lens 10cm away from the volunteer's back (measured using a pre-cut candle). With the back of the volunteer still covered with UV-opaque cloth, the

Waldmann UV meter (section 2.7.2) was placed on their back, the simulator shutter opened and the irradiance of the lamp measured. The meter was then set aside and as the UV-opaque cloth was pulled off the volunteer's back, timing of the solar simulator irradiance was started. The simulator was allowed to run for 25 minutes (provided the irradiance measured by the Waldmann meter was the standard 15.1 mW/cm^2) to give the following UV-dose series (mJ/cm^2):

68	54	43	35	27
6.6	8.2	11	14	18

At the end of exposure, the simulator shutter was closed, the MED template removed and the skin quickly marked before the template impressions faded. Volunteers were then asked to come back the next day (at preferably the same time) to asses the MED test results visually and using the Diastron erythema meter.

The standard irradiance output of the solar simulator (as measured by the Waldmann meter) was 15.1mW/cm^2. This irradiance output usually drops slowly and steadily with usage of the solar simulator. Any large change in irradiance output of 5-10% or more was reported to the physicist at the Photobiology Unit. Otherwise, to correct for any difference from 15.1mW/cm^2 (which equates to an exposure time of 25 minutes i.e. 1500 second) a new exposure time was calculated as follows:-

Corrected time (sec) = (1500 sec x 15.1 mW/cm^2) / Waldmann reading in mW/cm^2

This ensured that the UV-dose received by each aperture remained the same.

2.8.3 MED test using the TL 12 MED Tester

After switching on the TL 12 MED tester and leaving it to warm up for 5 minutes, it's irradiance output was measured using the TL 12 Lamp Sensor followed by the IL1400A radiometer (section 2.7.3), to ensure irradiance consistency and detect any variations as early as possible. Meter readings were between 0.29 - 0.31 mW/cm^2 using the TL 12 lamp sensor (260 -280mW/cm^2 with the IL 1400A meter). With the volunteers lying flat on their stomach, the TL 12 lamp was then applied to the left lower back; twice, on two rows, using two different exposure times to give a geometric series of ten doses (mJ/cm^2) as follows:

4min. 15 sec.	→	70.4	47.9	41.2	29.1	24.0
1min. 3 sec.	→	17.4	11.8	10.2	7.2	5.9

At the end of exposure, the volunteer's skin was quickly marked before the TL 12 aperture impressions faded and they were asked to come 24 hours later to assess and measure erythema at the irradiation sites. During the assessment visit, the volunteers were rested

flat on their stomach for 10 minutes, followed by a visual and Diastron erythema meter assessment of the MED.

2.8.4 MED test using the UV 800 lamp and MED template

The UV-800 lamp was switched on and allowed to warm up for 3 minutes. Irradiance of the lamp was then measured using two meters; the Waldmann UV meter (section 2.7.2) and the IL1400A meter (section 2.7.3) after removing the TL 12 MED tester adapter and replacing it with a UVB detector. Meter readings were 3.10 - 3.40mW/cm^2 using the Waldmann UV meter (10 - 11 mW/cm^2 using the IL 1400 meter). With volunteers lying flat on their stomach, the 10 aperture MED plate was then secured to the upper back with 3M Micropore^{TM} hypoallergenic tape. All other areas of the volunteer's skin and eyes were protected with UV-opaque cloth and UV goggles respectively. After adjusting couch height to keep the lamp at a distance of 20cm from the volunteer's back (measured using a pre-cut candle) the UV 800 was switched on and applied for 2 minutes and 35 seconds, to give the following UV-doses (mJ/cm^2):

63	50	40	33	25
6.2	7.7	11	13	17

At the end of exposure, the volunteer's skin was quickly marked before impressions of the MED template faded. Volunteers were then asked to come back the next day (at preferably the same time) to read the MED test results visually and using the Diastron erythema meter.

2.8.5 The Diastron Reflectance Erythema Meter

In addition to visually assessing the erythema response, the skin's erythema intensity was also measured objectively using a reflectance spectrophotometer; the Diastron erythema meter (designed by Professor Brian Diffey, University of Newcastle, UK) (Figure 2.13). The meter shines white light on the skin using a fibre optic probe and then, using narrow pass interference filters (546nm and 632nm), measures the reflected green and red light from the skin. Haemoglobin, the primary cause of skin redness absorbs the green light emitted from the Diastron meter whereas the red light absorption hardly changes. The results are expressed as the Erythema Index (EI) which is calculated as follows:

Erythema Index = Log 10 (reflected red light / reflected green light)

All meter EI readings were taken in triplicates in a sequential rather than a consecutive pattern i.e. each site reading was only repeated after all the other sites were measured once. Readings from both measured skin as well as unirradiated adjacent skin were taken. The mean reading of the adjacent skin was then subtracted from that of the measured skin to give a background corrected EI reading. This method was preferred

over measuring baseline skin EI beforehand and using that later to calculate background corrected readings as it avoided variation seen in vasodilatation on a day to day basis.

Figure 2.13 - The Diastron Erythema Meter

2.9 Application of 4X MED

For the assessment of UV-induced LC migration and secretion of immunomodulatory cytokines, a UV-dose of 4x MED was applied to the volunteer's unexposed upper buttocks skin. After irradiance output inconsistencies were seen with the TL 12 MED tester, it was replaced with another UVB- rich light source employing x10 identical TL-12 bulbs: the Waldmann UV 800 (section 2.6.3). The expected increase in irradiance output was accounted for by decreasing the lamp's application time:

Initially, the lamp's irradiance output was measured and used to calculate it's erythemally weighted irradiance:

Erythemally weighted irradiance of UV 800: 0.451 mW/cm^2
Erythemally weighted irradiance of TL 12 MED tester: 0.276 mW/cm^2

Using the erythemally weighted irradiance, the exposure time to apply the 10 UV doses (mJ/cm^2) of the MED test was calculated:

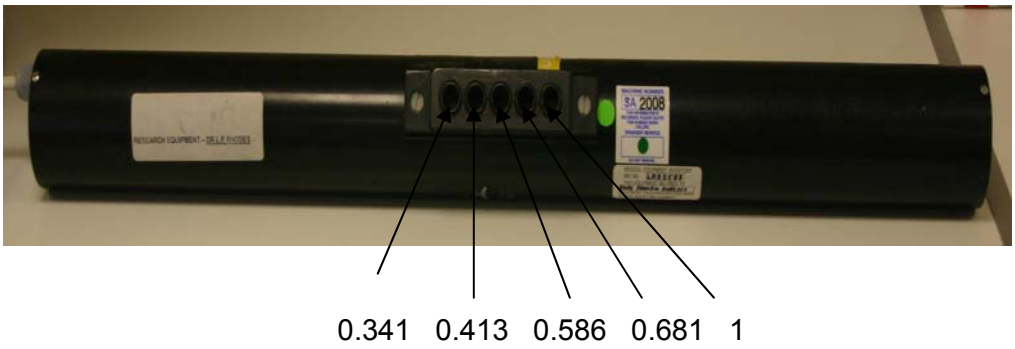
UV 800 exposure time (through the 10 aperture MED template):

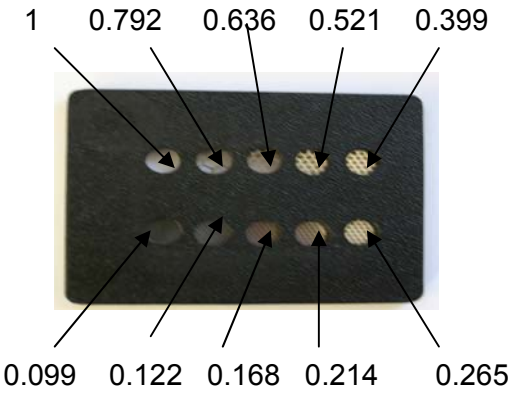
2min 35sec → 69.7 55.2 44.3 36.3 27.8
6.9 8.5 11.7 14.9 18.5

TL 12 MED tester exposure time (over two applications):

Upper row = 4min. 15 sec. → 70.4 47.9 41.2 29.1 24.0
Lower row = 1min. 3 sec. → 17.4 11.8 10.2 7.2 5.9

For both the TL 12 MED tester (drainpipe) and the MED template, the open aperture was designated a transmission factor of 1. Relative to this, each of the meshed apertures was given a fraction of this transmission factor as follows:





Using the transmission factor, an application time to give 4xMED through the open aperture was calculated:

Using the TL-12 MED Tester

Dose (mJ/cm^2)	Time needed to give 4X MED through open aperture (min:sec)
70.4	17:00
47.9	11 :35
41 .2	9:58
29.1	7:01
24.0	5:48
17.4	4:12
11.8	2:52
10.2	2:28
7.2	1:44
5.9	1:26

Using the UV 800 and MED template

Dose (mJ/cm^2)	Time needed to give 4X MED through open aperture (min:sec)
69.7	10:18
55.2	8:11
44.3	6:34
36.3	5:23
27.8	4:7
18.5	2:44
14.9	2:13
11.7	1:44
8.5	1:16
6.9	1:14

2.10 Initial Ni Patch Test Protocol

Prior to commencing the study, each volunteer had a Ni patch test to confirm their allergy to Ni and to determine the Ni concentration that will be used on them for the post supplementation, post UV Ni patch test; a concentration that gave moderate confluent erythema. The Ni concentrations (0.15% - 5%) and patch orientation were as shown in Figure 2.14 (on the volunteer's upper left back – just above their bra strap):

- Applying the patches in a vertical manner with the volunteer sitting up was more consistent with body contour and movement and helped prevent the patches from coming off prematurely.
- In addition to the 5 Ni patches, an empty Finn chamber was applied to exclude skin reactions to pressure or hypersensitivity to aluminium.
- The Finn chambers used were 8mm in diameter. The amount of Ni in each chamber was kept constant at 25mg (approx one line of Ni across the Finn chamber diameter). The Finn chambers were held in place using Micropore hypoallergic tape.
- 5% is the standard Ni concentration used at the contact dermatitis clinic and was the one diluted in white soft paraffin to get the remaining four concentrations. For the first three, a serial two-fold dilution of the 5% was used and for the final dose; the fourth, 0.6% concentration was diluted by four-fold to get 0.15%.

Figure 2.14 - Orientation and concentration of Ni patches on left upper back

2.11 Post-UV Ni Patch Test Protocol

Post-UV Ni patch tests were used as our clinical model for PI. In addition to setting up the model during the pilot study (section 3.3), the only other time the post-UV Ni patch tests were used was when the volunteers came back following 3 months of supplementation. For 3 consecutive days, the volunteers received a geometric 3-UV-dose series of SSR on their right upper backs, as follows (Figure 2.15):

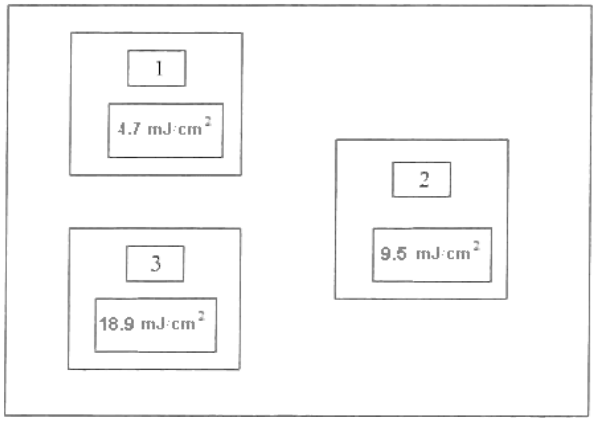


Figure 2.15 - Distribution of Geometric UV-Dose Series applied to volunteer's uppper back

The three UV doses (4.7, 9.5 and 18.9mJ/cm^2) were applied using the solar simulator (section 2.6.2) and a 0.5 cm thick, black rubber mask measuring 22x25 cm with three 1x1 inch openings cut out (Figure 2.16). Following a 10 minute warm up, the irradiance of the solar simulator was measured using the Waldmann UV meter (section 2.7.2). The standard irradiance output of the solar simulator (as measured by the Waldmann meter) was 15.1mW/cm^2. The solar simulator beam shutter was then closed and the couch height adjusted to keep the distance between the lamp and rubber mask at 8 cm (measured using a pre-cut candle). This was followed by re-opening the beam shutter to begin applying the UV-dose series. The doses of UV given were controlled by closing each rubber mask-opening at a different time point, using small radiopaque squares cut out from the same rubber that was used to make the mask, as follows (Figure 2.16):

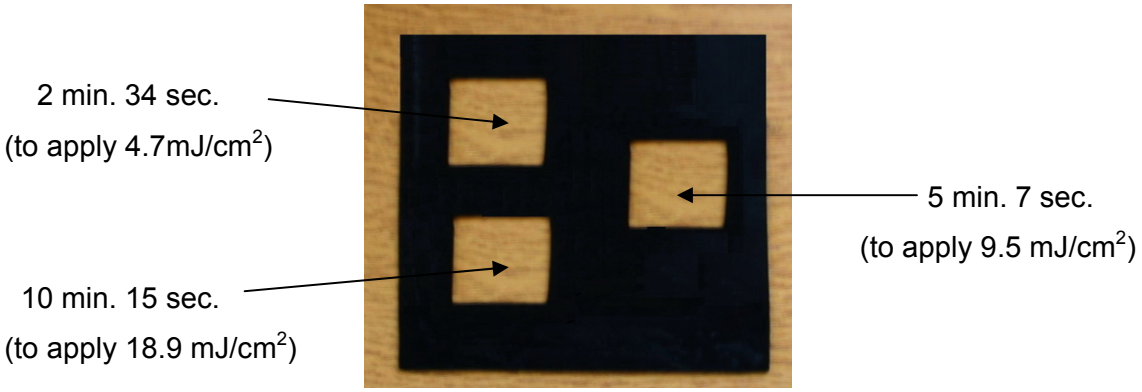


Figure 2.16 – Black rubber mask with exposure times for the 3 UV-doses

As outlined in section 2.6.2, the 3 UV doses given were erythemally weighted. As calculated by Dr. Donald Alan; Medical Physics Department, SRFT Hospital, the conversion factor for switching from total UV to erythemally weighted was 2.49 E-03.

Table 2.3 - Conversion of Erythemally Weighted to Total UV

Erythemally Weighted UV	Total UV
4.7 mJ/cm^2	1.89 J/cm^2
9.5 mJ/cm^2	3.82 J/cm^2
18.9 mJ/cm^2	7.59 J/cm^2

To reach this conversion factor, each wavelength in the spectral irradiance of the lamp was measured using a spectroradiometer and as per the CIE erythemal action spectrum (Figure 1.7) was accordingly multiplied by it's relative erythemal efficacy. As can be seen from this conversion factor, the relative erythemal efficacy of the solar simulator irradiance spectrum (96.68% UVA) is very weak and accordingly does not correlate with it's photoimmunosuppressive properties. For this reason; the same absolute UV-dose was used for all participants. The highest dose of UV used was 70% of the mean MED of 10 patients (28mJ/cm^2) from one of the photo investigation series studies conducted at Salford Royal Hospital (patients in this series were Fitzpatrick's skin type I - III). The dose was then decreased by a factor of two for two additional doses.

In addition to assessing the Ni responses visually using the ICDRG scale (table 2.4), erythema index (EI) readings from each patch area and adjacent skin (that was also within the solar simulator exposure field but did not have any Ni applied to it) were taken using the Diastron meter (section 2.7.1) and a background corrected EI reading was calculated. In addition, readings from the three control patches (Ni without prior UV-irradiation) along with baseline skin (neither nickel nor UV applied to it) were used to calculate a background corrected EI reading for the control patches. Thus a graph could be constructed with four background corrected data points; a point for the mean of the control, unirradiated patches and a point for each of the three UV-irradiated Ni patches. Using these background corrected readings, the % PI for each of the three UV-doses could be calculated as follows (Damian et al. 1997):

$$\% \text{ PI} = [(\text{EI unirradiated} - \text{EI irradiated}) / \text{EI unirradiated}] \times 100\%$$

Table 2.4 - The ICDRG Grading Scale

(Memon and Friedmann 1996; Fuchs and Packer 1999)

-	Negative reaction
?	Doubtful reaction; faint macular erythema only
+	Weak (non-vesicular) positive reaction; erythema; infiltration, possibly papules
++	Strong (vesicular) positive reaction; erythema, infiltration, papules, vesicles
+++	Extreme positive reaction; bullous reaction
IR	Irritant reaction

2.12 Blood Collection Protocol

Blood samples were sent to Bradford University for analysis of erythrocyte fatty acid profile by gas chromatography (section 2.14.3); PUFA content of erythrocyte membranes reflects compliance and the nutritional n-3: n-6 fatty acid ratio.

The blood was collected in two 3.4 ml sterile EDTA (ethylene diamine tetra acetic acid) tubes. It was centrifuged soon after collection at a speed of 1500 RPM, temperature 4^{o}C for 15 minutes. A plastic Pasteur pipette was then used to remove the plasma layer (the buffy coat layer formed between plasma and red blood cells) which contains cell debris etc; this was discarded. Blood samples were then labelled and stored in clean cryogenic vials, in an upright position at -80^{o}C and sent to Bradford University for analysis.

2.13 Punch Biopsy Protocol

With the patient lying down on her stomach, the lower back skin was cleaned with a sterile aqueous solution containing chlorhexidine gluconate 0.015% w/v and cetrimide 0.15% w/v. Lidocaine Hydrochloride 1% was then injected subcutaneously at the sites where the biopsies are to be taken (until the volunteer could not feel a needle prick; volume varies from one volunteer to the next). Both sides were anaesthetized at the same time (UV-irradiated and unirradiated). Using a dermal biopsy punch with a diameter of 5mm, the punch biopsies were then taken. The biopsy wounds were sutured with non absorbable stitches & a dry dressing applied. The volunteers were given dressings to take home and asked to come back in 7-10 days to remove the stitches.

In total, 4 biopsies were taken from each volunteer; 1 from irradiated and 3 from non-irradiated skin. One unirradiated skin biopsy was snap frozen in liquid nitrogen and taken to Bradford University for bioavailability studies which are not presented in this thesis. Two biopsies (one from irradiated and one from unirradiated skin) were used to count LCs in the epidermis (for details please section 2.8). The final biopsy (from unirradiated skin) was put in RNA later, snap frozen and stored at -70 ^{o}C for gene analysis at a later date.

2.14 Suction Blister Protocol

Four suction blisters were raised pre and four post supplementation; 2 from UV-irradiated and 2 from contralateral, unirradiated skin of the upper buttocks. The 4 suction blister cups were placed where the blisters were to be raised with Vaseline being used to help seal the cup rims and facilitate the creation of a vacuum (figure 2.17). Negative pressure was then applied to the skin at 250mm Mercury (Hg) Vacuum using the suction pumps and the skin was observed until the blisters were fully raised (normally within 1 and a half to two hours). Once the blisters were raised, the vacuum pressure was released and the blister fluids were aspirated using a U-100 0.5 ml insulin syringe, placed in a Nunc vial and snap frozen in liquid nitrogen. Blister fluid from two of the blisters (one irradiated and one un-irradiated) were sent to Bradford University for biochemical analysis not discussed in this thesis while two were kept in Manchester for cytokine analysis pre and post-UV before and after three months of supplementation.

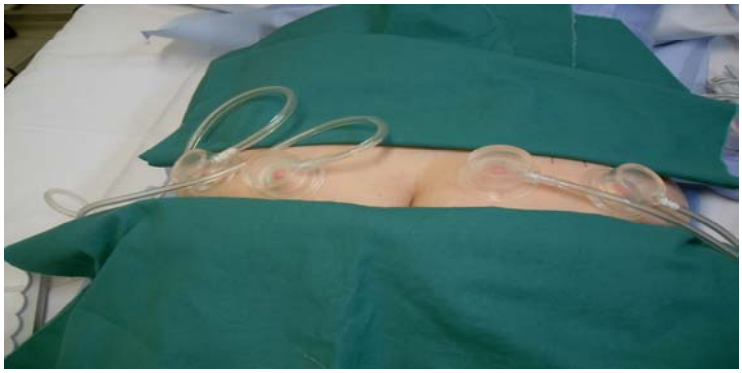


Figure 2.17 – The Raising of Suction Blisters

2.15 Laboratory Analysis Protocols

2.15.1 Langerhans Cell Counting Protocol

Epidermal sheets from 2 of the punch biopsies taken (1 UV-irradiated and 1 unirradiated) both pre and post supplementation, were used to immunohistochemically count LCs. Once taken, the biopsies were placed in vials containing a 0.02M solution i.e. 0.152 gm of ethylene diamine tetra acetic acid (EDTA) suspended in 20 ml of phosphate-buffered saline (PBS) – The EDTA was weighed out and suspended in the PBS just prior to use. After incubating the biopsy vials for two hours in 0.02M EDTA at a temperature of 37 ^{o}C (to facilitate the separation of epidermis from dermis), with the use of a forceps the epidermis was carefully peeled from the dermis. The epidermal sheets were then washed in PBS, fixed in acetone at -20 ^{o}C and then re-washed in PBS to remove any acetone residues. If desired, after this stage, the epidermal sheets could be stored and the staining & counting done at a later date. Epidermal sheets were then incubated in mouse monoclonal antibody to CD1a: clone NA1/34; IgG2a (diluted to 10ug/ml in PBS containing 0.1 % BSA; 30ul CD1a + 570ul BSA/PBS) for 60 minutes, washed three times in PBS and

then incubated for a further 60 minutes in fluorescein isothiocyanate (FITC) conjugated goat anti mouse (GAM) immunoglobulin (diluted to 1 in 100 in 0.1% BSA/PBS; 5ul GAM + 495ul BSA/PBS). Finally, the sheets were washed in PBS and mounted on a slide in citiflour with nail varnish sealing the slide. LCs were then counted using an Olympus Bx50 fluorescent microscope fitted with an eyepiece graticule (50 fields/graticules were counted per sheet of epidermis).

2.15.2 Cytokine Analysis of Blister Fluid

In half the volunteers participating in the study, both before and after 3 months of supplementation, suction blister aspirates from 1 unirradiated and 1 irradiated blister were used to asses skin cytokine expression pre and post-UV respectively. Skin cytokine levels were measured using a luminex multiplex cytokine assay system. Multiplex analysis of blister fluids allows the measurement of multiple cytokines in a small sample volume.

The Luminex Multiplex Cytokine Assay System

Following protein concentration analysis of the samples, the simultaneous quantification of cytokines in suction blister fluids was performed using a fluorescent microbead-based flow cytometry system: the Millipore Milliplex® Human Cytokine/Chemokine kit. The cytokines looked for were: IL-8, IFN-γ, TNF-α, IL-1β, IL-4 and IL-17.

The luminex assay kit uses internally colour-coded microspheres (beads) with two fluorescent dyes. Through the precise concentration of these dyes, 100 distinctly coloured bead sets can be created, each coated with a specific capture antibody. After an analyte from a test sample is immobilized through capture by the antibody-coated beads, a biotinylated detection antibody is introduced. The resulting reaction mixture is then incubated with a reporter molecule, the Streptavidin-Phycoerythrin (PE) conjugate in order to complete the reaction on the surface of each bead. The beads are then passed through a cytometer (fluorometer) where they are excited by two different laser beams. Rapidly passing the beads through the first laser (red) excites their internal fluorescent dye markers (fluorophores) which allows spectral address decoding and the subsequent decoding of the analyte measured. The second laser (green) then excites PE, the fluorophore on the Streptavidin-PE conjugate of the reporter molecule which in turn results in analyte quantification.

o Preparation of Human Cytokine Standards

As with the protein assay, standard curves had to be generated prior to commencing the analysis. In order to do this, the human cytokine standard supplied with the kit was reconstituted and used to prepare working standard dilutions.

Approximately 250 µL of deionized water were used to construct a 10,000 pg/ml concentration of standard for all cytokines. After mixing and centrifuging for 10 seconds,

the vial of reconstituted standard was allowed to stand for 5-10 minutes. The reconstituted standard was then diluted in 5 microfuge tubes containing 200ul of assay buffer each to prepare working standard dilutions of 2000, 400, 80, 16, and 3.2pg/ml as follows:

- 200ul Assay Buffer + 50ul reconstituted standard (10,000pg/ml) → 2000 pg/ml
- 200ul Assay Buffer + 50ul working standard (2000 pg/ml) → 400 pg/ml
- 200ul Assay Buffer + 50ul working standard (400 pg/ml) → 80 pg/ml
- 200ul Assay Buffer + 50ul working standard (80 pg/ml) → 16 pg/ml
- 200ul Assay Buffer + 50ul working standard (16 pg/ml) → 3.2 pg/ml

In addition, 200ul of Assay Buffer were used as the background standard with a concentration of 0pg/ml.

o Immunoassay Procedure

The Microliter filter plate was set on a plate holder and pre-wet by pipetting 200ul of assay buffer into each well. The plate was then sealed and placed on a plate shaker for 10 minutes. Excess assay buffer was blotted from the bottom of the plate with an absorbent pad. The placement of standard concentrations (0 -10,000 pg/ml), background and samples were mapped on a worksheet and 25ul of each were accordingly placed into the appropriate wells. The bead mixing bottle was then vortexed and, while intermittently shaking to avoid settling of the beads, 25ul of the mixed beads was added to each well. The plate was then sealed with a lid sealer and covered with a lid. A rubber band was then wrapped around the plate holder, plate and plate lid and left to incubate at room temperature for 1 hour. The plate was then washed with wash buffer (200ul/well) and excess blotted from the bottom of the plate with an absorbent towel. Next, 25ul of detection antibodies were added to each well and again, the plate was sealed and incubated on the plate shaker for 30 minutes. Finally, 25ul of Streptavidin-PE conjugate was added to each well and after sealing and covering with the lid, the plate was incubated once more on the plate shaker for 30 minutes. The plate was then washed with 200ul of wash buffer/well and 150ul of sheath fluid added to all wells. To re-suspend the beads, the plate was placed on the shaker for 5 minutes and then run on the luminex analyzer (Figure 2.18).

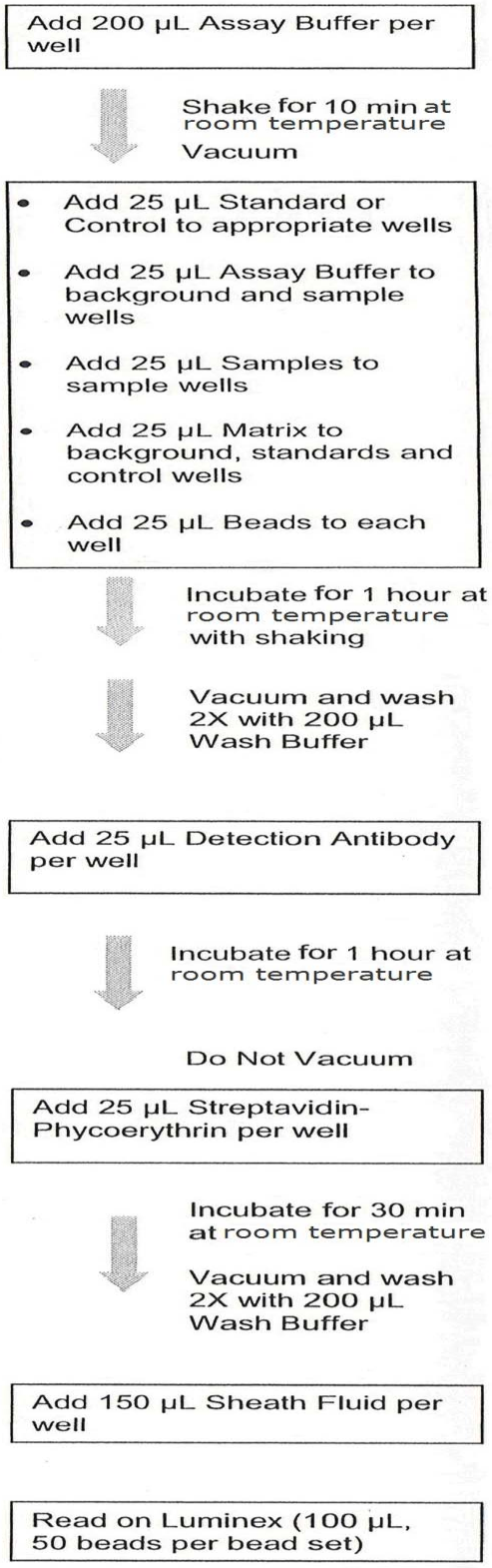


Figure 2.18 - Scheme of Multiplex Luminex Analysis

2.15.3 Gas Chromatography Analysis of RBC Membrane PUFAs

In contrast to proteins, membrane lipids can be extracted by vigorous methods without the risk of being denaturated. One method that is commonly used to extract and analyze membrane lipids is gas chromatography (GC). An aqueous suspension of the membranes is homogenised in a polar solvent mixture of chloroform and methanol (2:1, v/v) and

injected into the GC where it is vaporized into it's various constituents and mobilised through the GC glass coil by a carrier Helium gas. Through the intensity of signals given off by the compound at the other end, the GC reads off the different constituents and their relative amount using a flame ionisation detector (FID). The FID consists of a burning hydrogen flame that substances leaving the column will burn in, producing ions that can be detected by measuring the electric conductivity of the flame (Figure 2.19).

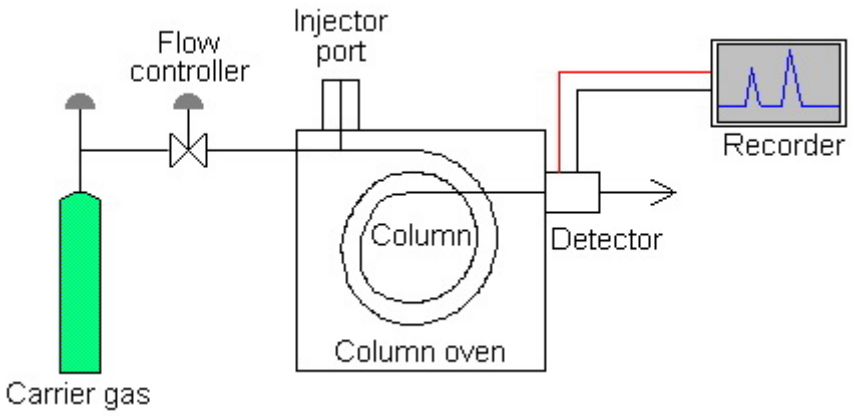


Figure 2.19 - Schematic diagram of a gas chromatograph

Blood treatment and dissolving in solvent

0.5 ml of blood was transferred into a clean pre-labelled microcentrifuge eppendorf tube and 1ml of water was added. The diluted blood was then vortexed using a Whirlimixer Vortex mixer (Fisher, U.K.) for 1 min and allowed to cool in ice for another 1 min. This was repeated 3 times before the blood was transferred to an extraction tube containing 4 ml of ice cold 0.01% butylated hydroxytoluene (BHT) in a 2:1 Chloroform: Methanol (v/v) solvent mixture. The emulsified mixture was then injected into a 6850 Series, Agilent technologies Gas Chromatogram-FID to start the lipid extraction and analysis. The GC autosampler was a 6850 network GC systems; serial no. US10613001 and the chemstation used was Agilent technologies, revision B2.01. The GC column was BPX-70, (Phenomenex, serial no. 9934A05, Part no. CG0-5513), 0.25mm ID 0.25μm film 60m L.

Gas Chromatography Analysis

The Milli-Q water level of the Hydrogen generator tank was checked to ensure enough water was present for a complete run (at least half full). The generator was then switched on and, once de-pressurization was complete, the start button was pressed to begin hydrogen production. As pressure began to build, the black valve on the outlet pipe behind the generator was opened. Final pressure was kept between 5-6 bars. The valves for the air and helium flow were then opened in full. The valve flow rate was pre-set and no adjustment was needed. The GC was switched on next together with the computer system and the 'Chemstation online' started.

2.16 Statistical Analysis

Statistical analysis of the study was performed using GraphPad Prism v5.02 for Windows, GraphPad Software Inc; San Diego, California, USA. All data values were checked for normality prior to analysis using the D'Agostino-Pearson omnibus test.

Analysis of UV-induced immunosuppression of the Ni CHS Response

For the clinical model of PI using Ni CHS, percentage of immunosuppression of the Ni CHS response was calculated in both active and control groups following three months of supplementation (for more details on calculating percentage PI please see section 2.11). The difference in PI caused by each of the three UV-doses applied was then compared between active and control groups using an unpaired Student's t test. A significance level of $P < 0.05$ was used.

Analysis of UV-induced LC Depletion and Cytokine Secretion

UV-induced changes in LC count and cutaneous levels of immunomodulatory cytokines were assessed both pre and post supplementation. To compare post supplementation changes between the groups, an unpaired Student's t-test was performed. For intragroup comparisons (comparisons within the same volunteers pre vs. post supplementation) a paired Student's t-test was employed.

3 PILOT STUDY EVALUATION OF CLINICAL PHOTOIMUNOSUPPRESSION MODEL

3.1 Introduction

UVR was found in several animal and human models to induce the inhibition of immune responses in a local as well as systemic manner (Kripke et al. 1992; Bestak and Halliday 1996; Kripke et al. 1996; Nghiem et al. 2001; Kuchel et al. 2005; Narbutt et al. 2005; Matthews et al. 2010). In experimental animal models, UVR-induced immunosuppression has been strongly implicated as one of the key factors in the promotion of skin cancer (Fischer and Black 1991; Gonzalez-Maglio et al. 2010). In addition, an individual's susceptibility to PI of their CHS responses was reported to directly correlate with their risk of developing skin cancer (Yoshikawa et al. 1990).

CHS and DTH responses are two examples of secondary cell-mediated immune responses that have frequently been used for the *in vivo* assessment of PI (Damian et al. 1999; Damian and Halliday 2002; Narbutt et al. 2005). In murine studies, it was shown that protecting against the UV-induced suppression of CHS/DTH correlates with protection against photocarcinogenesis (Fischer and Black 1991; Nghiem et al. 2002; Sime and Reeve 2004; Black and Rhodes 2006). Furthermore, in humans the ability of certain topical and systemic agents to protect against PI was demonstrated using DTH and CHS responses. Those agents were the dietary supplements β-carotene (Fuller et al. 1992), nicotinamide (Yiasemides et al. 2009) and a combination of Vitamin C and E (Fuchs and Packer 1999), as well as the topically applied agents; nicotinamide (Damian et al. 2008) and broad spectrum sunscreens (Fourtanier et al. 2005).

CHS is a form of cell-mediated immunity that occurs when an antigen is topically applied to the skin's surface (Schwarz 2002). Compared to DTH, which involves dermal introduction of an antigen, CHS provides a less invasive clinical model for the *in vivo* assessment of UV-induced immunosuppression. One of the most widely used models of CHS is nickel (Ni) allergy. Ni allergy is common in the general population, affecting approximately 10-15% of women and 1-5% of men; women are more exposed to Ni due to their higher prevalence of jewelry use (Fourtanier et al. 2005; Matthews et al. 2010).

The clinical assessment of PI in human subjects requires a dose-related and reproducible model. In a range of assessments, Memon et al. (Memon and Friedmann 1996) examined the reproducibility of Ni CHS responses. This involved looking at CHS responses from different Ni sulphate ($NiS0_4$) concentrations ranging from 0.01% - 5% as well as responses from Ni-containing coins on each side of different body sites: the upper and

lower back and forearm and re-examining them 6 months later. Response were assessed using the ICDRG clinical scoring scale for eczematous responses (please see table 2.4 for details) and the Diastron reflectance meter (section 2.7.1) for measuring erythema responses. When clinically assessed using the ICDRG scoring scale and pooling identical results with those showing a difference of +1, the reproducibility of Ni CHS responses to Ni-containing coins was found to be up to 95%. When responses from the different Ni-concentrations were quantified using the erythema meter, the dose-response curves from both sides of the back were near-identical. Moreover, in another study by Fuchs and Packer (1999), Ni allergy was found to be well characterized, can be suppressed by total or partial body UV-irradiation and is a good, reproducible model for PI in humans.

As standard, the endpoint assessment of Ni CHS responses is performed using the ICDRG grading scale (for details, please see table 2.4) (Memon and Friedmann 1996; Fuchs and Packer 1999). In an attempt to provide a more detailed assessment and look at grades in between those of the ICDRG scale, Damian et al. (Damian and Halliday 2002) used a more detailed, 0-10 scoring system to asses Ni CHS responses (for details, please see table 3.1). Furthermore, to make the evaluation more quantitative, other methods have been employed for the assessment of Ni CHS responses including the use of reflectance erythema meters to measure the erythema response (for details on the Diastron reflectance meter, please see section 2.8.5) and to measure Ni-induced skin induration, ultrasonography has been employed. Ultrasonography measures skin thickness enabling the assessment of skin induration post-Ni patch testing on the relatively tight, stretched human skin (as compared to the looser skin of mice whose skin induration is more easily assessed using calipers). This assessment technique is particularly useful when measuring DTH responses that tend to have a bigger dermal component (Memon and Friedmann 1996; Damian and Halliday 2002).

Previously, UVB was assumed to be the main wavelength responsible for PI; UVB is approximately 1000 times more erythemogenic than UVA and was assumed to be substantially more potent in inducing PI. Recent studies however showed that skin type does not correlate with PI (Damian et al. 2001) and that UVA is more immunosuppressive than it is erythemogenic (Walker and Young 2007). UVA is more efficient at generating ROS in the skin, penetrates deeper into the upper dermis and is approximately 20 times more abundant than UVB in ambient solar radiation (Halliday et al. 1998; Nghiem et al. 2001). In view of this, light sources containing both UVA and UVB and thus closely resembling natural sunlight are being increasingly used to apply suberythemal UV-doses for the assessment of PI (Narbutt et al. 2005). These sources however require longer irradiance times to apply the same UV-doses and tend to be bulkier and more difficult to handle.

3.2 Objectives

The primary aim of the pilot study was to find an appropriate method for performing and assessing PI of Ni CHS responses. This involved:

- Finding an appropriate Ni concentration to use post-UV
- Choosing a suitable UVR source and dose series for UV-application.
- Selecting an appropriate criteria for the endpoint assessment of Ni CHS responses

3.3 Methods

A total of 22 volunteers were recruited for the pilot studies; 15 skin type II, 6 skin type III and 1 skin type I. Mean age of the volunteers was 43yrs, with an age range of 26-59yrs.

Finding an appropriate Ni concentration for clinical PI

Following volunteer recruitment, patch testing with a series of Ni concentrations was performed in all volunteers (n=22) to confirm Ni allergy and determine the optimum Ni concentration to use for the assessment of post-UV clinical immunosuppression. The Ni concentration looked for was one that produced a threshold CHS response with only moderate, confluent erythema. This would allow suppression of CHS responses resulting from this Ni concentration with a suberythemal UV-dose. Five different Ni concentrations were used; the maximum was 5% (the standard Ni concentration used at the Contact Dermatitis Clinic). This was then diluted in white soft paraffin to get the remaining four concentrations; 2.4%, 1.2%, 0.6% and 0.15% (for more details on the initial Ni patch test, please see section 2.10).

Choosing a suitable UVR source and dose series

After deciding on the protocol for selecting the optimal Ni concentration, a comparison was made between a UVB-rich light source and another light source emitting both UVA and UVB in 11 volunteers to decide on the most appropriate lamp to use for the clinical model of PI. The Waldmann UV6 lamp was selected as the light source with primarily UVB-irradiance (section 2.6.4) while the solar simulator (section 2.6.2) was chosen as the light source with both UVA and UVB-irradiance. To compare the two lamps, a geometric series of UV-doses was applied to both sides of the volunteer's backs for 3 consecutive days; the UV6 lamp was used to irradiate the left side and the solar simulator was used for the right. On the last day of UV-irradiation, Ni patches were applied to each irradiated site, using the already determined threshold Ni concentration. In addition to patch testing the UV-irradiated skin sites, 3 control patches of the same threshold Ni concentration were applied to unirradiated skin across the volunteer's back (Figure 3.1 and Figure 3.2). The patches were removed after 48hrs and 24hrs later, a 72hr Ni patch test assessment was made visually and using the Diastron reflectance erythema meter (section 2.8.5). The PI induced by each UV lamp was assessed by comparing skin reactions at the site of control Ni patches (un-irradiated skin) with those of each of the irradiated skin sites.

To decide on the best UV-dose series to use with both UV sources, each volunteer's MED was determined and used to apply a suberythemal, UV-dose series on each side of the volunteer's back. As previously outlined, the sunburn response does not directly correlate with PI and selecting a suberythemal UV-dose series allowed for an easier visual and erythema meter assessment of Ni CHS responses post-UV. Initially, a geometric 5 UV-dose series with a maximum of 90% MED was applied as follows: 90%, 60%, 45%, 30% and 22.5% MED. After testing this series on 2 volunteers, another series with a maximum UV-dose of 70% MED was tested on an additional 2 subjects: 70%, 50%, 35%, 25% and 17.5 % MED (Figure 3.1).

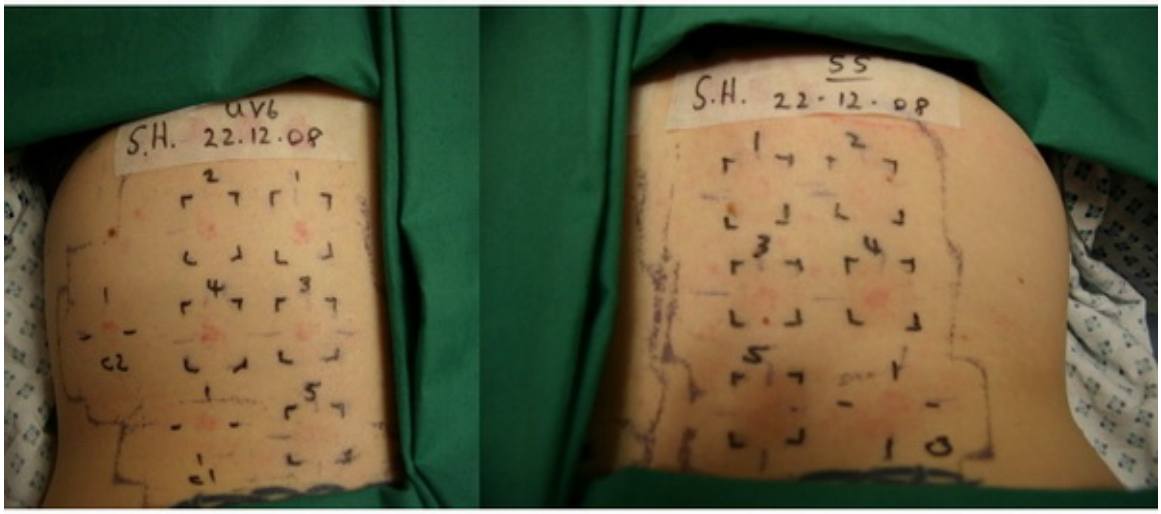


Figure 3.1 - Post UV Ni CHS following irradiation with a geometric 5 UV-dose series applied using the UV6 (left) and solar simulator (right) lamps

Patch no.1 received the lowest UV-dose; 17.15% of the subject's MED while patch no.5 received the highest at 70% MED. Patches C1, 2 & 3 were the control, unirradiated Ni patches that were used to calculate % PI for each of the 5 UV-doses. Response differences between the 1st and 2nd and the 3rd and 4th UV-dose increments was minimal. The number of UV-doses was accordingly reduced to 4 and later 3 UV-doses

After deciding on the less erythemogenic 70% MED UV-dose series, reducing the total number of UV-doses applied was then looked into. This was done to increase the space between the Ni patches and avoid potential overlap between the UV-doses applied. A geometric series of 4 UV-doses was tried on 4 volunteers using 70%, 35%, 25% and 17% of their MED (Figure 3.2). Another series using 3 UV-doses; 70%, 35% and 17.5% MED was also tested on 3 additional subjects. The 3-UV dose series was found most appropriate as response differences between the UV-dose increments was more clearly revealed.

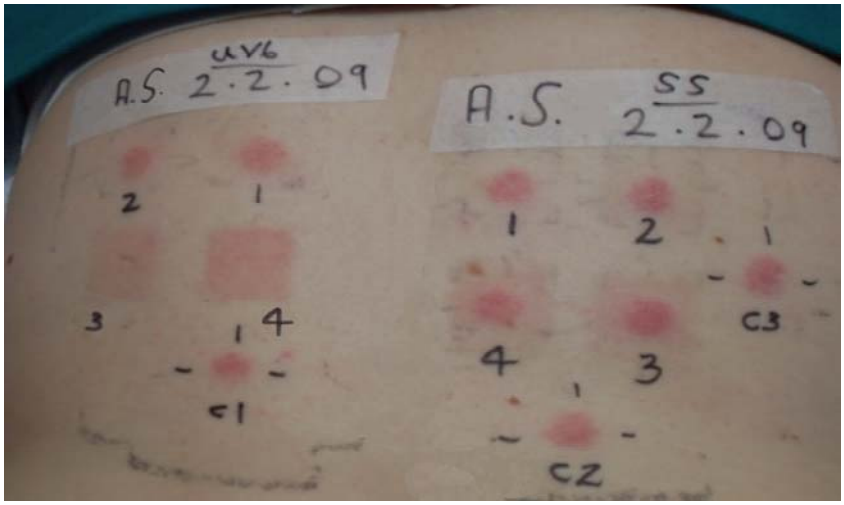


Figure 3.2 - Post UV Ni CHS following irradiation with a geometric 4 UV-dose series applied using the UV6 (left) and solar simulator (right) lamps

Patch 1 received the lowest UV-dose at 17.5% MED while patch 4 received the highest, 70% MED. C1, 2 and 3 were un-irradiated control Ni patches. Erythema caused by highest two UV-doses applied using the UV6 lamp camouflaged the erythema caused by the Ni patches.

Since all the UV-doses applied were based on a percentage of the MED, both light sources were compared to determine the most appropriate lamp to use for MED evaluation: the solar simulator and the Waldmann UV6 lamp. This was done by performing the MED test using both lamps on each side of 6 volunteer's backs (Figure 3.3). To perform the MED test using the solar simulator, a 10 UV-dose series was applied as follows (mJ/cm^2):

68	54	43	35	27
6.6	8.2	11	14	18

For MED testing using the UV6 lamp, the 10 UV-dose series applied was (mJ/cm^2):

63	50	40	33	25
6.2	7.7	11	13	17

For more details on MED testing using the UV6 lamp and the solar simulator, please see sections 2.8.1 and 2.8.2 respectively.

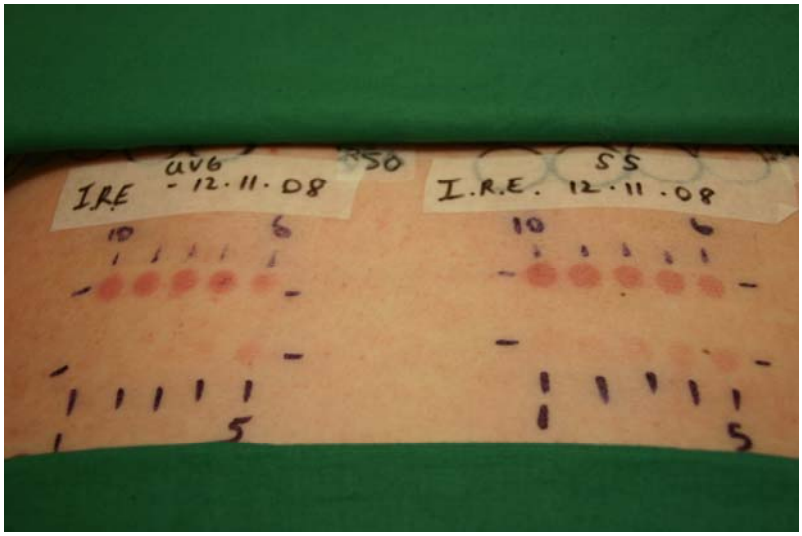


Figure 3.3 - MED assessment using the UV6 lamp (left) vs. the solar simulator (right)

As the solar simulator caused a more “brick red” erythema that could be confused with the tanning response, it was decided to use the UV6 lamp for MED testing and the lamp that most closely mimics ambient UVR, the solar simulator for the clinical PI model.

Next, applying the solar simulator for 3 and 5 consecutive days was compared in 5 volunteers. As per previous studies using the Ni CHS response to examine clinical PI (Fuchs and Packer 1999) the same UVR doses were given to all 5 volunteers. Using the mean MED of the 17 volunteers examined (28mJ/cm^2), a 3 UV-dose series with a maximum of 70% MED was given (18.9mJ/cm^2). This was then decreased by two-fold to give the three UV-doses as follows: 18.9mJ/cm^2, 9.5mJ/cm^2 and 4.9mJ/cm^2 (Figure 3.4).

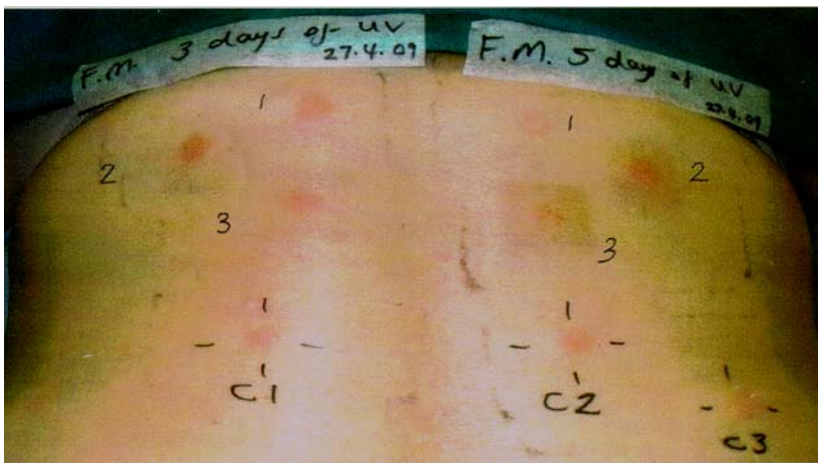


Figure 3.4 - Post UV Ni CHS responses following 3 and 5 consecutive days of SSR

The UV doses applied were 1.89 J/cm^2, 3.82 J/cm^2 and 7.59 J/cm^2 on areas 1, 2 and 3 respectively. C1, 2 & 3 were unirradiated control Ni patches. More UV-induced pigmentation could be seen at the highest two UV-doses following 5 days of SSR compared to 3 days

Selecting an appropriate criteria for the assessment of Ni CHS responses

After determining the method for selecting a threshold Ni concentration and deciding on the appropriate light source and dose series to use, options for endpoint assessment of the Ni CHS responses were examined. Several visits to the Contact Dermatitis Clinic at SRFT Hospital were conducted to observe and gain experience in visually assessing and grading Ni CHS reactions according to the ICDRG scale (Table 2.4). In addition, the feasibility of using the more detailed Damian and Halliday grading scale (Damian and Halliday 2002) was explored (Table 3.1). As per the protocol used in the Contact Dermatitis Clinic, the patches were removed at 48hrs and the skin was allowed to settle for 24hrs, after which a 72hr assessment was made. The optimal, threshold Ni concentration selected was one that gave a reaction closely resembling score no. 4 in the Damian and Halliday scale which corresponded to a + in the ICDRG scale.

Table 3.1 - Damian and Halliday Grading Scale for the Assessment of Ni CHS Responses

(Damian and Halliday 2002)

Score	Nickel reaction
0	No reaction
1	1 or 2 vesicles only
2	Limited area of induration within the patch area
3	Early but confluent induration
4	Moderate, confluent induration
5	As (4) but with <25% of patch area covered by vesicles
6	As (4) but with ~50% of patch area covered by vesicles
7	As (4) but with ~75% of patch area covered by vesicles
8	As (4) but with 100% of patch area covered by vesicles
9	As (8) but with the reaction spreading out beyond the patch area
10	Bullae
I (Irritant)	Erythema but no induration

To quantify the results, the responses were also assessed using the Diastron erythema meter (section 2.5.2.1) to measure EI of the Ni patches. In addition to assessing the Ni patches at 72hrs, two additional assessment times were examined in 3 volunteers; at 96hrs and 120hrs (Figure 3.5).

3.4 Results

Ni concentration to use for clinical PI model

By using the described 5%-0.15% Ni concentration series, a range was created in which the threshold Ni concentration fell. None of the tested volunteers (n=16) had an uncomfortable, excessively vesicular or irritant reaction nor failed to react to the concentrations used. Applying the patches in a vertical pattern with the volunteer sitting

up (just above the bra strap) was found to be more consistent with the patient's body contour and movement and helped prevent the patches from prematurely falling off.

Appropriate assessment of Ni CHS responses

Removing the patches after 48hr and assessing them after 72hr was found to be the most distinct and therefore the easiest to grade (Figure 3.5).

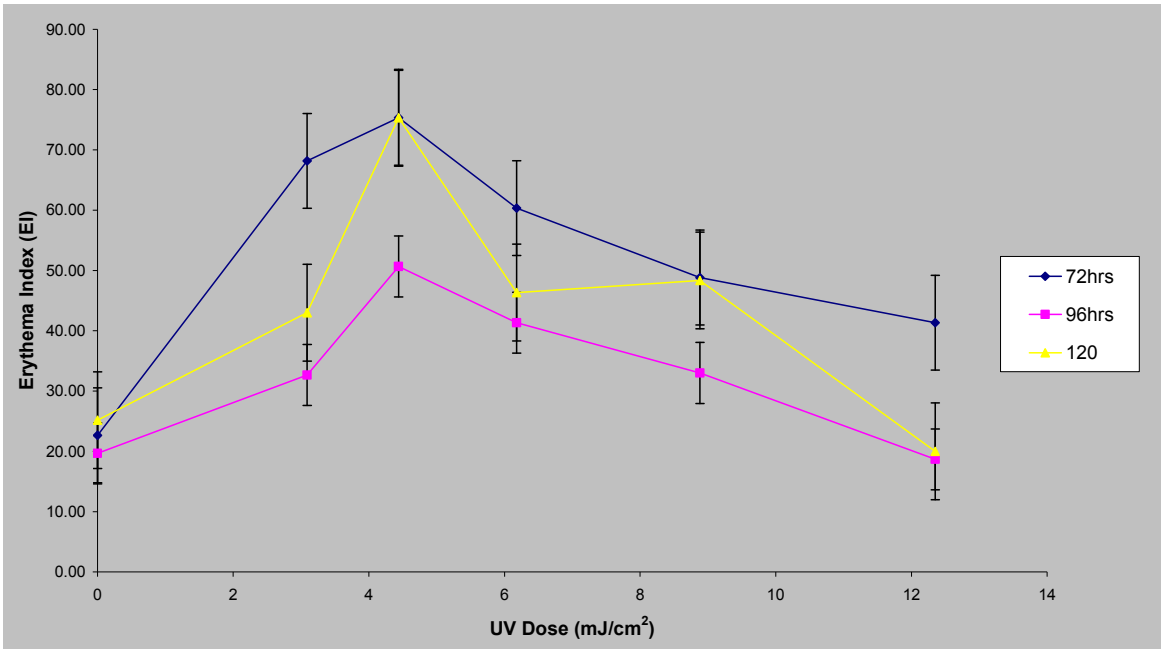


Figure 3.5 - Post-SSR Ni patch test assessment at 72h, 96h & 120h (n=3)

The most distinct UV-dose response curve and the clearest EI readings were seen at 72h.

Both the ICDRG and Damian and Halliday (2002) grading scales enabled the selection of a threshold Ni concentration that was suppressed by a suberythemal UV-dose. For assessing PI in post-UV Ni patch test readings however, the level of details found in the Damian and Halliday scale could not be identified within the Ni CHS models examined. This scale was therefore abandoned for the assessment of PI.

Suitable light source and UV-dose series

Although the 90% MED UV-dose series was suberythemal, applying it for 3 consecutive days resulted in skin redness that interfered with the visual and EI assessment of post-UV Ni CHS responses. For this reason, the UV-dose series with an upper limit of 70% MED was selected.

Using EI assessment, the solar simulator and UV6 lamp showed comparable immunosuppressive properties (Figure 3.6). The solar simulator was chosen for future use as it more closely resembles sunlight and is therefore more physiological.

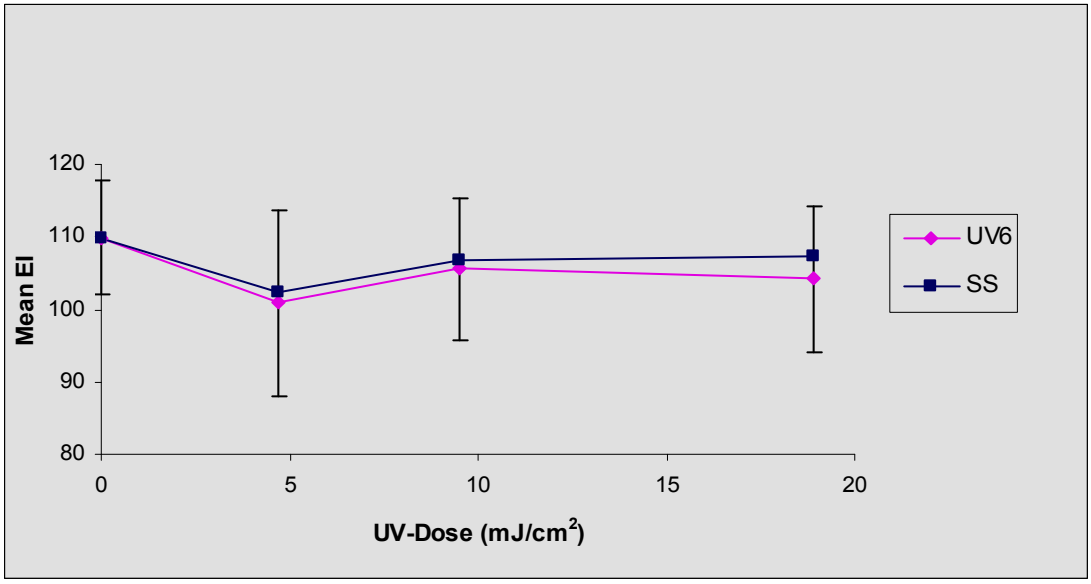


Figure 3.6 - Mean EI of Post-UV Ni CHS Responses Following UV6 vs. SS UVR Exposure (n=7)

Both lamps resulted in comparable PI. Since the irradiance output of the SS most closely resembles solar UV, it was selected as the lamp to use for the clinical model of PI

The ICDRG scale however, was found to be too insensitive for detecting evidence of PI (Figure 3.7)

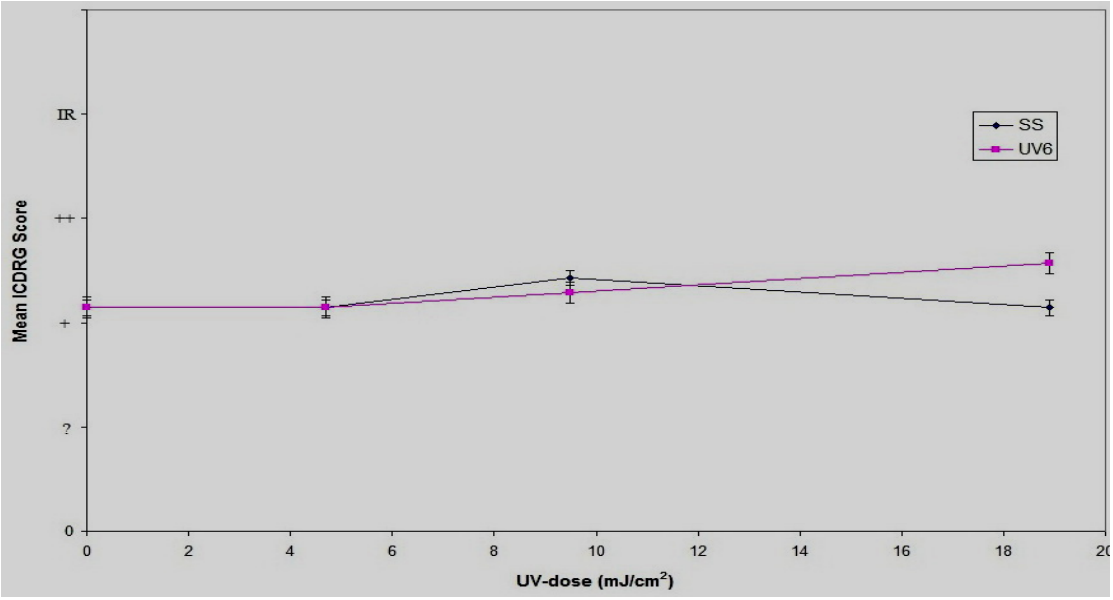


Figure 3.7 - Mean ICDRG Score of Post-UV Ni CHS Responses Following UV6 vs. SS UVR Exposure (n=7)

The crude nature of the ICDRG scale rendered it unsuitable for quantification of the Ni CHS responses

For MED testing however, the resulting skin responses from using the solar simulator were less distinct as they showed a restricted range (Figure 3.8). In addition, the time needed to apply the 10 UV-doses required for MED testing using the UV6 lamp was 8min whereas applying the 10 UV-doses that were calculated to be erythemally equivalent,

using the solar simulator took 25min. This longer application time was considerably less convenient for the volunteers.

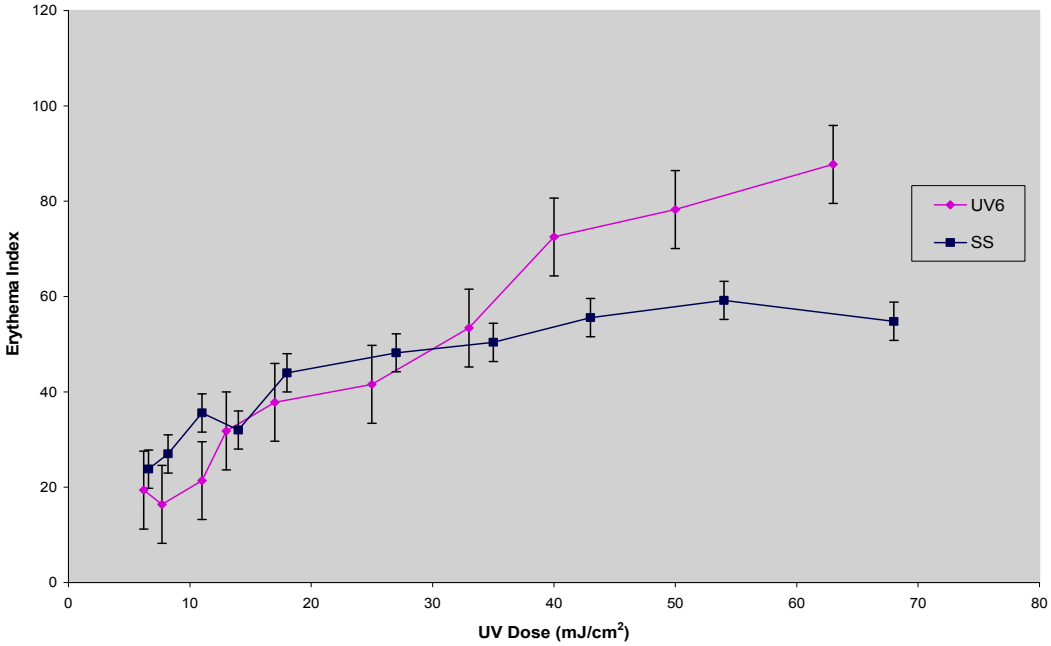


Figure 3.8 – UV-induced erythema dose response curves from MED tests performed with the solar simulator and UV6 lamps (n=6)

The higher portion of UVB in the irradiance output of the UV6 lamp resulted in a more distinct erythema dose response

With 5 consecutive days of SSR, more skin pigmentation was seen on the skin areas receiving the highest two UV-doses. This made the visual assessment of the Ni CHS responses more difficult to perform (Figure 3.9).

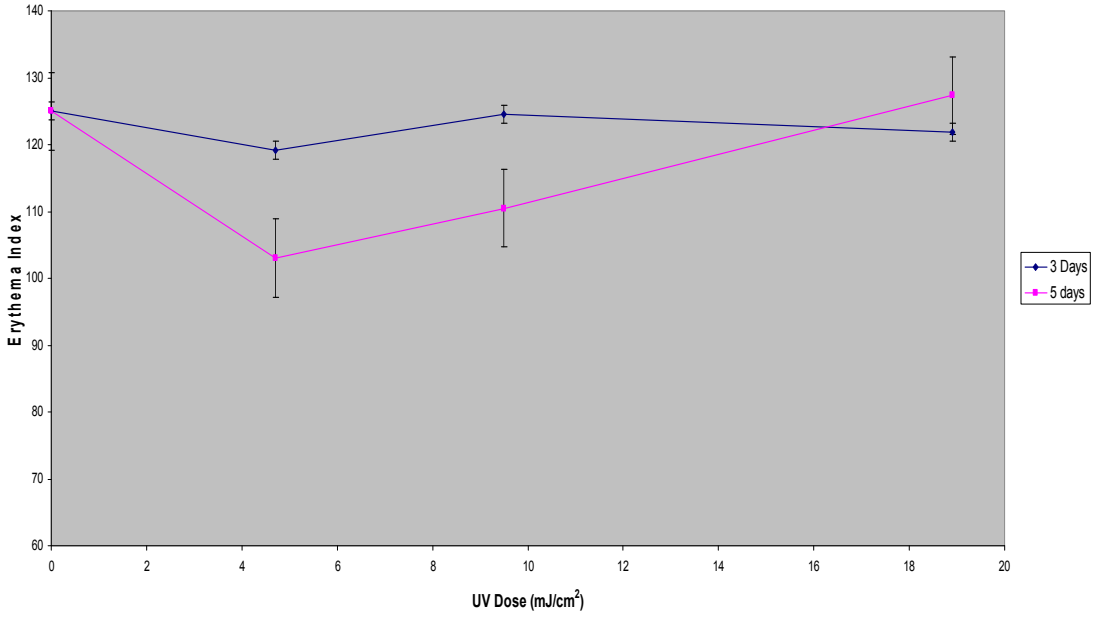


Figure 3.9 - Post-UV Ni CHS following 3 vs. 5 consecutive days of SSR (n=2)

After an initial drop, a steady increase in EI was noted with the 5 days application of SSR. This can most probably be attributed to excessive UV-induced erythema caused by the prolonged irradiation time; a similar increase in EI could not be seen with 3 days of SSR.

3.5 Discussion

For the initial Ni patch test, applying the patches in a vertical pattern on the upper left back was found to be more consistent with the patient's body contour and movement and helped prevent the patches from prematurely falling off. The range of Ni concentrations used (0.15% - 5%) ensured that most Ni allergies weren't missed and yet avoided excess, uncomfortable vesicular reactions. For both the initial Ni patch test and post-UV Ni CHS, removing the Ni patches after 48hrs and assessing the responses 72hr later was found to be most distinct and therefore the easiest to grade. The Damian and Halliday (2002) and ICDRG grading scales (Memon and Friedmann 1996) were both found suitable for selecting the threshold Ni concentration to use in the post-UV Ni CHS model. For assessment of post-UV Ni CHS responses however, the level of details found in Damian and Halliday scale could not be easily identified.

Since the repeated application of a 90% MED UV-dose for 3 consecutive days resulted in visible skin erythema, the less erythemogenic dose series with a maximum UV-dose of 70% MED was employed instead. Applying a total of 3 UV-doses using this series (70%, 35% and 17.5% MED) was found to be most suitable for allowing a more even distribution of UV-irradiated as well as unirradiated (control) Ni patches. This ensured that all Finn chambers were securely held in place on flat areas of the back away from body contours and flanks. Moreover, increasing the space between the irradiated-skin areas minimized the possibility of an overlap between effects of the various UV-doses applied.

The solar simulator and UV6 lamp showed comparable immunosuppressive properties (Figure 3.6). Since the solar simulator is the lamp that most closely resembles ambient UVR, it was selected as the light source for the clinical PI model. For MED testing however, the UVB-rich light source, the UV6 lamp was selected over the solar simulator. As compared to the UV6 lamp, skin responses to the MED test were less distinct using the solar simulator (Figure 3.8). In addition, the time needed to perform the test was considerably less using the more erythemogenic UV6 lamp. During the pilot study, the UV6 lamp showed an extreme variance in irradiance output (application times had to adjusted accordingly). This eventually led to it's exclusion from the main study; it was replaced by another UVB-rich light source; the TL 12 lamp (for details on the TL 12 lamp please see section 2.6.1).

When comparing application of the 3 UV-dose series of SSR for 3 vs. 5 consecutive days in the clinical PI model (the post-UV Ni CHS), the 5 consecutive days was found to cause more skin pigmentation on the areas receiving the highest two UV-doses (Figure 3.4). This made the visual assessment of Ni CHS responses more difficult to perform and it was therefore decided to apply SSR for 3 consecutive days.

Since skin type and the sunburn response do not directly correlate with UV-induced immunosuppression and in accordance with previous studies using the Ni CHS response to examine PI (Fuchs and Packer 1999), it was decided to keep the 3 UV-dose series given to all volunteer constant. Using the mean MED of the 22 volunteers examined (28mJ/cm^2), the maximum UV-dose of 70% MED was calculated. This was then decreased by two-fold to give the 3 UV-doses as follows; 4.7mJ/cm^2, 9.5mJ/cm^2 and 18.9mJ/cm^2.

Since the level of detail found in Damian and Halliday scale could not be easily identified in the post-UV Ni CHS responses examined, it was decided to use the ICDRG grading scale for visual assessment of the CHS responses. The EI readings taken with the Diastron reflectance erythema meter were used for quantification of the results.

Overall, the methods used were consistent with the methodology of published literature on the assessment of PI in humans using CHS responses in that Ni patches were utilized (Fuchs and Packer 1999; Fourtanier et al. 2005), 3 days of UV-exposure were given (Yiasemides et al. 2009), the lamp chosen for PI was a solar simulator (Poon et al. 2005), the Ni patches were read at 3 days post application (Damian et al. 1999), and that responses were assessed as reflectance meter quantified erythema (EI) (Damian et al. 1997). This method for the clinical assessment of PI was deemed appropriate for use in the human randomized control trial (RCT) to assess protection by dietary n-3 PUFAs against UV-induced immunosuppression (chapter 4).

4 RANDOMIZED CONTROLLED TRIAL TO EXAMINE THE EFFECTS OF DIETARY EPA ON CLINICAL PHOTOIMMUNOSUPPRESSION

4.1 Introduction

Skin cancer is a public health problem of growing concern. It is one of the commonest cancers and its incidence is increasing in white-skinned populations (Trakatelli et al. 2007). UVR is a complete carcinogen; it is a major initiator as well as promoter of skin cancer (Gonzalez Maglio et al. 2010). UVR initiates skin cancer by damaging DNA and promotes it's development via PI (Kripke et al. 1996; Berneburg and Krutmann 2000; Murphy 2009). In murine studies, UVR-induced immunosuppression was shown to be one of the key factors in the promotion of skin cancer (Walker and Young 2007). In addition, protection against PI of CHS in experimental animal models was found to protect against photocarcinogenesis (Sime and Reeve 2004; Black and Rhodes 2006). Moreover, in humans, individuals with a past medical history of skin cancer were found to be more susceptible to PI (Yoshikawa et al. 1990). Furthermore, SCCs in patients on immunosuppressive medication were found to be more aggressive (Damian et al. 2001) and immunosuppressed transplant patients showed an increased risk of developing skin cancer (Oberyszyn 2008; Murphy 2009).

Apart from protection via clothing and avoidance of intense sunlight, the use of sunscreens is the current method of skin protection against the damaging effects of solar-UV. In addition to being primarily designed to protect against sunburn and not PI, sunscreens are often inadequately applied and usually require re-application to provide protection throughout the time of UV-exposure (Diffey 2001; Fourtanier et al. 2005).

A new dietary approach, with the potential for a more continuous and even protection is therefore being examined as a potential adjuvant to topical sunscreens. An example of such dietary approaches is n-3 PUFAs. Previous studies by Moison et al. (Moison and Beijersbergen Van Henegouwen 2001; Moison et al. 2001) showed that in male BALB/C mice, both topically applied and dietary EPA can reduce UV-induced suppression of CHS reactions to DNFB and TNCB respectively. This indicates a potential for the use of fish oil supplements containing this long chain PUFA as a protective agent against UV-induced immunosuppression and skin cancer. Such results however, are yet to be demonstrated in human models (Black and Rhodes 2006).

As previously outlined, the use of secondary rather than primary immune responses allows the use of multiple test sites on each volunteer. In addition, each volunteer can act as his/her own unirradiated control (provided the UV-exposure is kept below that of systemic PI). In addition, since immune reactions and the development of memory cells

against a tumor most probably continue throughout the time of tumor development, UV-induced inhibition of secondary immune responses is likely to play an important role in photocarcinogenesis (Damian and Halliday 2002).

Amongst the secondary immune responses that have frequently been used to asses PI in humans are CHS responses to various antigens (Vink et al. 1998; Damian et al. 2001; Narbutt et al. 2005). Amongst the common sensitising chemicals are metals such as nickel and chromium. Nickel allergy is common in the general population, affecting approximately 10-15% of women and 1-5% of men; women are more frequently exposed to Ni through the use of jewelry (Fourtanier et al. 2005; Matthews et al. 2010). Patch testing Ni-allergic volunteers provides a convenient system of CHS (Damian and Halliday 2002). Prior UV-exposure of the sites facilitates a model for PI in humans.

4.2 Objectives

The primary aim of the study was to examine for the potential protective properties of supplemental n-3 PUFAs against PI in humans. This was done by comparing PI of Ni CHS responses in active (n-3 PUFA) and control (placebo) groups of Ni-sensitive female volunteers after three months of supplementation.

A secondary aim was to test for compliance and n-3 PUFA bioavailability by measuring n3-PUFA levels in RBC membranes.

4.3 Methods

To examine the potential protective properties of n-3 PUFAs against PI in humans, the UV-induced immunosuppression of Ni CHS responses were compared between active (4gm EPA/day) and control (GTCC placebo oil) groups of Ni-sensitive female volunteers after 12 weeks of supplementation. In a double-blinded, randomized placebo controlled study of healthy female volunteers with known Ni allergy, a repeated application of a 3 UV-dose series of suberythemal SSR was applied to the volunteer's backs for 3 consecutive days. This was then followed, on the third day of UV-irradiation with the application of Ni patches to the UV-irradiated and unirradiated (control) skin sites.

4.3.1 Volunteers and recruitment

A total of 79 volunteers with known Ni allergy were recruited for this study. All subjects were between the ages of 18-62 years, skin type I/II with no medical history of atopy, photosensitivity or skin cancer. Volunteers were required to not have sunbathed/used sunbeds in the past 3 months and not have any sunny holidays planned for the next 3 months. Fish meal consumption was restricted to no more than two meals/week and subjects were asked not to take fish oil or n-3 PUFA supplements. For more details on the

inclusion and exclusion criteria and recruitment methods used, please see sections 2.4.1 & 2.4.2 respectively.

4.3.2 Supplements and randomization

Both groups received five 1g capsules of supplement/day (taken as a single dose or in divided doses throughout the day according to each volunteer's personal preference). In the active group, this equated to 4g of EPA/day; a dose found to be effective in previous photoprotection studies (Rhodes et al. 2003).The control group received 5g/day of GTCC oil. For more details on the oil supplements used, please see section 2.5.

Volunteers were randomized into an active, n-3 PUFA group (n=40) and a control, GTCC oil group (n=39). Randomization of the participants was conducted using blocks of computer generated numbers (mixed blocks of four to six). All study allocations were randomized before any of the research group members had access to them.

4.3.3 Photoimmunosuppression Assessment

All volunteers were scheduled to have an assessment of clinical PI following 3 months of supplementation: the post-UV assessment of Ni CHS responses.

Application of UV and Ni Patches

Following 3 months of supplementation, a 3 UV-dose series was applied to the volunteer's upper back: 1.89, 3.82 & 7.59 J/cm^2 of total UV (4.7, 9.5 & 18.9 mJ/cm^2 of erythemally weighted UV) for 3 consecutive days, using a solar simulator fitted with a 1000 Watt xenon short arc lamp (for more details on the solar simulator please see section 2.5.2). The highest dose of UV in this series i.e. 7.59J/ cm^2 (18.9mJ/cm^2 of erythemally weighted UVR) was 70% of the mean MED of 10 patients (28mJ/cm^2) from a photo investigation study conducted at Salford Royal Hospital (patients in this study were Fitzpatrick's skin type I/II). This dose was then decreased by a factor of 2 for 2 additional doses. On the third day of UV-application, Ni patches of a threshold Ni concentration i.e. one causing moderate confluent erythema (determined individually for each volunteer when confirming their Ni allergy before commencing the study) were placed on the 3 UV-irradiated sites. In addition, 3 control Ni patches were placed on non-irradiated sites on the volunteer's back. The Finn chambers used for the Ni patches were 8mm in diameter and the amount of Ni per chamber was kept constant at approximately 25mg (one line of Ni across the diameter of the Finn chamber). The patches were secured in place using 3M Micropore^{TM} hypoallergenic tape. The volunteer was asked to remove the patches herself, after 48 hours and to return a day later for a 72 hour assessment, visually and using the Diastron reflectance erythema meter (section 2.8.5) (for more details on the post-UV Ni patch test protocol, please see section 2.11).

Assessment of Ni CHS Responses

Erythema meter readings were taken in triplicate, in a sequential rather than a consecutive manner i.e. each site reading was only repeated after all the other sites were measured once. In addition to measuring the erythema index (EI) at the site of the Ni patches, a second set of readings were taken from adjacent skin surrounding the patch test area (skin that was exposed to UV but not Ni). This background reading was then subtracted from the Ni patch test EI to give a background corrected erythema reading.

In addition, EI readings from the 3 control Ni patches were measured, summated and a mean taken. A background corrected reading for the control patches was then calculated by subtracting the mean of 2 readings from baseline skin (skin that was not exposed to UV or Ni) from the mean of the summated control Ni patches.

Sample Size Selection

Data from similar studies (Fuchs and Packer 1999; Damian and Halliday 2002) report an expected difference in means between groups of 15% with a standard deviation of 24. For a statistical power of 90%; a total of 56 subjects (28 per group) were required:

$$(15/24) \times 90 = 56.25$$

To allow for ~25% drop-out, 79 subjects were recruited to this intensive study.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism v5.02 for Windows, GraphPad Software Inc; San Diego, California, USA. Percentage immunosuppression of Ni CHS was calculated using the following formula (Damian et al. 1997):

$$[(EI\ unirradiated - EI\ irradiated) / EI\ unirradiated] \times 100\%$$

The difference in PI between active and control groups was assessed by an unpaired Student's t test, using a significance level of $P < 0.05$. All data values were checked for normality prior to analysis using the D'Agostino-Pearson omnibus test.

4.3.4 Measurement of EPA content in RBC cell membrane

To assess compliance and determine the bioavailability of EPA, blood samples were collected from each volunteer before and after 3 months of supplementation (for details on the blood sample collection protocol, please see section 2.11). Following centrifugation of the blood samples, an aqueous suspension of RBC membranes was created and homogenised in a polar solvent mixture of chloroform and methanol (2:1, v/v). This homogenised mixture was then injected into the gas chromatogram to measure the EPA content of RBC membranes, as percentage weight of total fatty acids (for more details on the gas chromatography analysis of RBC membrane PUFAs please see section 2.14.3).

4.4 Results

4.4.1 Volunteers and Compliance

The mean age of the volunteers was 42.8 yrs with an age range of 21-62 yrs. Apart from an increased “fishy” belching; no other adverse effects were reported by the volunteers. Six volunteers in the control group dropped out for reasons unassociated with the study. One volunteer in the active group showed very weak Ni CHS responses (on both irradiated and non-irradiated skin) and was excluded from the analysis of clinical PI. In addition, following analysis of EPA content in RBC membranes, 3 volunteers in the active group were found to be non-compliant and were omitted from the data analysis. The average change in membrane EPA content post supplementation in these 3 volunteers was a decrease of 27.7% ± 13.61 (SEM) compared to an average increase of 349.5% ± 38.54% (SEM) in other subjects of the active group. This left a total of 69 volunteers: 33 in the control group and 36 in the active.

4.4.2 Bioavailability of EPA in RBC membranes

In addition to excluding the 3 non-compliers, failure to inject one post supplementation blood sample into the gas chromatogram and the inability to draw blood from another volunteer post supplementation, left blood samples from 35 volunteers in the active group available for post supplementation analysis of EPA bioavailability in RBC membranes. The mean membrane EPA (measured as % of total fatty acid weight) in the active group (n=35) was 0.93% ± 0.07% (SEM) pre supplementation. This significantly increased to 3.61% ± 0.22% (SEM) post supplementation; the mean increase in EPA % weight post supplementation in the active group was 2.68% ($P<0.001$; 95% confidence interval: 2.24% to 3.12%). Similar to the active group, mean pre supplementation membrane EPA % weight in the control group was 0.9 % ± 0.07% (SEM) (n=33). This was unchanged at 0.93% ± 0.06% (SEM) post supplementation; the mean membrane EPA % weight increase post supplementation in the control group was 0.026% ($P<0.64$; 95% confidence interval: -0.138% to 0.086%) (figure 4.1). The mean difference in membrane EPA % weight between active and control group post supplementation was statistically significant: $P<0.0001$, difference between the means: 2.69% ± 0.23% (SEM), 95% confidence interval: 2.24% to 3.14%.

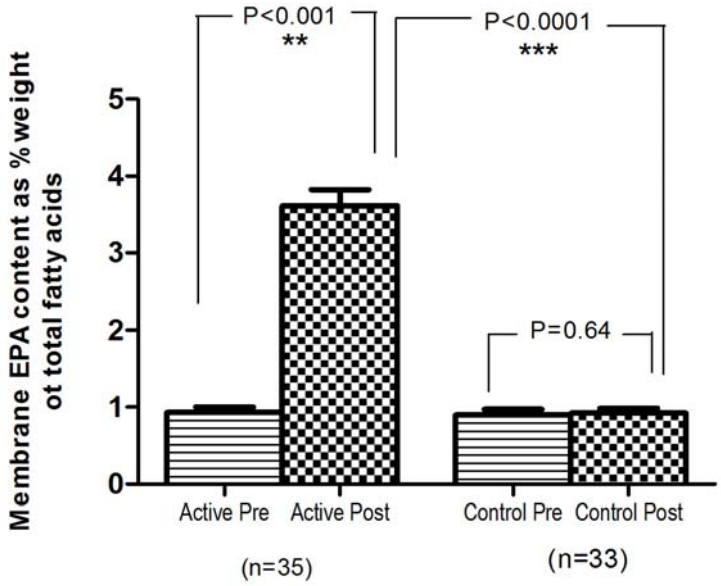


Figure 4.1 - Percentage weight of membrane EPA in active and control groups pre and post supplementation

Following 3 months of supplementation, a significant increase in membrane EPA could be seen in the active group (p<0.001) denoting bioavailability and compliance. Membrane EPA remained unchanged in the control group post supplementation

The mean % increase in membrane EPA post supplementation was 349.5% ± 38.54% (SEM) in the active group compared to 19.76% ± 11.60% (SEM) in the control group (figure 4.2). The difference between the mean increase in % EPA in the active and control groups was 329.7 ± 41.25 (95% confidence interval: 247.4% to 412.1%).

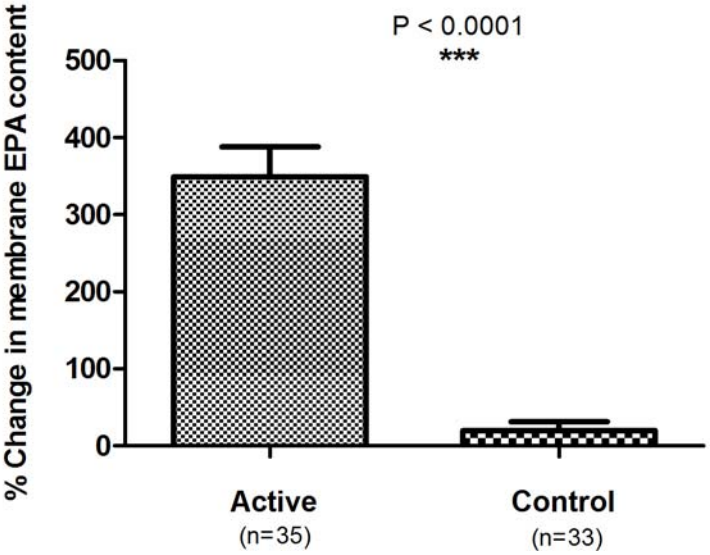


Figure 4.2 - Percentage change in membrane EPA post supplementation in active and control groups

A significant difference in the mean % increase in membrane EPA post supplementation could be seen between active and control group (P<0.0001)

4.4.3 Difference in Percentage of PI between Active & Control Groups

Following exclusion of the 3 non-compliers and the volunteer showing very weak CHS responses, the total number of subjects left in the active group was 36. In the control group, after 6 volunteers dropped out (for personal reasons unrelated to the supplement), the total number of volunteers remaining was 33.

The percentage of PI caused by the 3 UV-doses applied (1.89, 3.82 and 7.59 J/cm^2) was calculated and the results compared between the active and control groups (Figure 4.2).Omega-3 PUFAs appeared to convey protection against PI that reached statistical significance at the middle UV-dose of 3.82J/cm^2 (P<0.04)* (table 4.1).

Table 4.1 - Differences in % PI between Active and Control Groups (n=69)

Total UV-dose (J/cm^2)	Mean % PI of Ni CHS (± SEM)		P Value	Difference Between Means	95% Confidence Interval
	Active (n=36)	Control (n=33)			
1.89	10.65 ± 4.84	14.47 ± 5.27	0.6	-3.82 ± 7.14	-18.08 to 10.45
3.82	10.06 ± 3.85	20.98 ± 3.48	0.04	-10.92 ± 5.22	-21.34 to -0.4911
7.59	40.40 ± 4.89	42.86 ± 3.91	0.7	-2.46 ± 6.34	-15.12 to 10.20

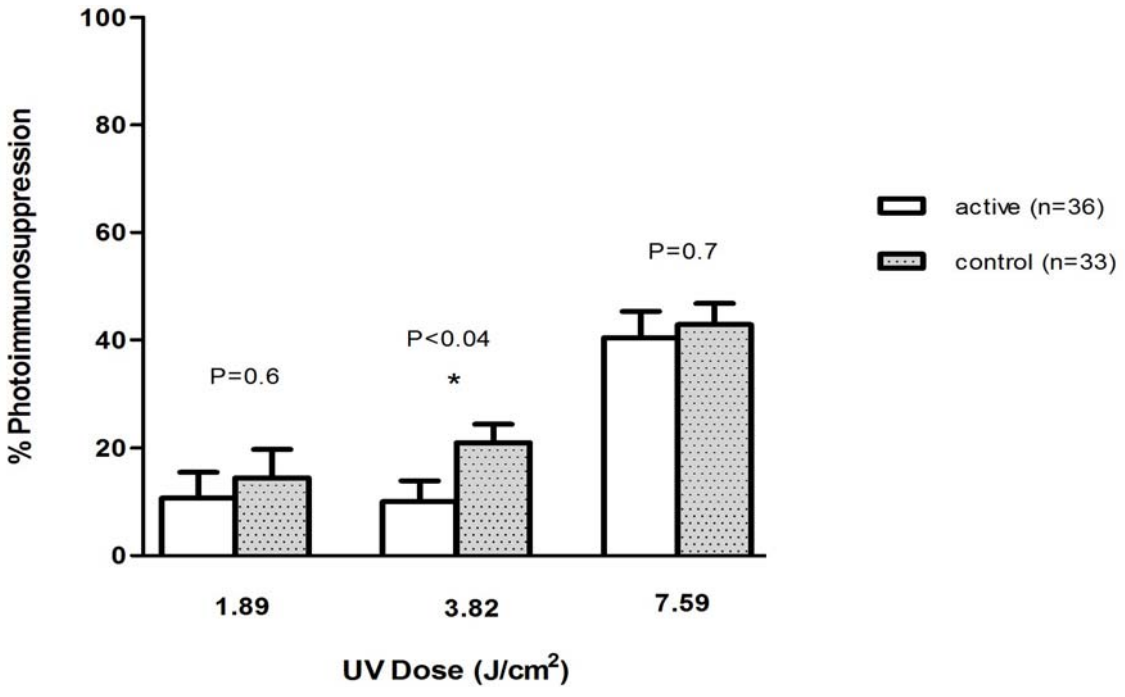


Figure 4.3% - % PI Post Supplementation in Active and Control Group (n=69)

Evidence of protection against PI by n-3 PUFA supplementation could be seen with all 3 UV-doses. This protection reached statistical significance at the middle dose of 3.82 J/cm^2 (P<0.04)

Possible effects of hormonal fluctuations of the menstrual cycle on CHS responses are debatable (Tamer et al. 2003; Bonamonte et al. 2005). Using the drug history taken from all volunteers prior to commencing the study, the possible suppressive effect of oestrogen on Ni CHS responses was examined. The PI caused by the 3 UV-doses was re-assessed after excluding all volunteers that were on hormonal medications (hormonal replacement therapy or oral contraceptives): 7 active and 2 control subjects (figure 4.4). This left a total of 60 volunteers: 29 in the active group and 31 in the control group (table 4.2).

Table 4.2 - Differences in % PI between Active and Control Groups after the exclusion of non-compliers and those on hormonal medication (n=60)

Total UV-dose (J/cm^2)	Mean % PI of Ni CHS (± SEM)		P Value	Difference Between Means	95% Confidence Interval
	Active (n=29)	Control (n=31)			
1.89	10.51 ± 4.48	16.17 ± 5.44	0.43	-5.66 ± 7.10	-19.88 to 8.56
3.82	10.97 ± 3.80	21.48 ± 2.99	0.033*	-10.51 ± 4.80	-20.12 to -0.89
7.59	42.85 ± 4.92	42.96 ± 3.91	0.99	-0.11 ± 6.24	-12.61 to 12.39

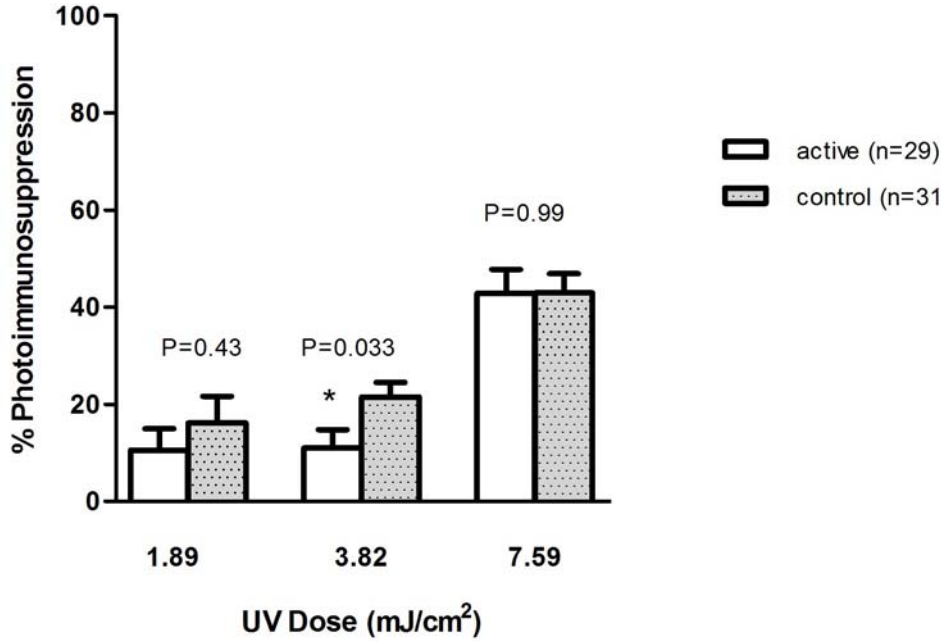


Figure 4.4 - % PI Post Supplementation following exclusion of subjects on hormonal medications (n=60)

An insignificant change in protection against PI by n-3 PUFA could be seen following exclusion of volunteers that were on hormonal medication (OCT and HRT) (n=7)

To exclude any inhibitory effects of n-3 PUFAs on the Ni CHS response (without UV-exposure), Ni-induced EI readings were compared in the unirradiated skin of the active group pre vs. post supplementation. No significant variation could be detected (using a paired t test: P=0.11) (Figure 4.5). This denotes that EPA did not have intrinsic effects on the Ni CHS response that might influence study outcomes.

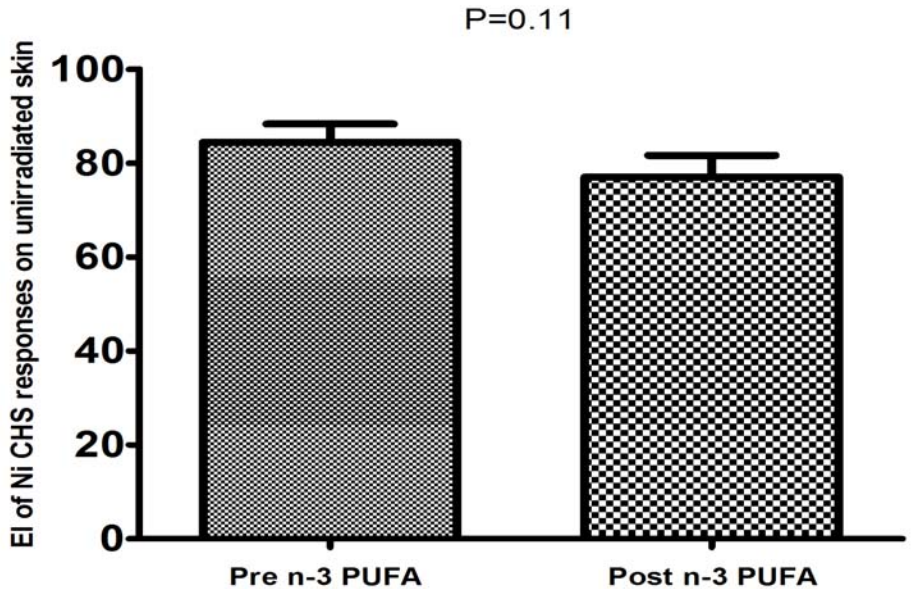


Figure 4.5 - Background Corrected EI of Ni Patch Tests on Unirradiated Skin Pre & Post n-3 PUFA Supplementation (n=36)

Ni CHS responses on the un-irradiated skin of volunteers in the active group were assessed before and after 3 months of n-3 PUFA supplementation; no significant difference could be seen. This denoted that n-3 PUFA did not have an intrinsic effect on the Ni CHS response

4.5 Discussion

Fatty acid levels in RBCs represent dietary intake and act as a general marker of tissue incorporation (Sands et al. 2005). Previous studies looking at EPA % weight in RBC membranes reported an average of 0.95% (95% confidence interval: 0.92% - 0.977%; mean age 46.6yrs; n=169) (Sands et al. 2005; Cohen et al. 2008). Similarly, the mean membrane EPA % weight pre supplementation in both active and control groups were found to be approximately 0.9%. At the given dose of 4gm EPA/day for 3 months, this significantly increased to an average of 3.61% in the active group. In comparison, control group subjects had effectively unchanged membrane EPA %weight post supplementation: 0.93% (Figure 4.2). In addition to compliance, this denoted bioavailability of the EPA supplement in the active group and demonstrated a significant increase in EPA levels between the active and control subjects post supplementation.

As outlined by the results, the hypothesis that n -3 PUFA has photoprotective properties against PI remains a possibility that needs to be further explored. At 4gm of EPA/day for

three months, a tendency towards photoprotection and support of the hypothesis that fish oil supplements can reduce UV-induced immunosuppression was seen. This was particularly noted at the lower two UV-doses, reaching statistical significance at the middle dose of 3.82J/cm^2. At 35% and 15% MED, these UV-doses more closely resemble average daily solar UV-exposure (Thieden et al. 2004) compared to the highest 70% MED dose. Moreover, even with the milder photoprotection seen at the lowest and highest UV-doses, over a person's lifetime this small effect might lead to a significant reduction in a person's risk of developing skin cancer (Black and Rhodes 2006). Furthermore, compared to UV-irradiation with the solar simulator, daily UV-exposure is usually less intense and more spread throughout the day which could possibly allow for more n-3 PUFA photoprotection. It should also be noted that protection from PI by n-3 PUFA is not intended as a replacement for the more commonly used topical sunscreens but rather as a valuable adjuvant to provide more even continuous photoprotection.

Similar to previous studies examining protection against PI by oral nutrients, a low-dose SSR protocol was employed in this study to suppress cell-mediated immune responses (Fuchs and Packer 1999; Yiasemides et al. 2009). The level of protection seen against PI in this study was less than that reported for oral nicotinamide (Yiasemides et al. 2009) and Vitamin C and E (Fuchs and Packer 1999). This may be explained by different agents having different mechanisms of action. It should also be noted that the combined dose of Vitamin C and E used in the Fuchs and Packer (1999) study was considerably higher than the average daily intake of these antioxidants; at 2g of tocopherol with 3g of ascorbic acid. In addition, in the Yiasemides study (2009) lower UV doses were applied; 1, 2 and 4J/cm^2 compared to UV-doses of 1.89, 3.82 and 7.59J/cm^2 employed in this study. Other factors that may contribute to the observed differences are different emission spectra of the lamps. Despite the use of a 1000W xenon arc solar simulator in these studies, the use of different filters resulted in different irradiance spectra. Fuchs and Packer (1999) used a 1.0 mm Schott WG-320 filter with two dichroic mirrors to give an irradiance spectrum of 280-400nm range. Although the same combination of filters used by Yiasemides and Halliday (2009) were employed in this study, examination of light source details revealed less UVB was filtered off in the Yiasemides study with a spectral irradiance of 290nm-400nm compared to a spectrum of 295-400nm used in this study. Furthermore, different clinical protocols that involve different APCs were used by Yiasemides and Halliday for the assessment of clinical PI i.e. DTH responses to PPD compared to CHS responses to Ni used in this study.

In previous murine studies that examined the photoprotective properties on dietary n-3 PUFA against PI (Moison and Beijersbergen Van Henegouwen 2001), a statistically significant reduction in % PI that reached 96% in comparison to the control group was seen. Several factors could have contributed to this such as the closely controlled

environment in which the study was performed (compared to human subjects that are free living and could have been exposed to variable levels of environmental factors including sun exposure). Moreover, an identical diet was fed to all the mice which would exclude any external effects of other dietary constituents. Furthermore, all the mice used were male which would exclude any potential suppressive effects of oestrogen. In addition, all the mice used were of the same strain (BALB/c) which ensured more consistent results through an identical pool of genes.

Several studies have examined the relationship between hormonal fluctuations of the menstrual cycle and the changing degree of allergic contact dermatitis. Results from these studies have been inconclusive. In clinical practice, varying patch test responses have often been reported in different stages of the menstrual cycle. Patients with allergic contact dermatitis commonly notice an increase in skin sensitivity during the days directly preceding menstruation (when oestrogen is at a low). Examining Ni CHS responses in both ovulatory and luteal phases of the menstrual cycle revealed a significant reduction in the intensity of responses in the ovulatory stage (characterized by high oestrogen levels). This suggests that high levels of oestrogen have a significant inhibitory role on CHS reactions (Bonamonte et al. 2005). In addition, oestrogen was found to impair the skin barrier via oestrogen-mediated water retention and subsequent increase in skin thickness (Eisenbeiss et al. 1998). Furthermore, although Ni sensitivity was found to be independent of the menstrual cycle phase, it's severity was intensified in the pre-menstrual stage (days 20-24) compared to post-menstrual (days 7-10) (Tamer et al. 2003). Aktan et al. (1998) on the other hand, found the reproducibility of Ni patch tests in 28 Ni allergic female volunteers to be unaffected by the hormonal changes seen in the follicular and ovulatory stages of the menstrual cycle. Rohold et al. (1994) confirmed Aktan's findings by patch testing 20 Ni allergic female volunteers; no premenstrual increase in sensitivity to Ni was found. Despite the desirability of standardizing Ni patch tests to a specific stage of the menstrual cycle and avoiding the potential influence of oestrogen, this may be difficult to logistically achieve. Menstrual cycles can be irregular with unpredictable durations that make the planning of a UV-irradiation schedule difficult to perform. Practices of other units were therefore followed, in not taking the menstrual cycle into account when looking at clinical PI. In the same way that variations in temperature and perspiration are controlled for by the simultaneous measurement of test and control sites in each subject, the potential hormonal influence on patch test severity can also be accounted for in a similar manner by taking both irradiated and unirradiated measurements (Damian and Halliday 2002). To account for the possible suppressive effects of oestrogen on CHS responses, PI was examined with and without subjects on hormonal medications: no significant difference was found (section 4.4.3). Possible reasons for this could be the minimal effect that hormones had on CHS responses. Alternatively, because the study had a large

number of subjects and only a minority were on OCT or HRT (a total of 9 out of the 69 volunteers used for PI assessment) this could have resulted in an insignificant effect.

As previously outlined in section 1.5, several mechanisms are involved in PI. This includes interactions between UV and lipid and protein mediators as well as cellular components. Several studies have shown that n-3 PUFAs can compete with n-6 PUFAs for metabolism by COX and LOX as well as competition for the incorporation and release from cell membranes. Subsequently this can lead to modification in cell signalling as well as a decrease in the production of immunomodulatory eicosanoids like PGE_2 which can in turn influence synthesis of immunomodulatory cytokines (for more details on the mechanisms of photoprotection by n-3 PUFAs please see section 1.6.3.2.6). In the next chapter, certain elements of this will be explored, i.e. the potential influence of supplemental n-3 PUFAs on UV-induction of LC trafficking and immunomodulatory cytokines.

5 EFFECT OF DIETARY EPA ON CELLULAR AND BIOCHEMICAL MARKERS OF PHOTOIMMUNOSUPPRESSION

5.1 Introduction

In the previous chapter, the impact of dietary n-3 PUFAs on clinical expression of PI was examined, in a double-blind randomised controlled study. In the studies in the current chapter, evidence was sought for the potential impact of the dietary supplementation on the cellular and cytokine mediation of UV-induced immunosuppression. Half of the human volunteers in each supplementation group (active and control) undergoing the clinical studies, had skin biopsies taken for assessment of the effect of UV on epidermal LC numbers. The other half had skin blisters raised for assessment of the effect of UV on levels of immunomodulatory cytokines in blister fluid. The rationale for these assessments is as follows.

LCs are dendritic antigen-presenting cells found in the epidermis. In conjunction with keratinocytes, LCs form a tight cellular network that covers body surfaces (van Beelen et al. 2007). Upon ingestion of an antigen, LCs migrate through the afferent lymphatics to reach draining LNs where they present Ag on their surface to naïve T cells (Romani et al. 2006). Depending on cytokines secreted by LCs and the surrounding micro environment, this Ag presentation results in a series of inflammatory/immunoregulatory responses (van Beelen et al. 2007). Signals from at least two cytokines are required for proper maturation and migration of LCs following skin sensitization; tumor necrosis factor-α (TNF-α) and interleukin 1beta (IL-1β) (Griffiths et al. 2005). Furthermore, in animal models PGE_2 has also been implicated in LC migration and maturation (Kabashima et al. 2003).

LCs can be identified immunohistochemically using antibodies against a number of antigenic surface markers. Upon UV-irradiation/inflammation of the skin however, a number of these markers are either lost or become simultaneously expressed by other cutaneous cells. An example of this is the loss of ATPase activity following UVA/PUVA treatment. Furthermore, while HLA-DR is specifically expressed by LCs in healthy human skin (Romani et al. 2006), it becomes co-expressed by keratinocytes (Schwarz. 2003), macrophages and vascular endothelial cells (Romani et al. 2006) in inflamed skin. A very useful marker for the detection of LCs in humans is CD1a which is exclusively expressed by LCs in both healthy and inflamed skin (Pena-Cruz et al. 2001; Schwarz. 2003).

UV-irradiation of the skin leads to a significant drop in number, change in morphology and altered Ag presentation of LCs (Schwarz et al. 1996; Vink et al. 1998). UV-induced LC depletion is largely implicated as the main cause of local PI while altered Ag presentation leads to the induction of IL-10 secreting Tregs and Ag specific tolerance (Schwarz 2010).

UV-induced DNA damage was found to play a major role in LC depletion by stimulating their migration to draining LNs. Moreover, Ag presentation by DNA-damaged yet viable LCs results in the induction of UV-Tregs and antigen specific tolerance. Repairing DNA photodamage of LCs significantly reduces their UV-induced depletion and restores their Ag presenting capabilities (Vink et al. 1997; Schwarz et al. 2005). When suction blisters were raised on UV-irradiated skin to study LC migration however, the number of DNA-damaged LCs in suction blister roofs did not correlate with the number of migrating DNA-damaged LCs trapped in suction blister fluid. Factors like blister cross section examined and LC adherence to dermal blister roof can partially explain this (Kolgen et al. 2002). In addition, LC-specific surface markers can be lost due to UV-induced cell membrane damage (Friedmann et al. 1983; Obata and Tagami 1985).

As previously outlined in section 1.5.4.2, a shift in activation of T cells from Th1 to a Th2 is postulated as one of the contributing cellular mechanisms of PI. The major signal for Th2 differentiation is IL-4. UV-irradiation induces serum IL-4 in a dose dependent manner (Ullrich 1996; Shreedhar et al. 1998). Compared to normal controls, T cells from UV-irradiated mice secrete less IL-2 and IFN-γ and more IL-4; indicating a shift toward a Th2 like reaction along with an inhibition in the development of Th1 cell (Shreedhar et al. 1998). There is no evidence however to support the secretion of IL-4 by UV-irradiated keratinocytes. Instead, it has been hypothesized that prostaglandins released by UV-irradiated keratinocytes are responsible for the induction of serum IL-4 and subsequently IL-10 (Shreedhar et al. 1998): one of the key immunomodulatory cytokines that was found to be pivotal for PI and antigen specific tolerance is IL-10 (through secretion by UV-Tregs) (Schwarz et al. 2000).

TNF-α secretion by skin keratinocytes and fibroblasts is normally elevated following UVB but not UVA exposure and has been shown to play a significant role in PI (Werth et al. 2003). In addition, TNF-α can produce morphological changes in LCs identical to those induced by irradiation with UVB (Kurimoto and Streilein 1992) and together with IL-1β, was found essential for epidermal LC migration (Cumberbatch et al. 2003).

In addition to inflammation, IL-8 was found to stimulate the keratinocyte synthesis of the PAF with resulting PAF-induced gene transcription for two important mediators of systemic PI, COX-2 and IL-10 (for more details on the immunosuppressive properties of PAF, please see section 1.5.3.1) (Konger et al. 2008).

The role of the novice Th17 cell and it's hallmark cytokine IL-17 in pro/anti tumor immunity remains controversial (Martin-Orozco and Dong 2009). The expression of IL-17A has been detected in several human tumors including prostate, breast and gastric cancer. Furthermore, mice deficient in the IL-17 receptor (IL-17R) showed inhibited tumor growth. Other studies however have shown an anti-tumor role for IL-17; hematopoietic tumor cells

over expressing IL-17 showed significant inhibition in tumor growth when implanted in syngeneic mice and in IL-17 deficient mice, injecting adenocarcinoma cells resulted in a 5 times or more increase in subcutaneous growth and metastases compared to control, wild-type mice (Ngiow et al. 2010).

5.2 Objectives

A. To examine and compare UV-induced LC trafficking in active and control groups before and after 3 months of supplementation with n-3 PUFAs and GTCC respectively. This would allow for assessment of the protective properties of n-3 PUFA against UV-induced LC depletion and offer an insight into the mechanisms underlying the clinical results seen in chapter 4.

B. To measure and compare changes in the levels of key immunomodulatory cytokines in suction blister fluids post UV-irradiation; within and between groups, both pre and post supplementation. This would allow for the assessment of n-3 PUFA effects on the cutaneous immunomodulatory cytokine environment.

5.3 Methods

5.3.1 Langerhans Cell Studies

Half the subjects in each group (n=20 active; n=19 control) had UV-induced LC trafficking assessments both pre and post supplementation. For every assessment, two punch biopsies, taken by the study's research nurse, from the upper buttocks skin of each volunteer were used: one from un-irradiated skin and another from contralateral skin taken at 24hrs post UV-irradiation (with 4x MED). To keep the UV-challenge constant, the same 4x MED dose used pre-supplementation was also used post-supplementation (for the punch biopsy and MED testing protocols, please see sections 2.12 and 2.8.3 respectively). An epidermal sheet was then prepared from each biopsy and indirect IHC was performed to visualise CD1a+ LCs. Using a fluorescent microscope fitted with a graticule eyepiece, LCs were then counted (for more details on the punch biopsy protocol and LC staining and counting, please see sections 2.12 and 2.14.1 respectively).

During the application of 4x MED, inconsistencies were observed in the irradiance output of the TL 12 MED tester (the irradiance output of all lamps used in this study was measured prior to every use). Accordingly, this lamp was replaced with another lamp using x10 identical TL12/20W bulbs: the Waldmann UV 800. A total of 10 active and 10 control biopsy subjects used this lamp (for more details on the Waldmann UV 800, please see section 2.6.3). The expected increase in irradiance output was accounted for by decreasing the lamp application time. A previous study examining UV-induced LC

trafficking observed an insignificant variation in LC depletion when an identical UV-dose (1.5 MED) at one tenth the irradiance was used (Murphy et al. 1993).

5.3.2 Cytokine Studies

For cytokine assessment in suction blister fluid, half the subjects in each group (20 active and 20 control) had suction blister raised on UV-irradiated (4x MED) and contralateral unirradiated skin of the upper buttocks both pre and post supplementation. Using luminex multiplex cytokine assay kits (the Millipore Milliplex® Human Cytokine/Chemokine kit), cytokine levels in aspirated suction blister fluids were simultaneously quantified and differences were evaluated using a paired Student's t test. The cytokines looked for were: IL-8, IL-10, IFN-γ, TNF-α, IL-1β, IL-4 and IL-17 (for more details on the raising of suction blisters and cytokine analysis of blister fluids, please see sections 2.13 and 2.14.2 respectively).

5.4 Results

5.4.1 Results of the Langerhans' Cells Studies

5.4.1.1 Volunteers

Active Group

A total of 20 volunteers in the active group were assigned to have punch biopsies pre and post n-3 PUFA supplementation. The mean age of the volunteers was 45.2yr (age range: 24yr – 60yr). One volunteer declined post supplementation biopsies and one sample was excluded due to an incubator malfunction. In addition, the TL 12 MED tester failed during the application of 4x MED post supplementation in another volunteer. Data from this volunteer were omitted (for more details please see section 5.1.3). This left data sets from 17 active volunteers for the examination of LC trafficking post-UV.

Control Group

In the control group, a total of 19 volunteers were set to have punch biopsies. The mean age of the volunteers was 45.6yr (age range: 25yr – 58yr). Five subjects dropped out. Another 2 were excluded due to an incubator malfunction in 1 and failure to separate the epidermis from the sub-epidermal tissue in the other. Data from 1 volunteer was excluded due to failure of the TL 12 MED tester. This left data from a total of 11 control subjects for the post-UV assessment of LC trafficking.

5.4.1.2 Post Supplementation Changes in Baseline LC Count

The average CD1a+ LC count/mm^2 at the start of the study, before the volunteers were UV-irradiated and before they began supplementation was 898.1 ± 39.20 (SEM) (n=17) in the active group and 934.9 ± 40.81 (n=11) in the control group. When the volunteers

returned following 3 months of supplementation, the change in baseline (pre-UV) LC count/mm^2 within each groups was insignificant: P=0.27 in the active group (n=17; mean difference 42.3; 95% confidence interval: -35.58 to 120.3) and P=0.4 in the control group (n=11; mean difference: 57.65; 95% confidence interval: -89.00 to 204.3). This indicated that 3 months of supplementation with n-3 PUFA or placebo did not have a significant effect on baseline LC count (figure 5.1)

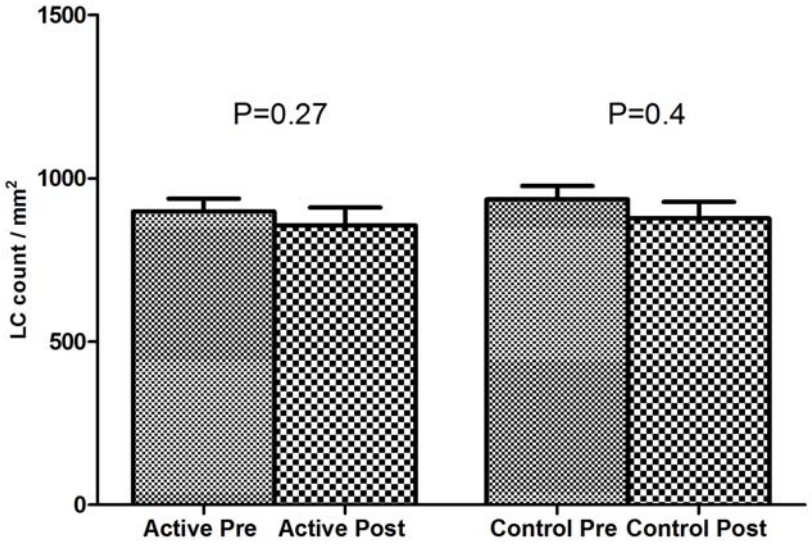


Figure 5.1 - Baseline LC count before and after 3 months of supplementation in active and control groups

An insignificant change in baseline (unirradiated skin) LC count post-supplementation was seen in both active control groups

5.4.1.3 UV-induced LC Depletion in active and control groups

Active Group

The average pre supplementation LC count/mm^2 was 898.1 ± 39.20 (SEM) (n=17) pre UV-irradiation. This significantly decreased to 305.4 ± 47.82 (SEM) post UV-irradiation (P< 0.0001; difference between means: 592.7 ± 61.83; 95% confidence interval: 482.0 to 703.4) (figure 5.2a).

The average post supplementation LC count/mm^2 was 855.8 ± 54.53 (SEM) (n=17) pre UV-irradiation. This significantly decreased to 191.3 ± 25.65 (SEM) post UV-irradiation (P< 0.0001; difference between means: 664.5 ± 60.26; 95% confidence interval: 541.7 to 787.3) (figure 5.2b).

Control Group

The average pre supplementation LC count/mm^2 was 934.9 ± 40.81 (SEM) (n=11) pre UV-irradiation. This significantly decreased to 346.3 ± 73.07 (SEM) post UV-irradiation (P< 0.0001; difference between means: 588.6 ± 83.70; 95% confidence interval: 414.0 to 763.2) (figure 5.3a).

The average post supplementation LC count/mm^2 was 877.3 ± 50.03 (SEM) (n=11) pre UV-irradiation. This significantly decreased to 229.2 ± 46.39 (SEM) post UV-irradiation (P< 0.0001; difference between means: 648.1 ± 68.23; 95% confidence interval: 505.7 to 790.4) (figure 5.3b).

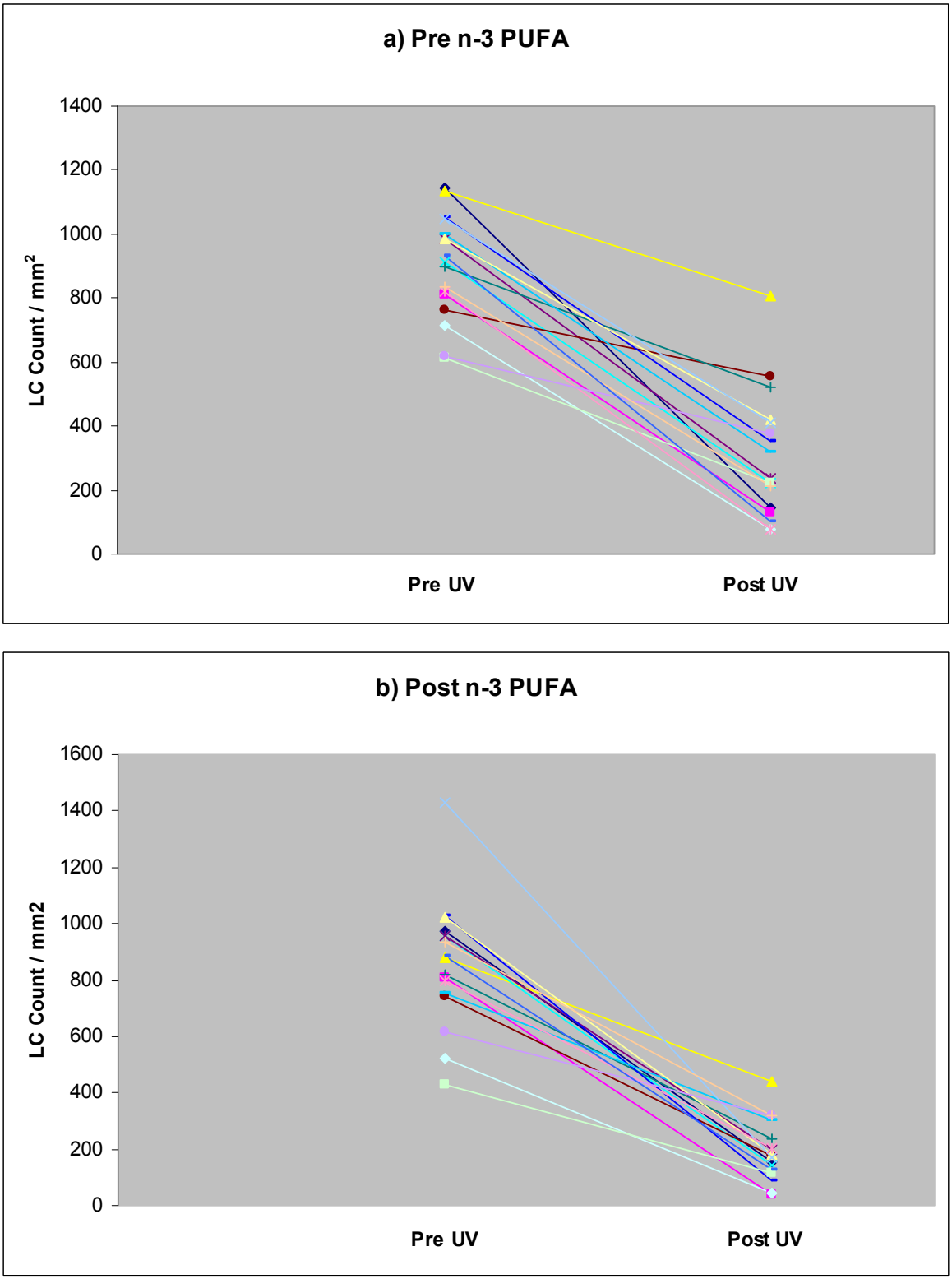
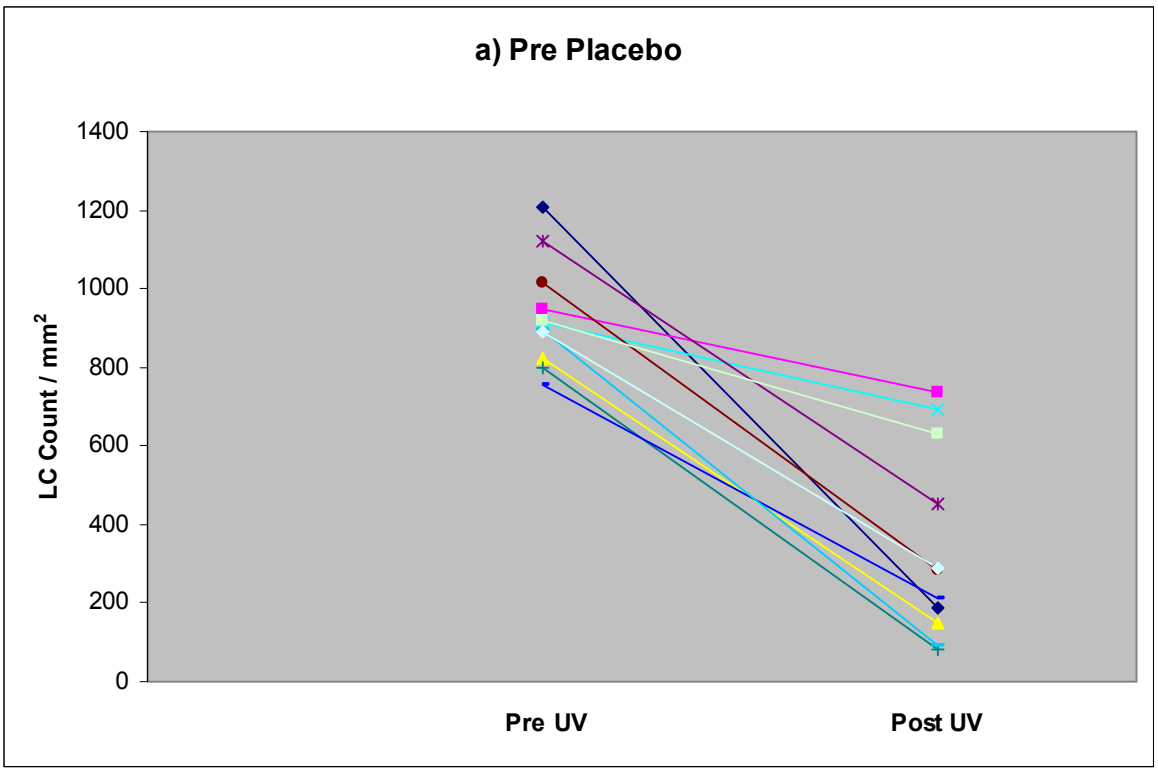


Figure 5.2 - UV-induced LC depletion in active group (n=17)

a) pre and b) post supplementation

A significant decrease in LC count in the active group was seen post UV-irradiation both pre and post supplementation (P<0.0001)



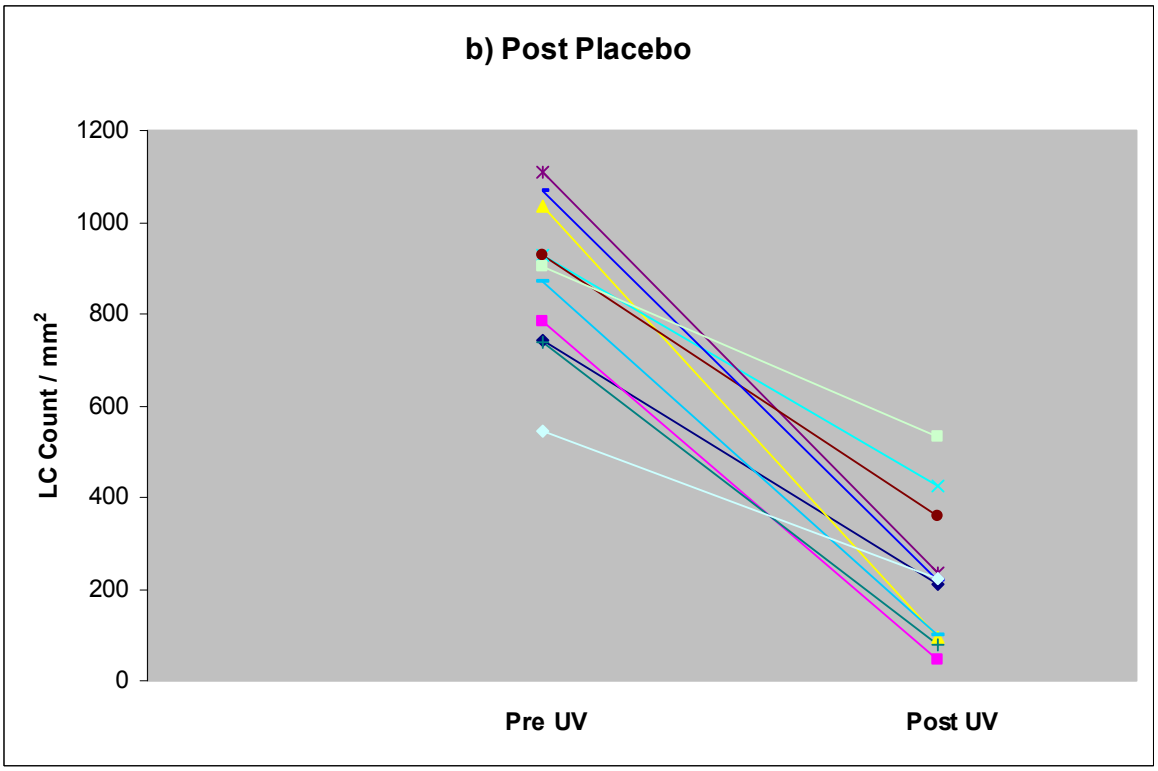


Figure 5.3 - UV-induced LC depletion in control group (n=11)

a) pre and b) post supplementation

A significant decrease in LC count in the active group was seen post UV-irradiation both pre and post supplementation (P<0.0001)

5.4.1.4 Intragroup Comparison of UV-induced LC depletion Pre vs. Post Supplementation

A comparison was made within each group to look at UV-induced % decrease in LC count pre vs. post supplementation.

Active Group

The mean pre supplementation % decrease in LC count 24hrs post UV-irradiation with 4x MED was 65.84 ± 5.09% (SEM). This increased to 76.61 ± 3.39% (SEM) post supplementation (n=17, P=0.018, mean of differences: -10.76%; 95% confidence interval: -19.43 to -2.09%) (figure 5.3 & 5.4).

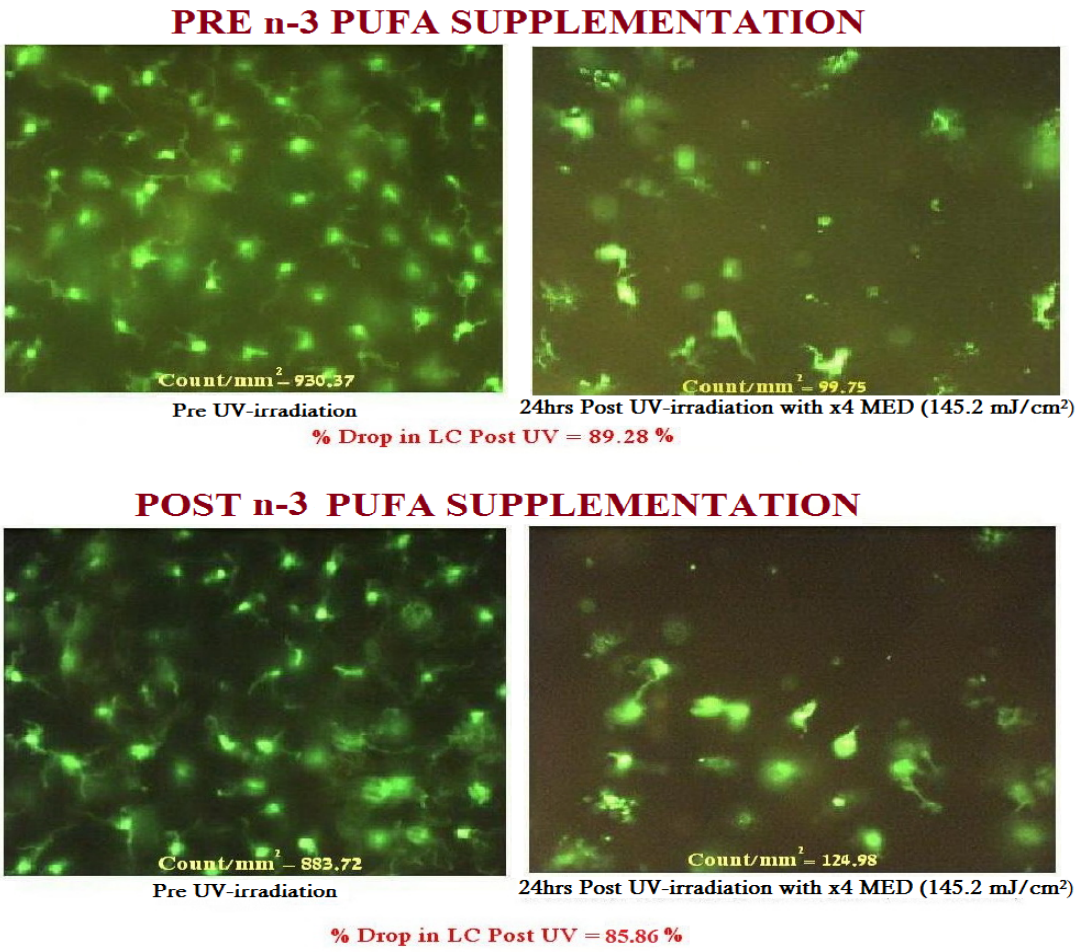


Figure 5.3 - UV-induced LC depletion and loss of dendrites pre & post three months of n-3 PUFA supplementation

Following UV-irradiation (4x MED) a significant decrease in number and change in morphology could be noted in the active group both pre and post supplementation

Control Group

The mean pre supplementation % decrease in LC count 24hrs post UV-irradiation with 4x MED was 63.14 ± 7.75% (SEM). This increased to 73.52 ± 5.24% (SEM) post supplementation (n=11, P=0.18, mean of differences: -10.38%, 95% confidence interval: -26.55 to 5.793%) (figure 5.4).

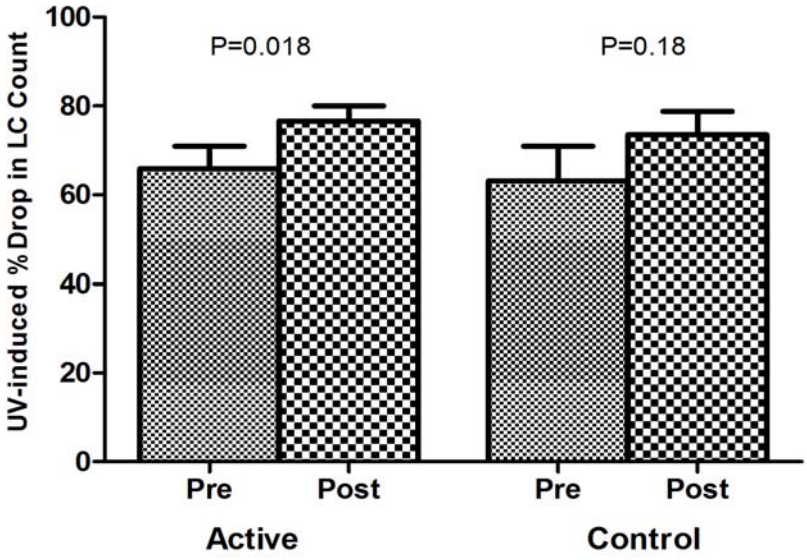


Figure 5.4 - UV-induced % drop in LC count pre and post supplementation in active (n=17) and control (n=11) groups

An increase in % drop of LC count was noted in both active and control groups following 3 months of supplementation. This increase reached statistical significance in the active group (P=0.018)

5.4.1.5 Comparison of UV-induced LC Depletion between Groups Following Three Months of Supplementation

The % change in LC count post-UV was compared between the active and control groups after 12 weeks of supplementation. No significant difference in UV-induced LC depletion was noted between the active and control groups (figure 5.5): P=0.61 (difference between means: -10.76 ± 6.112 (SEM), 95% confidence interval: -23.22 to 1.690).

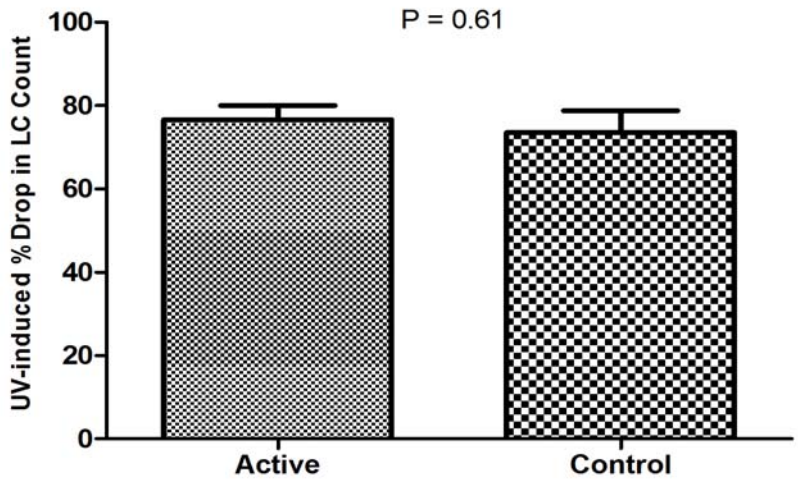


Figure 5.4 - UV-induced % decrease in LC count post supplementation in active (n=17) and control (n=11) groups

The difference in UV-induced % drop in LC count post supplementation between the active and control groups was insignificant (P=0.61). In the active group the post supplementation UV-induced % drop was 76.61 ± 3.39% (SEM) compared to 73.52 ± 5.24% (SEM) in the control group

5.4.2 Results of the Cytokine Studies

5.4.2.1 Volunteers

Active Group

A total of 20 volunteers in the active group were assigned to have suction blisters. The mean age of the volunteers was 41.2yr (age range: 25yr – 60yr). Following analysis of membrane EPA in RBCs, 3 of the volunteers were found to be non-compliant and were excluded from the data analysis. This left data sets from 17 active volunteers for the examination of immunomodulatory cytokines in suction blister fluids.

Control Group

In the control group, a total of 20 volunteers were set to have suction blisters. The mean age of the volunteers was 45.1 (age range: 32yr – 63yr). One subject dropped out. Another 2 were excluded due to irradiance inconsistencies of the TL 12 MED tester. This left data from a total of 17 control subjects for the assessment of cytokines in blister fluids.

5.4.2.2 Intra and intergroup comparison of cytokine levels pre and post supplementation

When the human cytokine standards where run against suction blister samples (n=34), 4 of the 7 cytokines analyzed were found to be out of range (low): IL-17, IL-1β, IFN-γ and IL-4. This left data for 3 cytokines to be analyzed: IL-10, TNF-α, and IL-8. Cytokine levels (pg/ml), pre and post UV were measured and compared within and between groups before and after supplementation.

- **IL-10**

IL-10 levels were found to be out of range (low) in samples from 2 active and 4 control subjects. This left data sets from a total of 15 active and 13 control volunteers for the analysis of IL-10 levels in blister fluid.

Active Group

Before supplementation, mean pre-UV IL-10 level was 91.67pg/ml and this increased to 115.62pg/ml post-UV (P value=0.16; mean of difference: -23.95; 95% confidence interval: -58.17 to 10.27) (n=15). Following 3 months of supplementation, the mean pre-UV IL-10 level became 81.56pg/ml and this increased to 105.01pg/ml post-UV (P value=0.14; mean of difference: -23.45; 95% confidence interval: -55.35 to 8.46) (n=15) (figure 5.4).

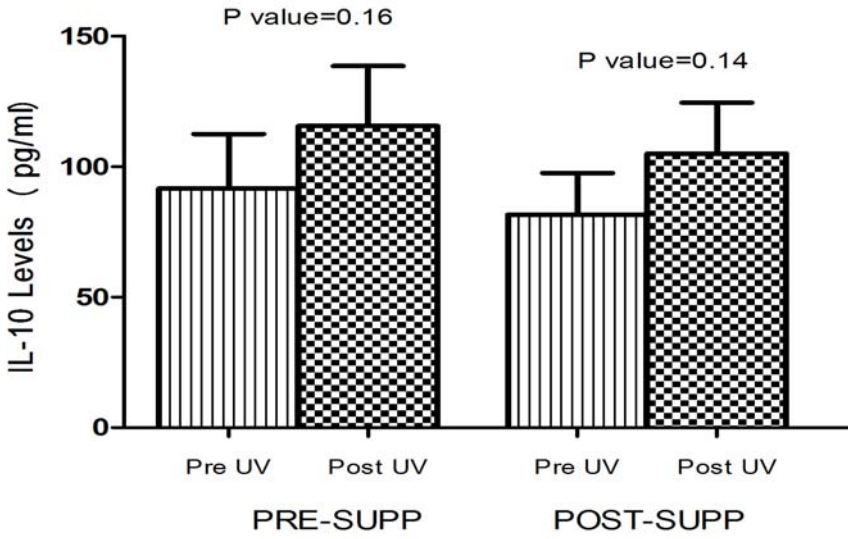


Figure 5.5 - IL-10 levels (pg/ml) in active group subjects (n=15) pre and post supplementation

IL-10 levels in the active group insignificantly increased post UV-irradiation both pre (P=0.16) and post (P=0.14) n-3 PUFA supplementation

Control Group

Mean IL-10 level before supplementation and prior to UV-irradiation was 111.23pg/ml and this increased to 116.19pg/ml post-UV (P value=0.85; mean of difference: -4.96; 95% confidence interval: -61.19 to 51.28) (n=13). Following supplementation, the mean pre-UV level of IL-10 was 98.49pg/ml and this increased to 141.36pg/ml post-UV (P value=0.22; mean of differences: -42.87; 95% confidence interval: -114.2 to 28.46) (n=13) (figure 5.5).

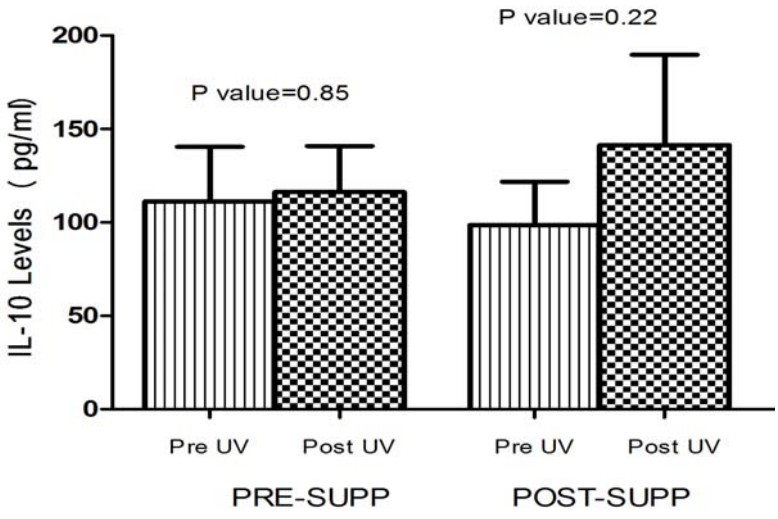


Figure 5.6 - IL-10 levels (pg/ml) in control group subjects (n=13) pre and post supplementation

IL-10 levels in the control group insignificantly increased post UV-irradiation both pre (P=0.87) and post (P=0.94) supplementation

When the UV-induced increase in IL-10 levels was compared between the active and control groups following 12 weeks of supplementation, no significant difference was noted: P=0.58 (difference between means: -19.42 ± 34.35 (SEM), 95% confidence interval: -90.04 to 51.20) (figure 5.7).

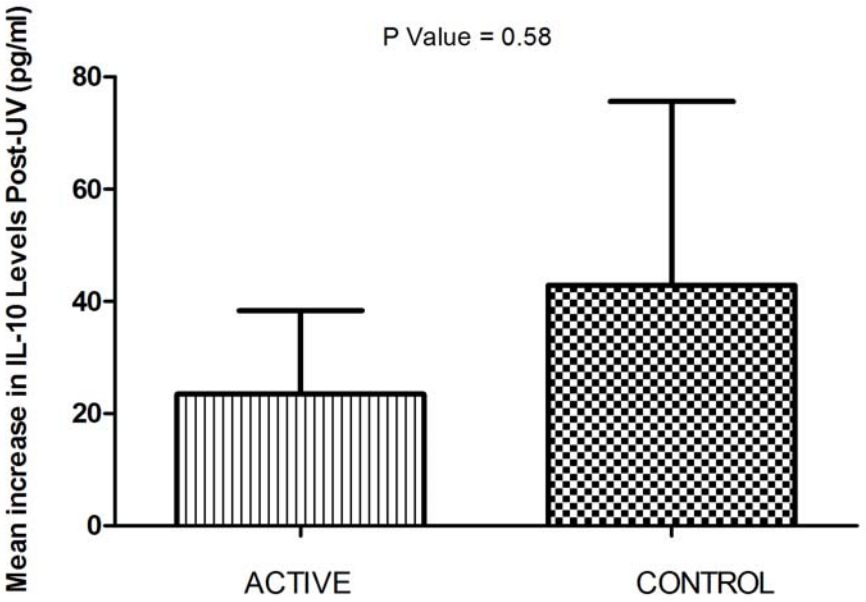


Figure 5.7 - Mean UV-induced increase in IL-10 post supplementation in active (n=15) and control (n=13) groups

Post supplementation, IL-10 levels increased post UV-irradiation in both active and control groups. The difference in this UV-induced increase was insignificant between groups (P=0.58)

- **TNF-α**

TNF-α levels were found to be out of range (low) in samples from 2 active and 2 control subjects. This left data sets from a total of 15 active and 15 control volunteers for the analysis of TNF-α levels pre and post UV.

Active Group

Mean pre supplementation level of TNF-α was 86.36pg/ml pre-UV and this increased to 91.99pg/ml post-UV (P value: 0.87; mean of differences: -5.64; 95% confidence interval: -76.55 to 65.28) (n=15). Post supplementation, the mean pre-UV TNF-α level was 98.58pg/ml and this increased to101.78pg/ml post-UV (P value=0.94; mean of difference: -3.2; 95% confidence interval: -95.22 to 88.82) (n=15) (figure 5.6).

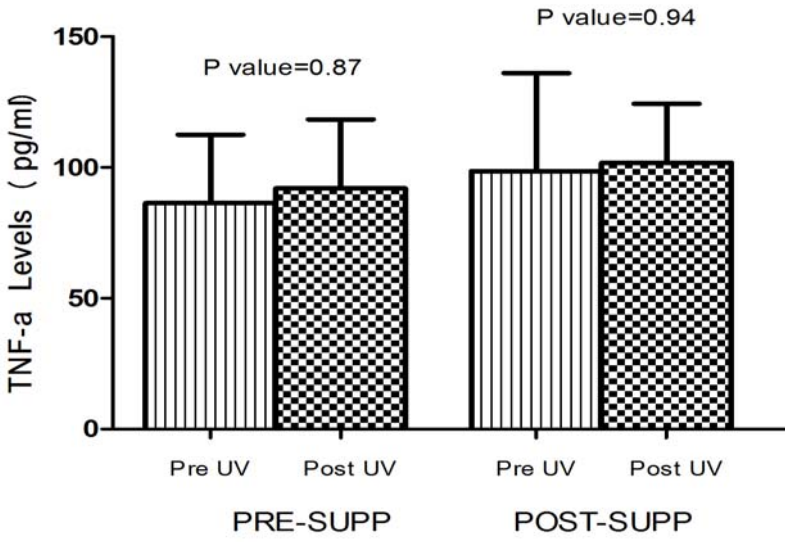


Figure 5.8 - TNF-α levels (pg/ml) in active group subjects (n=15) pre and post supplementation

TNF-α levels in the active group insignificantly increased post UV-irradiation both pre (P=0.87) and post (P=0.94) n-3 PUFA supplementation

Control Group

Mean TNF-α level before supplementation and prior to UV-irradiation were 95.96pg/ml and this increased to 110.57pg/ml post-UV (P value=0.66; mean of difference: -14.61; 95% confidence interval: -84.39 to 55.16) (n=15). Following supplementation, the mean pre-UV level of TNF-α was 110.57pg/ml and this increased to 11.7pg/ml post-UV (P value=0.97; mean of differences: -1.13; 95% confidence interval: -58.72 to 56.46) (n=15) (figure 5.7).

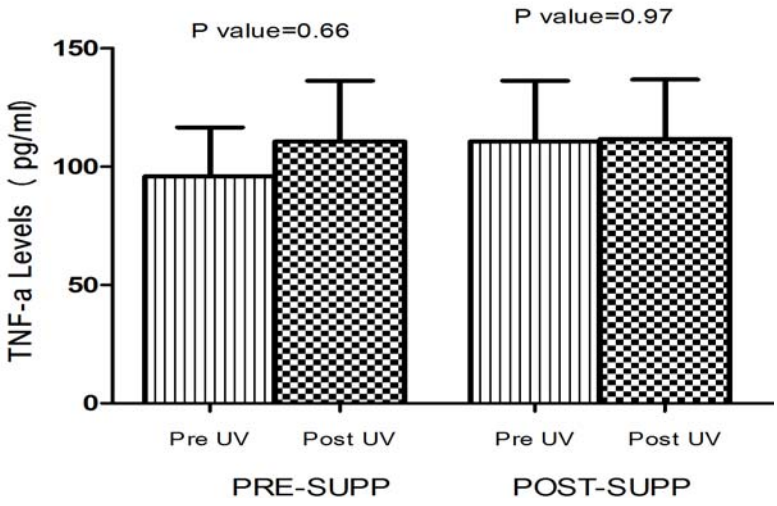


Figure 5.9 - TNF-α levels (pg/ml) in control group subjects (n=15) pre and post supplementation

TNF-α levels in the control group insignificantly increased post UV-irradiation both pre (P=0.66) and post (P=0.97) supplementation

When the UV-induced increase in TNF-α levels was compared between the active and control groups following 12 weeks of supplementation, no significant difference was noted: P=0.97 (difference between means: 2.071 ± 50.61(SEM), 95% confidence interval: -101.6 to 105.7) (figure 5.10).

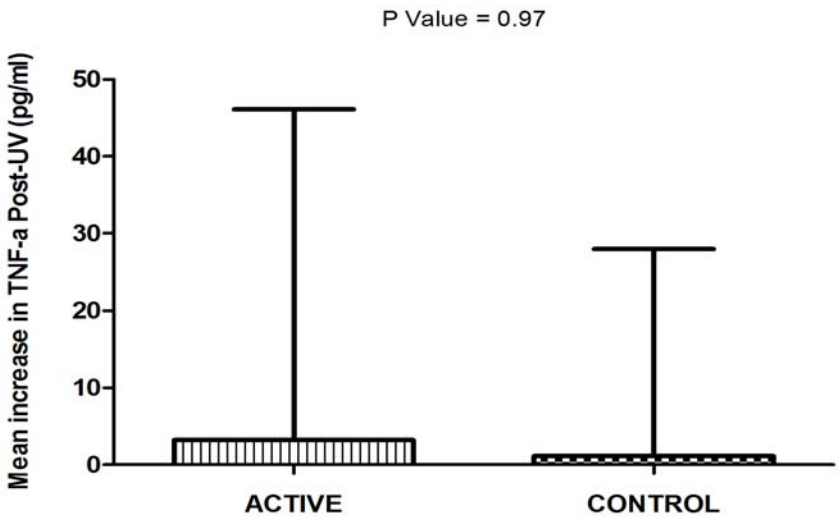


Figure 5.10 - Mean UV-induced increase in TNF-α post supplementation in active (n=15) and control (n=15) groups

Post supplementation, TNF-α levels increased post UV-irradiation in both active and control groups. The difference in this UV-induced increase was insignificant between groups (P=0.97)

- **IL-8**

IL-8 levels were found to be out of range (low) in samples from 2 active and 2 control subjects. This left data sets from a total of 15 active and 15 control volunteers for the analysis of IL-8 levels pre and post UV.

Active Group

Mean pre supplementation level of IL-8 was 296.81pg/ml pre-UV and this significantly increased to 831.02pg/ml post-UV** (P value=0.002; mean of differences: -534.2; 95% confidence interval: -839.8 to -228.6) (n=15). Post supplementation, the mean IL-8 level was 313.79pg/ml pre-UV and this significantly increased to 725.63pg/ml post-UV* (P value= 0.037; mean of difference: -411.8; 95% confidence interval: -794.5 to -29.22) (n=15) (figure 5.8).

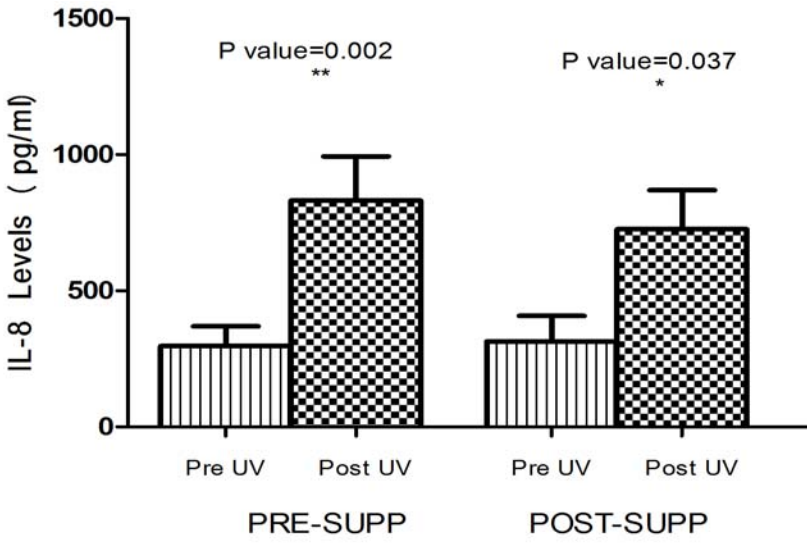


Figure 5.11 - IL-8 levels (pg/ml) in active group subjects (n=15) pre and post supplementation

IL-8 levels in the active group significantly increased post UV-irradiation both pre (P=0.002) and post (P=0.037) supplementation

Control Group

Mean pre supplementation level of IL-8 was 392.87pg/ml pre-UV and this significantly increased to 931.36pg/ml post-UV*** (P value=0.0008; mean of differences: -538.5; 95% confidence interval: -811.7 to -265.2) (n=15). Post supplementation, the mean IL-8 level was 250.76pg/ml pre-UV and this significantly increased to 947.80pg/ml post-UV*** (P value<0.0001; mean of difference: -538.5; 95% confidence interval: -811.7 to -265.2) (n=15) (figure 5.9).

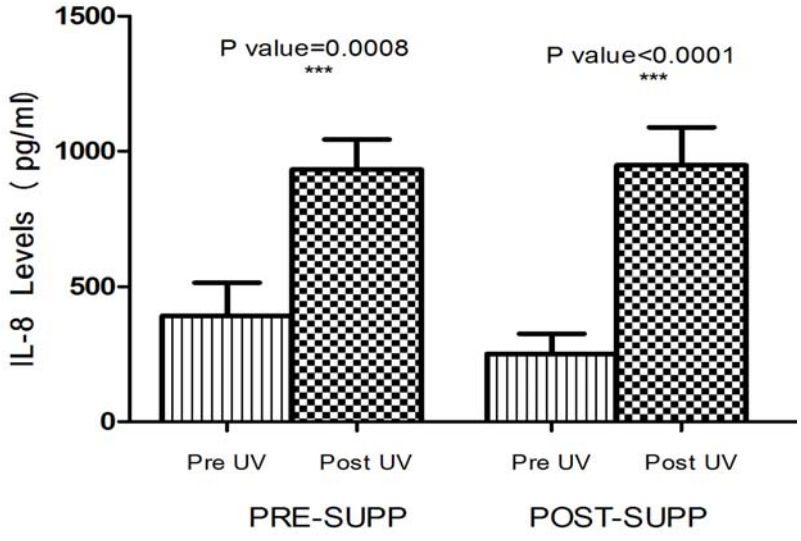


Figure 5.12 - IL-8 levels (pg/ml) in control group subjects (n=15) pre and post supplementation

IL-8 levels in the control group significantly increased post UV-irradiation both pre (P=0.0008) and post (P<0.0001) supplementation

When the UV-induced increase in IL-8 levels was compared between the active and control groups following 12 weeks of supplementation, no significant difference was noted: P=0.17 (difference between means: -285.2 ± 202.3 (SEM), 95% confidence interval: -699.4 to 129.0) (figure 5.13).

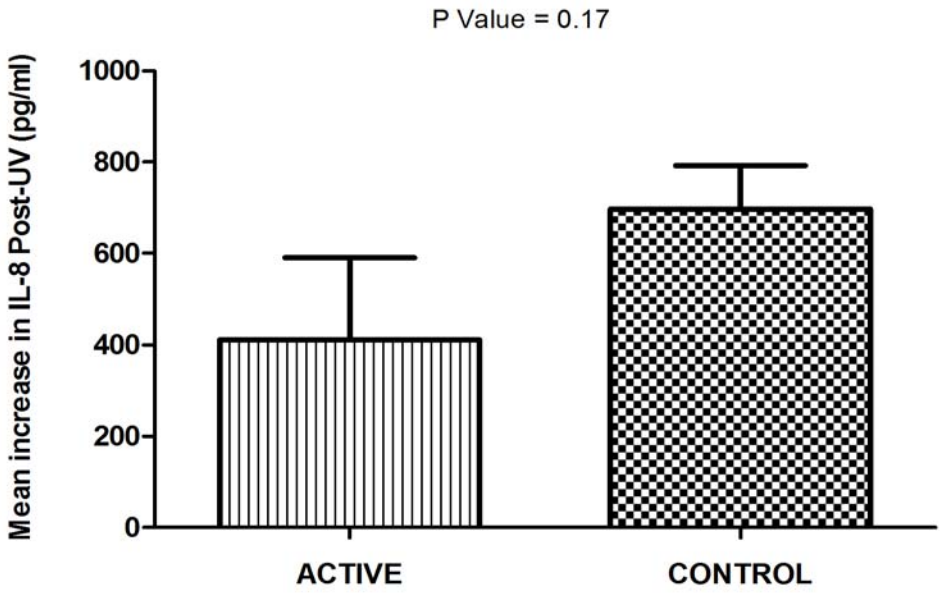


Figure 5.13 - Mean UV-induced increase in IL-8 post supplementation in active (n=15) and control (n=15) groups

Although the UV-induced increase in IL-8 levels post supplementation was significant in both active and control groups, the difference in this increase between groups was insignificant (P=0.17)

5.5 Discussion

The average LC count/mm^2 at the start of the study, before volunteers were UV-irradiated and before they began supplementation was 898.1 ± 39.20 (SEM) (n=17) in the active group and 934.9 ± 40.81 (n=11) in the control group. These baseline LC counts are in agreement with previous studies examining LC counts in normal human skin (Chen et al. 1985; Cruchley et al. 1994; Dearman et al. 2004).

As detailed in section 5.4.1.2, the change in baseline LC counts following 3 months of supplementation was insignificant in both the active and control groups: P=0.27 in the active group and P=0.4 in the control group (figure 5.1). This indicated that 3 months of supplementation with n-3 PUFA or placebo GTCC oil did not have a significant effect on baseline LC counts (figure 5.1)

As with previous animal and human studies (Murphy et al. 1993; Bestak and Halliday 1996; McLoone et al. 2005), cutaneous UV-irradiation led to a significant decrease in count and alteration in morphology with loss of dendrites in all subjects examined: in the active and control groups both pre and post supplementation. Based on individual MED

assessments, erythemally equalized doses of UV were given to each volunteer (4x MED). The degree of LC depletion observed in both active and control groups of this study were comparable to that of other studies examining LC depletion in human skin using a UVB-irradiation dose of 4x MED (also applied using a TL 12 tube): ~70% (Cooper et al. 1992).

The technique used to identify and count LCs in this study involved stripping of epidermal sheets, monoclonal antibody labelling and counting using indirect immunofluorescence. LC identification was on the basis of CD1a expression which was found to be exclusive to LCs in human epidermis (Cumberbatch et al. 1999). Compared to using full thickness cross sections of skin, epidermal sheets offer better identification of LCs with less transection of cells and reduced cellular background (Emilson and Scheynius 1995). Dearman et al. (2004) previously demonstrated successful monitoring of cutaneous cytokine levels using suction blister fluid. When suction blister roofs were used to assess LC migration however, a reduced LC count was seen in comparison to LC counts from epidermal sheets separated from punch biopsies (in a similar manner to the one used in this study). Mechanical trauma from the raising of suction blisters with resulting stimulation of LC migration has been postulated as the reason for reduced LC count in blister roofs. Given these findings, the use of epidermal sheets from punch biopsies can be regarded as a more favourable method for counting LCs (Dearman et al. 2004).

Unexpectedly, the UV-induced % drop in LC count increased in both active and control groups following 3 months of supplementation (figure 5.3): 11% increase in the active group and 10% in the control group. No statistical difference in this post supplementation increase could be seen between groups. In the active group, this post supplementation increase in UV-induced LC drop reached statistical significance. As previously outlined, long chain n-3 PUFAs are more susceptible to lipid peroxidation with subsequent generation of ROS (Rhodes et al. 1994) and reduction in antioxidant status (Moison and Beijersbergen Van Henegouwen 2001) with possible oxidative DNA damage (for more details please see section 1.6.3.2.1.1). Since UV-induced LC depletion has been linked to DNA photodamage (Kolgen et al. 2002) and recovery of LCs is linked to nucleotide excision repair of this damage (Jimbo et al. 1992), this might partially explain the significant increase UV-induced LC depletion seen in the active group following 3 months of supplementation. In addition, confounding factors such as seasonal changes could have also contributed to the increase in UV-induced LC depletion in both groups post supplementation.

Although the clinical assessment of PI showed evidence of photoprotection by n-3 PUFAs (chapter 4), this protection does not appear to be linked to LCs. Protection by n-3 PUFAs might be acting in a non-LC dependant manner via an influence on other PI mediators. In addition, the different light sources used for the clinical model of PI and UV-induced LC depletion might have influenced the results observed. For clinical PI, the primary light

source was the solar simulator: a lamp with an irradiance output of 96% UVA. In comparison, the two light sources employed for the UV-induced LC depletion model were UVB-rich lamps: the TL12 MED tester and Waldmann UV 800. Furthermore, the maximum dose of UV used in each model varied significantly: 70% MED for clinical PI compared to 4x MED in the LC depletion model. Similar to the maximum dose of SSR used in the clinical model of PI (70% MED given for 3 consecutive days which equates to an accumulated dose of 2.1 MED), this 4x MED dose of UVB could have exceeded the photoprotective abilities of n-3 PUFAs: in a previous study by Cooper et al. (Cooper et al. 1992), a single exposure to a UV-dose of 4x MED was found to induce systemic PI.

To examine biochemical mechanisms that might be involved in n-3 PUFA protection against PI, cytokine levels in suction blister fluid were examined. These included cytokines that were found to induce LC migration: IL-1β and TNF-α (Cumberbatch et al. 1997; Cumberbatch et al. 1999; Cumberbatch et al. 2003) which could help in understanding the observed n-3 PUFA effects on UV-induced LC depletion. Not finding a statistically significant difference between cutaneous TNF-α levels in the active n-3 PUFA group and placebo group and not being able to detect IL-1β in the examined blister fluids (out of range, low) correlates with the observed absence of a significant difference in UV-induced LC depletion between the active and placebo groups.

IL-10 is a key soluble mediator of PI. As expected, a post-UV increase in mean IL-10 levels was seen post-UV in both active and control groups pre and post supplementation. This increase was not significantly affected by 3 months of n-3 PUFA supplementation. This finding might imply that the protective properties seen by n-3 PUFAs against PI (chapter 4) are induced by a mechanism unrelated to modifications in UV-induced IL-10 levels.

IL-8 is a proinflammatory cytokine that has been implicated in UV-induced skin inflammation (Baggiolini and Clark-Lewis 1992). When the effects of EPA and DHA on UV-induced IL-8 secretion by fibroblast and keratinocyte cell lines were examined, a respective 66% and 65% reduction was seen (Storey et al. 2005). In accordance with this, a reduction in post-UV increase in IL-8 levels was seen in the active group of this study: from a mean UV-induced increase of 534.2 pg/ml pre-supplementation to a mean increase of 411.8 pg/ml post-supplementation with n-3 PUFA. The mean UV-induced increase in IL-8 remained unchanged in the control group at 538.5pg/ml.

Shahbakhti et al. (2004) examined the effects of 4g EPA taken for 3 months on IL-8 and TNF-α levels in suction blister fluids post-UVB irradiation: a dose of 3x MED was applied to buttocks skin using a TL-12 lamp and suction blisters were raised at 16hrs post-UV. Mean baseline IL-8 levels reported in this study (n=14) were 15.4pg/ml ± 3.3 (SEM) and this significantly increased to 37.5pg/ml ± 4.2 (SEM) ($P<0.001$). No significant changes in IL-8 levels were noted post-supplementation. Similarly, TNF-α levels were significantly

elevated post-UV: 6 pg/ml ± 0.7 (SEM) at baseline to 14.5 ± 2 (SEM) post-UV and this elevation was not significantly affected by EPA supplementation. It should be noted that cytokine levels in this study were analyzed using an ELISA kit following a UV-dose of 3x MED at 16hrs post-UV which could explain the noticeable differences observed in baseline and post-UV cytokine levels.

Another study (Barr et al. 1999) employing 3x MED (using a 1000W solar simulator) to assess UV-induced cytokine levels in suction blister fluid at different time points found a maximum increase in TNF-α, IL-1β and IL-1-α at 15hr post-UV. TNF-α rapidly decreased after this time point whereas, IL-1β and IL-1-α remained raised for 72hr (although at lesser levels than those observed at 15hrs). IL-10 on the other hand increased to a maximum 2 fold between 15 and 24hrs post UV: around the same time that TNF-α and IL-1β levels began to decline. Mean baseline IL-10 and TNF-α levels observed in unirradiated upper buttock skin, using an ELISA technique were 39pg/ml and 65pg/ml respectively (compared to respective means of ~111.23pg/ml and ~90pg/ml measured in this study). Following UV-irradiation with 3xMED SSR, TNF-α levels reached a maximum of ~700pg/ml at 15hrs post-UV while IL-10 increased to a maximum of ~70pg/ml at 15hrs post-UV. In comparison, post-UV TNF-α and IL-10 levels in this study, 24hrs post UVB-irradiation with 4x MED reached ~116 and 101pg/ml respectively. The relatively small increase in IL-10 (maximum 2 fold increase) suggests that 3x MED is the threshold dose for it's induction and may warrant the use of a higher UV-dose. In addition, the reported cytokine levels at different time points, suggests that earlier aspiration and examination of suction blister fluids, before 24hrs post-UV might yield better results.

Accordingly, when Teunissen et al. (2002) investigated the hypothesis that UV-irradiation leads to an influx of IL-4 secreting cells (Bacci et al. 1998), unexposed buttocks skin was irradiated with a single dose of 4x MED SSR. This was followed by examination of cytokine levels (to assess Th1/Th2 balance) in fluid of suction blisters raised at 1 and 2 days post-UV using ELISA. In addition, in order to stain for IL-4^{+} cells, punch biopsies were taken at 5, 10 and 24hrs post-UV. IL-4+ cells reached a maximum at 24hrs post UV in the dermis and at 3 days in the epidermis indicating a UV-induced intrinsic influx of cells intro the epidermis. IL-6, IL-8 and TNF-α levels were markedly increased at 24 and remained high at 48hrs post-UV. Only IL-4 levels continued to increase beyond the 24hr mark though (probably due to the continued influx of IL-4+ cells into the epidermis) whereas levels of IL-6, IL-8 and TNF-α began to decline at 24hrs post-UV. The sharpest decline was seen with TNF-α which reached baseline levels at 48hrs post-UV. Maximum post-UV levels of IL-6, TNF-α and IL-8 (at 24 hrs) were ~ 650, 270 and 1000pg/ml respectively. Maximum IL-4 levels were seen 48hrs post-UV: ~23pg/ml.

Overall, the laboratory studies of the current chapter did not find evidence of an underlying change in cellular or cytokine mediator expression post-UV that could explain

the dietary abrogation of clinical PI. However, as described above, this could be explained at least partly by the methods and techniques selected. Moreover, this was not a fully comprehensive study. LCs are not the only dendritic Ag presenting cells found in steady-state skin (Henri et al. 2010) and their presence is not required for the induction of CHS responses (Noordegraaf et al. 2010). Furthermore, the role of LCs in PI is controversial. While Wang et al. (2009) showed that UV-induced suppression of CD8+ T cell expansion and PI of CHS responses could be induced in the absence of LCs, Schwarz et al demonstrated that LC are essential for PI (Schwarz et al. 2010). In addition, other immunoregulatory cells such as Tregs were not examined.

The cytokine levels found in this study differed significantly to those reported by other researchers (Barr et al. 1999; Teunissen et al. 2002; Shahbakhti et al. 2004). Such differences can be attributed to factors like the sampling method, UVR dose spectrum and time course. This makes direct comparisons across studies difficult and suggests that the most meaningful interpretation of data are comparisons between control and active groups. In the present study there was no evidence of an influence of n-3 PUFA supplementation on IL-10, TNF and IL-8 that could explain the observed photoprotective effects seen against PI.

6 CONCLUSIONS

The overall aim of the work presented in this thesis was to examine for potential photoprotective properties of dietary n-3 PUFAs against PI and by implication, the UV-induced promotion of skin cancer, in human subjects. This was performed by examining the effects of dietary n-3 PUFAs on UV-induced suppression of clinical CHS responses and on cellular and biochemical biomarkers of PI. To do this, a clinical model of PI was refined for use through pilot studies described in chapter 3. In chapter 4, this protocol was used to study the protective effects of n-3 PUFAs against UV-induced suppression of CHS responses, in a double-blind randomised controlled oral supplementation study. In addition, to confirm compliance and bioavailability, n-3 PUFA levels were assessed in RBC membranes. Finally, in chapter 5 the effect of dietary n-3 PUFAs on UV-induced LC trafficking and modulation of immunoregulatory cytokines was examined using punch in cutaneous samples taken from the volunteers in chapter 4.

Bioavailability of n-3 PUFAs

Fatty acid level in RBC membranes was shown to be a good indicator of dietary EPA intake and acted as a general marker of tissue incorporation. In accordance with previous studies (Sands et al. 2005), the % weight of EPA in RBC membranes at the start of the study was ~0.9% in both active and control groups. This remained unchanged in the control group, indicating that the placebo GTCC oil had no effect on tissue n-3 PUFA levels. In the active group on the other hand, the % weight of EPA significantly increased which indicated compliance and bioavailability of n-3 PUFA supplements and enabled the detection and exclusion of non-compliant subjects. As observed in previous studies (Rhodes et al. 2003) the given dose of 4gm EPA/day for 12 weeks was enough to produce significant EPA tissue incorporation in the active group and result in a significant difference in EPA levels between groups; thus potentially any observed differences in responses between study groups might be attributable to this. Furthermore, non-compliant subjects (n=3) were identified and excluded from data analysis using this method.

Effect of dietary EPA on clinical photoimmunosuppression

This is the first human study to examine the potential protective properties of dietary n-3 PUFAs against PI. As outlined in section 4.4.3, the hypothesis that n-3 PUFAs might be used to protect against UV-induced immunosuppression and promotion of skin cancer in humans is a possibility that needs to be further explored. At 4gm of EPA/day for 3 months, a tendency towards protection against PI of CHS was seen at the lower two UV-doses of 1.89J/cm^2 and 3.82J/cm^2. This photoprotection reached statistical significance at the middle UV-dose of 3.82J/cm^2 ($P<0.04$) (section 4.4.3). At 35% and 15% MED, these lower UV-doses more closely resemble average daily solar UV-exposure (Thieden et al. 2004)

compared to the highest dose of 70% MED, which may make them more applicable for the assessment of photoprotection. Moreover, over a person's lifetime this small effect could potentially lead to a significant reduction in the risk of developing skin cancer (Black and Rhodes 2006). Furthermore, compared to UV-irradiation with the solar simulator, daily UV-exposure is usually less intense and more spread throughout the day which could possibly allow for more n-3 PUFA photoprotection. It should also be noted when assessing protection from PI by n-3 PUFA that it is not envisaged as a replacement for the commonly used topical sunscreens, which are designed to protect primarily from sunburn, but can also exhibit protection from UV-immunosuppression, but rather as an adjuvant to provide more even and continuous skin protection.

Effects of Dietary EPA on UV-induced Langerhans Cell Depletion

All volunteers taking part in this study were confirmed to be allergic to Ni prior to study entry. Baseline LC counts in these volunteers were found to be similar to those of normal human subjects (Chen et al. 1985; Cruchley et al. 1994; Dearman et al. 2004) (section 5.1.5). As previously demonstrated in both animal and human models, cutaneous UV-irradiation led to a significant decrease in count and alteration in morphology of LCs (Murphy et al. 1993; Bestak and Halliday 1996; McLoone et al. 2005). The degree of LC depletion observed in both active and control groups of this study were comparable to that of other studies examining LC depletion in human skin using a UVB-irradiation dose of 4x MED: ~70% (Cooper et al. 1992).

Although subjects on dietary n-3 PUFA showed evidence of protection against PI of Ni CHS responses, a similar protective tendency could not be observed when examining the effects of n-3 PUFA on UV-induced LC depletion. This might be explained by several factors including the use of different UVR sources and different UV-doses when examining PI of Ni CHS and UV-induced LC depletion. For PI of Ni CHS, the solar simulator (96% UVA irradiance) was used to apply a maximum dose of 70% MED for 3 consecutive days whereas when examining protection by n-3 PUFA against LC depletion, a TL-12 lamp (56% UVB & 43% UVA) was used to irradiate the skin with a single dose of 4x MED. Similar to the maximum dose of SSR used in the clinical model of PI (70% MED for 3 consecutive days, which equates to a cumulative dose of x2.1 MED), this 4x MED dose of UVB could have exceeded the photoprotective abilities of n-3 PUFAs: in a previous study by Cooper et al. (Cooper et al. 1992), a single exposure to a UV-dose of 4x MED was found sufficient to induce systemic PI.

Moreover, the UV-induced % drop in LC count increased in both groups following 3 months of supplementation, reaching significance in the active group (section 5.4.1.4). This significant change might possibly be explained by an increase in lipid peroxidation, as has been reported in some though not all, previous studies examining this aspect

(Rhodes et al. 1994). This in turn could lead to oxidative DNA photodamage with resulting induction of LC migration (Kolgen et al. 2002). In addition, confounding factors such as seasonal changes could have affected the observed increase in UV-induced LC depletion seen in both groups.

The role of LCs in PI is controversial. While Wang et al. (2009) showed that UV-induced suppression of CD8+ T cell expansion and PI of CHS responses could be induced in the absence of LCs, Schwarz et al demonstrated that LC are essential for PI (Schwarz et al. 2010). It should also be noted that these studies examined APCs in the skin of mice and are probably not an ideal model for humans.

Effects of Dietary EPA on Cutaneous Immunomodulatory Cytokines

To examine biochemical mechanisms that might be involved in n-3 PUFA protection against PI, cytokine levels in suction blister fluid were examined (section 5.2). These included cytokines that are known to induce LC migration: IL-1β and TNF-α (Cumberbatch et al. 1997; Cumberbatch et al. 1999; Cumberbatch et al. 2003), the proinflammatory cytokine, IL-8 (Baggiolini and Clark-Lewis 1992) and the key immunosuppressive cytokine, IL-10 (Aubin 2003).

Similar to a previous study by Shahbakhti et al. (2004), 4gm EPA/day for 3 months did not have a significant effect on blister fluid levels of IL-8 and TNF-α post-UVB. In addition to the insignificant difference in TNF-α levels between the active and control groups, IL-1β could not be detected in the examined blister fluids (out of range, low). Since both IL-1β and TNF-α are key cytokines in the induction of migration of LCs, these findings are not surprising in the light of the insignificant difference seen between UV-induced LC depletion in the active and placebo groups (section 5.1.4.2).

IL-8 is a key proinflammatory cytokine that is implicated in UV-induced skin inflammation and the sunburn response (Baggiolini and Clark-Lewis 1992). EPA and DHA were shown to reduce UV-induced IL-8 secretion in cultured skin cells (Storey et al. 2005). In concordance with this, a reduction in the post-UV increase in IL-8 levels was seen in the active group of this study whereas the post-UV increase in IL-8 remained unchanged in the control group (section 5.2.4.2).

Strengths of the Study

This is the first time that the protective properties of n-3 PUFAs against PI have been explored in human subjects *in vivo.* Whereas cultured cells (Pupe et al. 2002) and mouse models (Moison and Beijersbergen Van Henegouwen 2001) have provided promising data, these systems are very different to the human and can produce quite different

results. Fortunately skin is more accessible to scientific study than many other human organs.

The study design was “gold-standard” in that it was double-blind, randomised and controlled; this involved considerably more work than an open study, but helped avoid spurious results, as the placebo group acted as a comparator for the actively treated group. Using only female subjects within a specified age group in this study meant the study population was kept relatively homogenous. In addition, an appropriate sample size was estimated for detection of statistically significant results.

The UV doses applied in the clinical assessment of PI resembled doses that a person could receive from casual sun exposure. Despite taking a longer time to apply these doses and being more cumbersome, the UVR source that most closely mimics natural sunlight was used, i.e. the solar simulator.

Both clinical and laboratory aspects of PI were explored, comprising UV-induced suppression of Ni CHS responses, LC depletion and cutaneous immunomodulatory cytokine levels. Furthermore, compliance was confirmed by measuring EPA % weight in RBC membranes before and after supplementation.

Dietary influence on results of the study were minimized by only recruiting volunteers who consumed a maximum of 2 oily fish meals/week and who were not taking n-3 PUFA supplements; they were instructed not to alter their diet during the study. The dose of EPA given to volunteers in the active group was realistic and could be consumed through dietary intake ± supplementation (100g portion of mackerel contains ~2.5g EPA).

To optimize the Ni CHS test system, input from experts in the field was obtained: A. Memon and P.S. Friedman were invited to our laboratory to discuss their Ni patch test protocol (Memon and Friedmann 1996) and the Damian and Halliday (2002) grading scale for contact dermatitis responses, was discussed with G.M. Halliday, who also provided extensive information regarding his laboratory’s human photoimmunosuppression protocols. The protocols of this study were subject to rigorous peer review by the AICR grant review board and their nominated external reviewers. Pilot studies were then performed before proceeding to the main study.

Weaknesses of Study

The Ni CHS test system used in this study was challenging to accurately perform, with the results being less reproducible compared, for example to the sunburn (UV-erythema) response. The Ni patches came off the skin prematurely on occasions, sometimes due to volunteers perspiring excessively after increased physical activity. To compensate for this, a large number of volunteers were used (n=79), and several control patches were used in

each volunteer. Despite the difficulty of setting up this test system, evidence of n-3 PUFA protection against PI was seen (section 4.4.3)

The effect of hormones on cell-mediated immunity is controversial and results from studies done to examine this are conflicting (Tamer et al. 2003; Bonamonte et al. 2005). Only a small number of the volunteers taking part in this study were on HRT/OCP (n=9: 7 active and 2 control). This potential weakness was accounted for by examining the results with and without subjects on hormonal medications: an insignificant difference was noted (section 4.4.3).

Even though the solar simulator is the UVR source most closely resembling natural sunlight, it is nonetheless more intense than average sun exposure and does not contain visible light which could possibly play a part in PI, although this is likely to be small.

After irradiance output inconsistencies were seen with the TL 12 MED tester which contained one tube, it was replaced with the Waldmann UV 800 unit fitted with ten TL-12 tubes. Thus although the spectral irradiance of the two sources was identical the irradiance of the Waldmann unit was considerably higher. To account for this increased in irradiance, exposure times were reduced accordingly.

Cytokine analyses were conducted using a fully-multiplexed assay system that involved mixing all cytokine bead sets, standards and detection Abs. While this enabled analysis of multiple cytokines simultaneously, it increased the risk of cross-reactivity or interference between assays. This is largely overcome by the use of a combination of antibodies against an individual target cytokine: the capture and detection Abs. Another drawback that may be encountered during multiplex analysis is the settling of beads while being run on the luminex analyzer. Mixing the samples on a plate shaker for 5 minutes before running on the luminex analyzer greatly reduces this. To enable cytokines in the suction blister samples to fall in the range of the human cytokine standards, samples are diluted before analysis. Since the cytokines to be analyzed are found in different concentrations, diluting the samples can result in one or more of the less abundant cytokines falling outside the range of it's standard curve. This was seen with 4 of the 7 cytokines analyzed in this study: IL-17, IL-1β and IFN-γ and IL-4 were all found to be out of range (low). This was unavoidable given the restricted sample size inherent in clinical studies.

Overall conclusions

As detailed in chapter 4, supplemental EPA was bioavailable and evidence of protection against clinical PI of Ni CHS was seen in the active group. However, as could be seen in chapter 5 this protection did not seem to be mediated by a reduction in UV-induced LC depletion: EPA protection against PI appeared to be acting via a LC independent manner.

In accordance with this, UV-induced changes in the levels of two cytokines that are involved in LC migration, IL-1β and TNF-α were insignificantly affected following 3 months of supplementation in both active and control groups. This novel study provides the first evidence that dietary n-3 PUFA may protect against clinical PI, and potentially skin cancer promotion, in humans. Further research is now warranted to confirm this finding and further explore potential underlying mechanisms for this observed photoprotection.

Future work

A different approach to the clinical assessment of PI can be employed in future studies, this time using the DTH system (which is induced by a different APC: DDC). The most commonly used DTH model is the Mantoux test, in patients previously inoculated with the BCG vaccine. This would allow for equal recruitment of male and female volunteers. Due to improved health care and social measures however, the incidence of TB has declined and the BCG vaccine is now only recommended for high risk individuals. This could make the recruitment of Mantoux-positive volunteers more difficult.

As seen with the highest dose of SSR (70% MED applied for 3 consecutive days to give an accumulative dose of x2.1 MED) during the evaluation of n-3 PUFA protection against PI of Ni CHS responses (chapter 4), the UVB-dose of 4x MED might be too intense for assessing the protective abilities of n-3 PUFA supplements against UV-induced LC depletion and secretion of immunomodulatory cytokines. For this reason, perhaps, in accordance with previous studies (Murphy et al. 1993; Barr et al. 1999; Novakovic et al. 2001), a lower UV-dose can be used for future work examining LC depletion and cytokine secretion post-UV. In addition to LC depletion, other cellular responses of PI can be examined in future work including post-UV induction of IL-10 secreting Tr1 cells (section 1.5.4.3).

Reported cytokine levels at different time points post-UV suggest that earlier aspiration and examination of suction blister fluids, before the 24hr time point might be worthwhile. Barr et al. (1999) noted a maximum increase in TNF-α, IL-1β and IL-1-α at 15hr post-UV; TNF-α rapidly declined beyond that time point. Teunissen et al. (2002) on the other hand found that IL-6, IL-8 and TNF-α levels reached a peak at 24hr post-UV and after which they began to decline. In addition to different time points, the use of the better validated, highly quantitative and reproducible cytokine measuring technique, ELISA might be employed (Leng et al. 2008).

In addition to cytokines, exploring eicosanoids, in particular the pivotal PI mediator, PGE_2 might aid in better understanding biochemical mechanisms underlying protection against PI. Further research is needed to confirm this finding, and to examine the underlying mechanisms, which could involve other immunoregulatory cells of the skin, such as

dermal dendritic cells and T regulatory cells and other mediators of UV-immunosuppression including the prostanoids, which may be modified by n-3 PUFA

Potential Clinical Application

As previously outlined, UVR promotes the development of skin cancer via PI (Kripke et al. 1996; Berneburg and Krutmann 2000; Murphy 2009). Several murine studies support that PI is one of the key factors in the promotion of skin cancer (Walker and Young 2007). In addition, protection against PI of CHS in animal models was found to protect against photocarcinogenesis (Sime and Reeve 2004; Black and Rhodes 2006). In humans, an individual with past medical history of skin cancer was found to be more susceptible to PI (Yoshikawa et al. 1990). In addition, SCC in patients on immunosuppressive medications was found to be more aggressive (Damian et al. 2001) and immunosuppressed transplant patients show increased skin cancer incidence (Oberyszyn 2008; Murphy 2009).

While these animal and human studies give support rather than provide proof, protection against PI could theoretically protect against skin cancer promotion in humans. Further studies are required, but this first evaluation of the impact of dietary n-3 PUFAs on photoimmunosuppression in humans supports that this approach may provide an adjuvant to physical measures in protecting people from skin cancer.

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