Assessing the influence of solar ultraviolet radiation exposure on the primary immune response to immunisation with a protein antigen in humans

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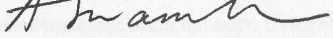
Declaration

"This thesis represents original work. No part of the work presented has previously been submitted for the purpose of obtaining another academic degree or qualification at any institution. Contributions made by others to this research have been explicitly acknowledged in the body of the work."

Signed: 

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Abstract

Ultraviolet radiation (UVR) is immunosuppressive, particularly to antigen-specific cell-mediated processes, acting via direct and indirect (e.g. vitamin D-mediated) pathways. This research aimed to examine the influence of acute and cumulative solar UVR exposure, at doses relevant to day-to-day activities, on the primary immune response to immunisation in humans.

The Australian Ultraviolet Radiation and Immunity (AusUVI) Study was a prospective, longitudinal, twin-centre immunotoxicological study. Healthy adults were immunised subcutaneously with the T-cell dependent antigen, keyhole limpet haemocyanin (KLH). Acute personal UVR exposure was measured by electronic UVR dosimeter worn on the wrist for ten days centred on the day of immunisation; and by sun diary. Cumulative UVR exposure was quantified by microtopographic analysis of silicone impressions of sun exposed skin. Variables that might confound the association between UVR and vaccine immune response were measured, including serum vitamin D (25(OH)D) level. Participants attended for five study visits over a period of 31 days, with recruitment spread over one year. Immune function outcomes were: anti-KLH IgG1 antibody levels measured by enzyme linked immunoassay; delayed-type hypersensitivity (DTH) response to KLH antigen (reflecting T helper cell-1 (Th1) processes); and quantification of T-helper cell subsets by flow cytometry-based methods. A pilot study trialled many components of the AusUVI Study protocol and immune assays.

The AusUVI Study was conducted in the Australian cities of Canberra (35°2'S) and Townsville (19°1'S) from July 2010 to August 2011. Two hundred and twenty two healthy participants were recruited (Canberra: 110; Townsville: 112). Participants' average age was 27.9 years (range: 18 – 40 years) and 63.5% were female. Participants with both parents of northern European ancestry (70.0%) predominated. 25(OH)D levels and personal UVR exposure varied by season and by site of enrolment. Townsville participants had higher 10-day clothing-adjusted UVR exposure compared with Canberra participants (2.5 *vs.*1.8 standard erythemal dose (SED); p=0.003). Higher cumulative UVR exposure was strongly associated with age, male sex, Townsville residence and northern European ancestry.

In multiple linear regression models, anti-KLH IgG1 response at day 21 post-immunisation was associated with age (antibody titre reduced by 1.6% per year of age; p=0.001) and sex (14.6% higher titre in females compared with males (p=0.004)). No association between KLH IgG1 response and acute or cumulative UVR exposure, or serum 25(OH)D levels was demonstrated.

Reduced DTH response to KLH recall challenge at day 21 post-immunisation was associated with higher acute UVR exposure on the day prior ('Day 5') to immunisation (p=0.015), and Days

5-8 and 5-9 (p=0.039 and p=0.025, respectively) that spanned the pre- and post-immunisation period. No association with cumulative UVR or serum 25(OH)D levels was demonstrated.

Change in T-helper 17 (Th17) cell percentage between pre- and post-vaccination time points differed in direction when comparing the low and high UVR exposure groups (-0.39% *vs.* 0.31%; p=0.004).

In conclusion, acute personal solar UVR exposure, at doses relevant to day-to-day activities, modulated the primary cell-mediated immune responses to KLH immunisation. Cumulative UVR exposure and serum 25(OH)D levels were not associated with immune function outcomes.

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Abbreviations

$1,25(OH)_2D$	1,25 hydroxyvitamin D or "calcitriol"
25(OH)D	25-hydroxyvitamin D
ANU	Australian National University
AusUVI	Australian Ultraviolet Radiation and Immunity Study
CHS	Contact hypersensitivity
CIE	International Commission on Illumination
DTH	Delayed-type hypersensitivity response
HREC	Human Research Ethics Committee
IFN	Interferon (cytokine)
IL	Interleukin (cytokine)
JCU	James Cook University
KLH	Keyhole limpet haemocyanin
MED	Minimal erythemal dose
NCEPH	National Centre for Epidemiology and Population Health
NH&MRC	National (Australian) Health and Medical Research Council
NIWA	National Institute for Water and Atmospheric Research (New Zealand)
PBMC	Peripheral blood mononuclear cell
SED	Standard erythemal dose
SMB	Safety Monitoring Board
SPF	Sun protection factor (used to mark sunscreens protection capacity)
SS-UVR	Simulated solar-UVR
TCH	The Canberra Hospital
TD	T-cell dependent (antigen)
TDAR	T-cell dependent antibody response
TGA	Therapeutics Goods Administration (of Australia)
Th1	T - helper cell - 1
Th17	T - helper cell - 17
Th2	T - helper cell - 2
TNF	Tumour necrosis factor
Treg	Regulatory T cell
UVI	Ultraviolet index
UVA / B / C	Sub-classification of UVR
UVR	Ultraviolet radiation

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Introduction

Solar ultraviolet radiation (UVR) is an important environmental exposure for terrestrial and aquatic life (1). UVR exposure is the major contributor to stores of vitamin D in humans; the active form of vitamin D is required for calcium metabolism, optimal musculoskeletal health and other critical physiological processes (2). Vitamin D deficiency has been associated with numerous disease states, including osteomalacia, autoimmune diseases and solid-organ cancers (3). Conversely, high UVR exposure leads to sunburn and is the strongest risk factor for several skin cancers and eye diseases (4). These adverse health effects are likely related, at least in part, to the immunomodulatory effects of UVR exposure.

What is known about photoimmunology?

The study of the effects of non-ionising radiation on the immune system is known as 'photoimmunology' (5) – in this thesis the term will be restricted to the effects of UVR (wavelengths 100nm – 400nm). The origins of this field can be traced to a series of seminal experiments conducted by Professor Margaret Kripke and colleagues in the 1970s relating to the control of skin tumours in mice. They elegantly demonstrated that irradiation with UVR induced an antigen-specific, cell-mediated and systemic immunosuppressive effect (6). Since that time, UVR-induced immunomodulatory effects have been demonstrated using different antigens and in numerous contexts, including contact hypersensitivity and delayed-type hypersensitivity responses, infection and vaccination (7,8).

Absorption of UVR by specific elements within the epidermis of the skin sets in train a complex cascade of processes that ultimately leads to both local and systemic immunosuppression. These steps include release of numerous chemical mediators (e.g. cytokines) and migration of immune cells (e.g. Langerhans cells) that lead to an immuno-tolerant environment within the draining lymph node (9). Regulatory T-cell lymphocytes have been shown to play an important mediatory role in this setting (10). Calcitriol - the biologically active form of vitamin D – has also been shown to influence both innate and adaptive immune processes and constitutes another (indirect) pathway for UVR-induced immunomodulation (11). The overall effects of both UVR exposure and calcitriol on adaptive immunity are to suppress antigen-specific cell-mediated ("Th1") immune processes and promote a regulatory immune environment (12).

Although the potential adverse clinical consequences for UVR-induced immunosuppression are obvious, namely increased risk of infection and cancer, the postulated benefits include reduced cutaneous reactivity (i.e. dermatitis) to non-pathogenic commensal organisms, increased time for cellular DNA repair and reduced rates of autoimmune disease (13,14).

These advantages constitute at least some of the evolutionary rationale for the human immune system's complex adaptations to a ubiquitous environmental exposure.

Limitations in current models used in photoimmunology

Much of what is currently known about photoimmunology has resulted from studies that have simulated conditions that are not relevant to typical UVR exposures experienced by humans. Many studies have used animal models of the immune system where UVR exposure has been derived from artificial sources, at non-solar spectra and/or delivered at supra-normal doses (8). Studies involving humans have often suffered from similar limitations, but additionally have been restricted by small numbers and/or non-consideration of other potential immunomodulatory factors (e.g. vitamin D status) (15,16). Many ecological studies have not directly measured UVR exposure at an individual level but instead used surrogates of UVR exposure such as latitude of residence or season (17,18), which can potentially lead to misleading inferences.

There has been very limited research assessing whether UVR exposure adversely affects the immune response to vaccination, despite this having important public health implications. In humans, there has been only one clinical trial involving vaccination that has taken into account UVR exposure at an individual level (19). Although well designed and assessing a range of immune outcomes, methodological limitations of this study that related to the vaccine used in particular narrowed the conclusions that could be drawn (7).

Immunotoxicology can inform human photoimmunological research

Immunotoxicology relates to the study of adverse influences of putative toxins on immune function and the resultant risks posed to health (20). The principles and practices underpinning this relatively nascent research field lend themselves well to better understanding the effects of UVR exposure on human immune function.

Immunotoxicological literature and guidelines emphasise the accurate quantification of both toxin exposure (at an individual level) and immune outcome (using a combination of immune tests), with measurement of potential confounding factors (21,22). Immunisation with a T-cell dependent antigen has been promoted as a safe, reliable tool for characterising and quantifying alterations in the human immune response to putative toxins (23). Processing of a novel vaccine antigen allows for primary cell-mediated and humoral responses to be generated, and, importantly, quantified using *ex vivo* and *in vivo* assays. Recent advances in UVR measurement with electronic dosimeters, in conjunction with diary data, enables the accurate quantification of personal UVR exposure (24,25).

Aims of this doctoral research

The aim of the doctoral research was to examine the effect of naturally-acquired solar UVR exposure, at doses relevant to day-to-day activity, on the primary immune response to immunisation in humans. The research relied heavily on the principles and methods espoused by the fields of immunotoxicology and clinical epidemiology, to robustly examine the association between UVR exposure and immune responses under 'real life' conditions. A major emphasis of this work was to measure and adjust for other potential immunomodulatory factors, a significant deficiency in previous studies. Additionally, the role of vitamin D status on immune response and the possible differential effects of acute versus cumulative UVR exposure were examined.

Research Hypotheses

Based on the current literature, the following hypotheses were generated:

- Higher personal acute UVR exposure is associated with impaired cell-mediated and/or humoral immune response to primary immunisation.
- Higher cumulative personal UVR exposure does not lead to photoadaptation to the immunomodulatory effects of UVR
- Vitamin D status modulates the antigen-specific response to immunisation

Specific research questions:

In the context of primary immunisation with a T-cell dependent antigen:

- Does acute personal UVR dose at the time of immunisation affect subsequent cell-mediated and / or humoral vaccine response?
- Does cumulative (long-term) personal UVR dose modulate the effect of acute personal UVR exposure on cell-mediated and / or humoral vaccine response?
- Does season or latitude of residence at the time of immunisation affect subsequent cell-mediated and / or humoral vaccine response?
- Does vitamin D status affect subsequent cell-mediated and / or humoral vaccine response?

Outline of thesis

A brief outline of the eight chapters contained within this thesis is provided here.

Chapter 1 introduces UVR concepts and terminology as they relate to terrestrial exposure, biologically relevant doses and effects on human health. The synthesis, physiological actions and the health effects of vitamin D are also discussed.

Chapter 2 reviews the current literature on photoimmunology, with particular emphasis on postulated mechanisms of immunomodulation and highlighting host resistance and clinical studies. Evidence supporting vitamin D-associated immunomodulation is also reviewed.

Chapter 3 outlines the field of immunotoxicology and discusses how it can inform an assessment of the effect of solar UVR exposure on human immune response. Important clinical epidemiology elements of conducting an immunotoxicological study are discussed.

Chapter 4 describes the choice of an appropriate T-cell dependent vaccine antigen for use in an immunotoxicology study, the development of immunisation and immune assay protocols, and the results of a pilot study.

Chapter 5 introduces the Australian Ultraviolet Radiation and Immunity (AusUVI) Study, a prospective, longitudinal, twin-city study that constitutes the main research vehicle for this thesis. This chapter discusses recruitment strategies, tools for measuring personal UVR exposure and other potential immunomodulatory variables, statistical considerations and study protocol.

Chapter 6 describes the results from the AusUVI Study including demographic details, participant characteristics, examination findings, vitamin D status and personal UVR exposure.

Chapter 7 describes the outcomes of immune function testing and their associations with personal UVR exposure and potential confounding variables. Multiple linear regression models examine their overall associations.

Chapter 8 discusses the findings of the AusUVI Study and their significance in the context of the broader literature. Limitations of the study and potential new avenues for investigation are canvassed.

Relevance of this research

It is hoped that a robustly designed and rigorously conducted epidemiological immunotoxicology study using a reliable vaccine antigen, comprehensive immune testing strategy and innovative UVR exposure monitoring can provide an original and valuable contribution to the field of photoimmunology. Conducting this study in a high ambient solar UVR environment such as Australia under real life conditions will additionally inform our understanding of appropriate sun exposure in this setting. This may have added relevance in the future if, under global change (especially climate change) conditions, the levels and patterns of solar UVR exposure change within and between populations.

Chapter 1: Ultraviolet radiation

Synopsis

This chapter reviews the literature on solar ultraviolet radiation (UVR) with particular attention given to its relevance to human health. Vitamin D, a steroid pro-hormone that relies on UVR radiation for synthesis and whose active metabolite has numerous critical physiological functions in humans, will also be discussed.

1.1 Ultraviolet Radiation

1.1.1 Classification

Ultraviolet radiation (UVR) constitutes a small component of electromagnetic radiation beyond the visible spectrum, with a wavelength spectrum ranging from 100 to 400 nanometers (nm) (26). In 1932, the Second International Congress on Light proposed a further sub-classification of UVR in recognition of varying biological effects observed at different spectral regions (27). These sub-divisions were: UVA (400-315nm), UVB (315-280nm) and UVC (280 – 100nm). Photobiologists have further refined these spectral definitions to better match photobiological phenomena: UVA (400 – 320nm), UVB (320 – 290nm) and UVC (290 – 200nm) (28). A wavelength of 290nm corresponds to the shortest wavelength of UVR present in terrestrial sunlight, whilst the UVA/UVB cut-off is more arbitrary. UVA is further divided into UVAI (340-400nm) and UVAII (320-340nm) in recognition of the greater photobiological activity of the shorter wavelengths (28). Table 1.1 describes the properties of the various UVR sub-types.

1.1.2 Factors influencing terrestrial UVR levels

The spectrum and intensity of ambient UVR that reaches Earth's surface ("terrestrial UVR") is dependent on the "solar altitude", defined as the elevation of the sun above the horizon. Solar altitude varies with latitude, season and time of day. In addition, differential absorption of UVR by the atmosphere also affects the spectral composition of terrestrial UVR (28).

Table 1.1 UVR subtypes and their properties

UVR sub-type	General Properties	Biological Properties
UVA (320-400nm)	• Not filtered by atmospheric ozone • Constitutes ~95% of terrestrial UVR • Passes through most glass	• Passes deeper into skin (dermis) • Contributes to photo-ageing and tanning • Immunosuppressive • Possible role in carcinogenesis
UVB (290 – 320nm)	• Extensively filtered by atmospheric ozone • Constitutes ~ 5% of terrestrial UVR • Does not pass through glass	• Superficial penetration (epidermis) • Catalyses synthesis of vitamin D pre-cursors • Causes sunburn, tanning, photo-ageing, immunosuppression • Carcinogenic – induces skin cancer
UVC (200 – 290nm)	• Completely filtered by atmospheric ozone and oxygen • Artificial sources only	• Carcinogenic • Causes sun burn

Adapted from: (26,29)

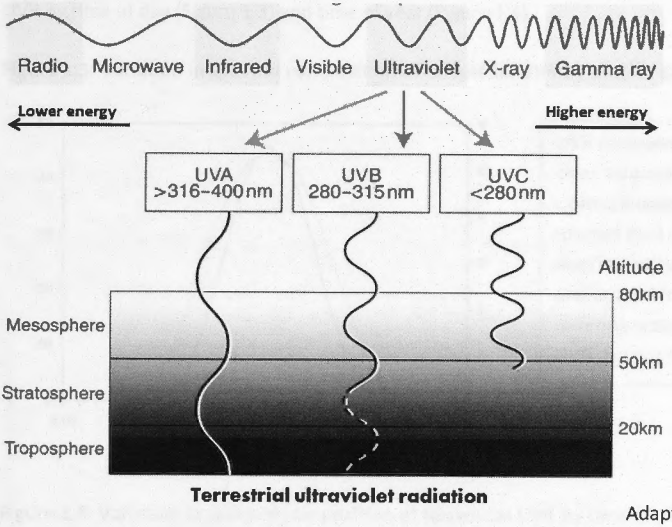
Atmospheric influences

Ozone (O_3), O_2 and N_2 molecules within the stratosphere (~10 – 50 km above sea level) absorb all UVC and the vast majority of UVB. In the troposphere (0 – 10 km above sea level), further absorption and / or scattering by pollutants such as ozone, NO_2, SO_2, soot and clouds occurs (30,31). Terrestrial UVR therefore is comprised mainly of UVA (~95%) with UVB making up the remainder, with some modest variation in proportion dependent on solar altitude (See Fig 1.1) (28).

Solar altitude and zenith angle

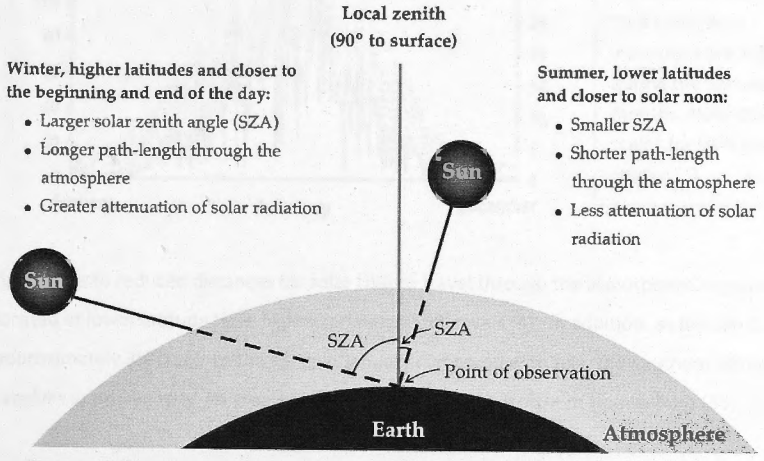
A measure of solar altitude is the 'solar zenith angle' (SZA). The SZA is the angle between the local vertical and the instantaneous position of the sun (32). A SZA of 0° indicates that the sun is directly overhead and represents the shortest travel distance for UVR to Earth's surface (See Figure 1.2). The likelihood of low SZA increases at low latitude, in summer months and the four hours centred around local or 'solar' noon (when 50-60% of summer UVR is received), and therefore terrestrial UVR is at its most intense under these conditions (28,30).

Figure 1.1 Electromagnetic spectrum and the filtering of ultraviolet radiation by Earth's atmosphere



Adapted from: (33)

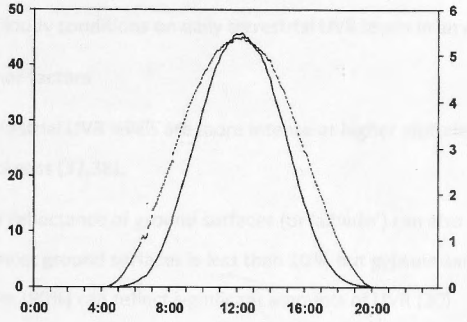
Figure 1.2: Solar zenith angle determines the length of the path ultraviolet radiation travels to the point of observation



Source: (34)

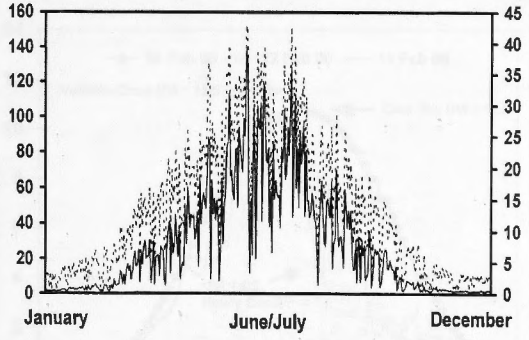
The spectral composition of terrestrial UVR also varies with solar zenith angle (and therefore with latitude, season and time of day). Terrestrial UVR has a higher proportion of UVB at smaller SZA since there is less atmospheric filtering of these shorter UVR wavelengths (28). Figures 1.3 and 1.4 illustrate the influence of SZA on the spectral composition of terrestrial UVR by time of day (Figure 1.3) and time of year (Figure 1.4).

Figure 1.3: Variation in spectral composition of terrestrial UVR by time of day.



UVR measurements on a clear summer day in the United Kingdom. UVA (dotted line) and UVB (solid line) intensities are higher at and around local noon. Note differing scales for UVA and UVB. Source (28)

Figure 1.4: Variation in spectral composition of terrestrial UVR by time of year



UVR measurements for Durham, UK (latitude 55°N) during 1994. UVA (dotted line) and UVB (solid line) intensities are higher during the summer months. Note differing scales for UVA and UVB. Source (3)

Again, due to reduced distances for solar UVR to travel through the atmosphere, regions located at lower latitude have higher terrestrial UVR levels (4). In addition, as the sun is approximately 3% closer to the earth in January compared with July, the Southern Hemisphere receives approximately 7% greater UVR intensity than the Northern Hemisphere (35).

Cloud cover

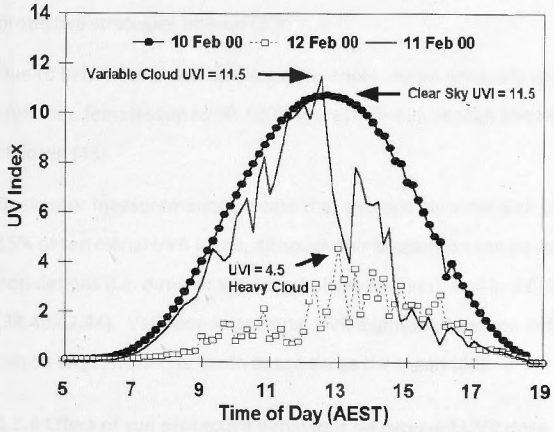
Clouds, which are composed of ice crystals or water, tend to scatter rather than absorb UVR and constitute the most important factor affecting UVR for a given SZA (36). Even in cool and cloudy summer conditions, there can be significant terrestrial levels of UVR as clouds are more efficient at absorbing infra-red radiation (heat) than absorbing UVR. As an approximation, cloud cover reduces annual terrestrial levels of UVR to two thirds of estimated clear sky levels in temperate latitudes, and 75% in tropical latitudes (30). Figure 1.5 demonstrates the effect of cloudy conditions on daily terrestrial UVR levels in an Australian temperate city (35).

Other factors

Terrestrial UVR levels are more intense at higher altitudes due to decreased atmospheric thickness (37,38).

The reflectance of ground surfaces (or 'albedo') can also affect terrestrial UVR. The reflectance of most ground surfaces is less than 10%, but gypsum sand (15-30%), choppy water (20%) and snow (90%) can reflect significant amounts of UVR (30).

Figure 1.5: Effect of clouds on terrestrial UVR



The variation of solar UVR measured in Melbourne, Australia (latitude 38°S) for three consecutive days in February 2000, showing the effects of cloud cover on the measured terrestrial UVR levels.
Dotted line: clear sky
Solid line: variable clouds
Broken line: Heavy clouds
Source: (35)

1.1.3 Personal UVR exposure and dose

For the purposes of this thesis, the following terms will be used to describe UVR exposure:

- **Terrestrial (or ambient) UVR** – The intensity of UVR reaching Earth's surface which is related to solar zenith angle, altitude and cloud cover.
- **Personal UVR exposure** – A function of the terrestrial (or artificial) UVR and the duration of exposure, e.g. time outdoors.
- **Personal UVR dose** – The dose of UVR a person receives which depends on personal UVR exposure and the area of skin exposed (which differs by clothing and sun protective strategies used, e.g. sunscreen, sunglasses).

Terrestrial UVR is measured as part of atmospheric monitoring in many locations around the world and in Australia using ground-based spectroradiometers (35). In addition, satellite data on atmospheric ozone is used to calculate clear-sky and cloud-corrected terrestrial UVR globally (NASA). Numerous studies have quantified personal UVR exposure using personal sun exposure diaries and/or dosimeters (e.g. polysulphone, digital) (24). Personal UVR dose relies on measurement of personal UVR exposure coupled with data on the nature of outdoor activity (e.g. use of shade), the coverage and type of sun protective clothing worn, and sun protective strategies utilised (30).

Due to behavioural and clothing differences, males generally receive higher personal doses of UVR than females (up to 50-100% more) (40–42), though this difference is less pronounced in children (43).

Dosimeter measurements indicate that average personal UVR exposure is approximately 3-15% of terrestrial UVR levels, although this proportion can be markedly higher in certain sub-populations (i.e. outdoor workers, holiday-makers), and in different seasons and regions (38,40,42,44). Variation in personal UVR exposure between individuals within a population can be large, from one-tenth to ten times the mean (40).

1.1.4 Effect of sun protective behaviour on received UVR dose

The Sun Protection Factor (SPF) is a measure of sunscreen efficacy. This is a laboratory derived ratio describing the relative protection from UVR-induced erythema provided by sunscreen application to the wearer above that offered by natural pigmentation (45). For example, an SPF rating of 15 indicates that sunscreen-applied skin could be exposed to 15 times the dose of UVR compared with no sunscreen, before erythema becomes evident. Indeed, an SPF 15 sunscreen would theoretically offer all day protection even under tropical sun conditions on

single application (46). However, SPF ratings are based on laboratory testing where sunscreen is applied at a concentration of 2 mg/cm^2. Application of 1mg/cm^2 or 0.5mg/cm^2 gives an exponentially lower level of protection (square or fourth-root, respectively) compared with baseline (47). Indeed, studies show that the actual amount of sunscreen applied varies from 0 to 1.2 mg/cm^2 after one application (48). In real-life situations therefore, a useful 'rule-of-thumb' is that the 'expected SPF' of applied sunscreen is typically one-third of 'laboratory tested SPF' (30). In addition, UVR protection from sunscreen is compromised by haphazard application and removal by water immersion, abrasion or towelling (30,46,49). Regular re-application of sunscreen or following water immersion or towelling is advised to compensate for initial under-application and / or to replace sunscreen removed with activity (30).

Sunscreen was initially developed to provide protection against erythema inducing UVB radiation, although broad spectrum (UVA and UVB) sunscreens are now common (50). A limitation of the traditional SPF rating is that it gives only an indication of the relative protection against a single erythema-inducing UVR dose, and as such, the degree of protection against sub-erythemal UVR doses or UVA exposure is not afforded by this measure (48). Recently however, standardised methods for also assessing UVA protection have been developed (51). In the United States, regulations are being introduced to ensure that there is comparable UVA and UVB protection offered by broad-spectrum sunscreen (52).

The regular use of sunscreen can reduce the incidence of several UVR-related diseases, including actinic keratosis and squamous cell carcinoma in organ transplant recipients (48). There are conflicting reports of a protective effect of sunscreen on the incidence of melanoma (53–55). Currently there is no evidence of a sunscreen protective effect against basal cell carcinoma, although this is based on data from a single study (56). Vitamin D deficiency does not appear to be more prevalent amongst those who regularly use sunscreen (57,58).

Clothing and hats

Clothing offers UVR protection, however the protective effectiveness is dependent on fabric weave, colour, weight, stretch and wetness (30). Some textiles have a greater 'ultraviolet protective factor' (UPF; analogous to SPF) than others – for example, polyester blends offer more protection than pure cotton, linen, acetate or rayon fabrics (59).

Driscoll et al found that 80% of summer clothing had a UPF of greater than 15, whilst another researcher reported one third of commercial summer clothing to have a UPF < 15 (60,61). The differences are likely to be explained by differing measurement methodologies (62). Clearly the skin coverage of clothing will also affect the received personal UVR dose, with long sleeve length attire offering greater protection than shorter length attire for the same fabric. Hats

can offer variable protection depending on type – legionnaire's hats (with coverage of the neck and ears) provide the best overall protection, whilst broad brimmed hats and baseball caps offer less (30,63).

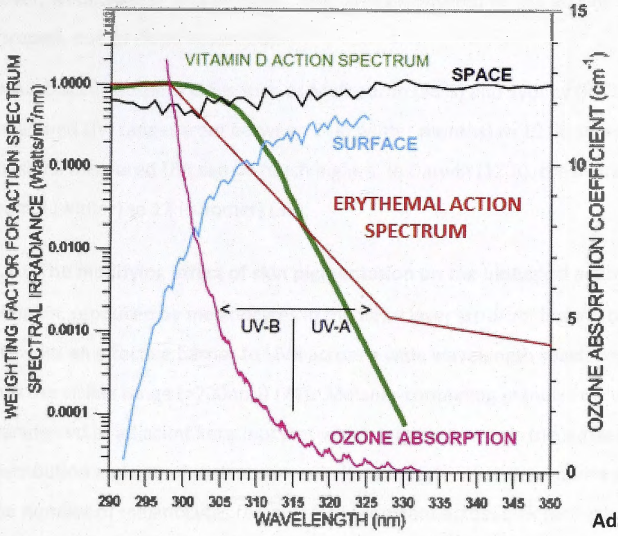
1.1.5 Biologically-relevant UVR spectra

UVR induces different biological effects at different wavelengths (38). In general, higher energy wavelengths of UVR have greater biological activity (i.e. UVB > UVA); however the penetration of UVR into the skin is greater at longer wavelengths (i.e. UVA > UVB) (26,64). An "action spectrum" describes the relative effectiveness of energy at different wavelengths in producing a specific biological response (65). Action spectra have been described for skin erythema (sun burn), squamous cell skin cancer, immunosuppression and vitamin D synthesis (66,67).

Erythema action spectrum

The McKinlay-Diffey erythemal action spectrum has been endorsed as the International standard (with minor modifications) and represents the erythema-inducing effectiveness of UVR at various wavelengths (68). This action spectrum is graphically represented as three linear lines (see Figure 1.6, lines in red) on a semi-log plot which was derived from mathematical equations to fit experimental data (69). This action spectrum is the basis for commonly used measures of erythemally-weighted energy in photobiology – minimal erythemal dose, standard erythemal dose and ultraviolet index; and against which default response spectra for UVR monitors are compared (68).

Figure 1.6: Action spectra for various UVR-associated biological responses



Adapted from (42)

Minimal Erythemal Dose

Minimal erythemal dose (MED) is the erythemally effective UVR sufficient to cause faint erythema at 24-hours post irradiation (70). As the development of skin erythema depends on numerous factors including skin pigmentation, anatomical site and previous UVR exposure, the MED can vary significantly between individuals (even of the same complexion) (71).

Standard Erythemal Dose

The standard erythemal dose (SED) was proposed in an effort to standardise reporting of erythemally effective UVR dosing (B. L. Diffey et al, 1997). One SED is equivalent to an erythemal effective radiant exposure of 100 J/m^2 and is independent of skin pigmentation. Therefore, for fair skin (skin phototypes I to IV (see Section 1.1.6), 1 MED is equivalent to between 1.5 – 6 SED (71).

UV Index

The UV Index (UVI) was developed to describe the intensity of terrestrial UVR (at erythemally effective wavelengths) in a manner that could be easily understood by the public (72). The forecast UVI is calculated by multiplying the biologically effective solar UVR (UVR_{eff}) by 40 (73). UVR_{eff} (Watts/m^2) is calculated by spectral-weighting of incident solar UVR with the erythemal response of human skin (based on the erythemal action spectrum) across the wavelengths 280 -400nm and summating the effects (35). The UVI, when used as a forecast, is calculated using

computer models which input various atmospheric parameters such as ozone, potential cloud cover, water vapour and aerosols. The UVI as measured on the ground is often less than that forecast, due to cloud cover (26).

In Australian southern cities such as Melbourne (38°S) and Sydney (33°S), the average monthly measured UVI ranges from between 2 (in winter months) to 10 (in summer), although the maximal measured UVI can be much higher. In Darwin (12°S), the average monthly UVI ranges from 8 (winter) to 12 (summer) (35).

1.1.6 The modifying effect of skin pigmentation on the biological action of UVR exposure

Melanin, produced by melanocytes in the basal layer (*stratum basale*) of the epidermis, provides an effective barrier to UVR across a wide wavelength spectrum (UVC (200-280nm) into the visible range (>700nm)) (74). Melanin-containing granules or 'melanosomes' are transferred to adjacent keratinocytes which migrate towards the surface. It is the density, distribution and size of melanosomes in keratinocytes that determines skin pigmentation, as the number of melanocytes remains fairly constant across different ethnic populations (75).

Constitutive skin pigmentation is controlled by a number of genes (e.g. human melanocortin-1-receptor (*MC1R*) gene, melanocyte differentiation antigen (*MATP*), *HERC2* gene) (76).

Tanning (or induced pigmentation) is the process of skin darkening resulting from UVR exposure, and is also influenced by genetic make-up (See also Fitzpatrick skin phototypes below) (77). Although both UVB and UVA lead to tanning, they induce different melanin responses characterised by differing time kinetics (78). Immediate pigment darkening (IPD) following sun exposure is due to UVA-induced oxidation of pre-existing melanin and melanin precursors (79). IPD typically lasts for 10-20 minutes but higher UVA doses (>10 J/cm^2) can lead to 'persistent pigment darkening' (PPD) which can remain for some days. Importantly, IPD and PPD are not protective against subsequent UVB-induced erythema (79). Delayed tanning (DT) is visible 72 hours post-UVR exposure, is responsive to wavelengths similar to the erythema action spectrum (i.e. UVB > UVA) and, in fair skinned individuals, follows erythema (78–80). UVR-induced DNA damage is thought to activate key enzymes required for melanocyte growth and proliferation, and increased transfer of melanosomes to keratinocytes (75,79).

The Fitzpatrick Skin Phototype scale is a practical and commonly used tool to classify skin types according to pigmentation and skin response to solar or artificial UVR exposure (see Table 1.2) (81,82). Individuals with low phototype classifications (i.e. fair complexions) are at increased risk of melanoma and non-melanoma skin cancers. Phototypes are also useful for estimating doses for UVR, PUVA treatment (for psoriasis) and laser therapy (83).

Table 1.2: Fitzpatrick's Skin Phototypes

Phototype	Sunburn & Tanning history (defines the phototype)	Immediate pigment darkening	Delayed tanning	Constitutive color (unexposed buttock skin)	UV-A MED (mJ/cm^2)	UV-B MED (mJ/cm^2)
I	Burns easily, never tans	None (-)	None (-)	Ivory White	20–35	15–30
II	Burns easily, tans minimally with difficulty	Weak ± (± to +)	Minimal to weak (± to +)	White	30–45	25–40
III	Burns moderately, tans moderately and uniformly	Definite +	Low +	White	40–55	30–50
IV	Burns minimally, tans moderately and easily	Moderate + +	Moderate + +	Beige-Olive, Lightly tanned	50–80	40–60
V	Rarely burns, tans profusely	Intense (brown) + + +	Strong, intense brown + + +	Moderate brown or tanned	70–100	60–90
VI	Never burns, tans profusely	Intense (dark brown) + + +	Strong, intense brown + + +	Dark brown or black	100	90–150

Source (83)

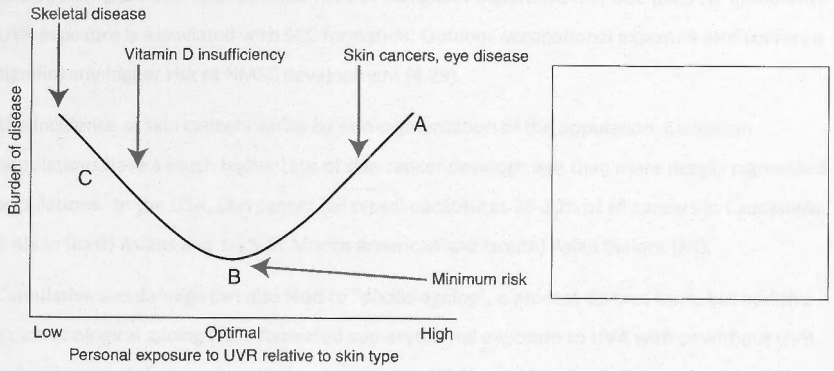
Increased skin pigmentation confers better erythema protection from UVR – an individual with high melanin density (e.g. Fitzpatrick phototype VI) has an MED 6 to 33-fold higher than a fair-skinned counterpart and corresponding inherent sun protection factor of up to 13.4 (64,84). Epidermal melanin in black skin filters 93% of UVB and 83% of UVA compared with 76% and 45% for Caucasian skin (84). Intermediate phototypes have step-wise lower levels of protection.

Section 1.2.4 discusses the relationship between vitamin D synthesis and skin pigmentation.

1.1.7 Health effects of UVR exposure

UVR exposure is associated with both beneficial and adverse health outcomes (extensively reviewed in (4)). UVR is required for the synthesis of vitamin D which influences a wide range of essential physiological functions (discussed in Section 1.2.6). Artificial sources of UVR (e.g. sun lamps) are used as effective therapeutic measures for certain inflammatory dermatological conditions including psoriasis, lupus vulgaris and dermatitis (85,86). Either via a direct effect of UVR, or indirectly via vitamin D synthesis, a number of cancers (e.g. bowel, breast, prostate), infectious diseases (e.g. tuberculosis), metabolic, cardiovascular and psychiatric disorders have been associated with low UVR exposure (3,4). However, the majority of direct health effects from cumulative UVR exposure are negative and primarily affect the skin and the eyes (87). An apparent 'U-shaped' relationship exists between burden of disease and UVR exposure, with both low and high exposures leading to increased risk of illness (see Figure 1.7) (33).

Figure 1.7 Relationship between ultraviolet radiation exposure and burden of disease



Source: (33)

Adverse effects of UVR on human health

UVR and skin disease

The potential adverse effects of UVR exposure on human health are most commonly and obviously seen in the skin. UVR penetrates approximately 0.1 to 1mm into human skin, with UVA radiation penetrating further than UVB radiation (26,88).

Acute UVR exposure can cause erythema leading to painful sunburn and peeling, and blistering in the most severe manifestation (79). Recurrent acute UVR exposure leads to damage to DNA, decreased DNA repair and apoptosis of damaged cells (via mutations to tumour suppression genes (e.g. p53)), oxidative cell stress, local immunosuppression (including of immune surveillance cells) and pro-inflammatory responses. All of these mechanisms contribute to skin cancer development and photo-ageing (29,89).

There is convincing biological and epidemiological evidence linking UVR exposure and the development of malignant melanoma and non-melanoma skin cancer (NMSC). Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) are the most common forms of NMSC, and indeed are the most common form of cancer amongst Caucasians in the western world (29). A recent estimate using Australian population data, showed that around 434,000 persons (253,000 males, 180,000 females) would be diagnosed with one or more NMSC in the year 2008 alone (90). In 2006, 410 Australians died from NMSC (Australian Bureau of Statistics, 2008).The ratio of BCC to SCC is approximately 4:1, although the risk of metastases and mortality is >10-fold greater with SCC. Melanoma causes 75% of skin cancer-related mortality in the USA despite constituting only ~3% of all skin cancers (29).

Studies have linked intermittent and severe UVR exposure (i.e. painful sunburn) in childhood and adolescence with later development of malignant melanoma and BCC (84,91). Cumulative UVR exposure is associated with SCC formation. Outdoor occupational exposure also confers a significantly higher risk of NMSC development (4,29).

The incidence of skin cancers varies by skin pigmentation of the population. Caucasian populations have a much higher rate of skin cancer development than more deeply pigmented populations. In the USA, skin cancer (all types) constitutes 20-30% of all cancers in Caucasians, 2-4% in (east) Asians and 1-2% in African American and (south) Asian Indians (84).

Cumulative sun damage can also lead to "photo-ageing", a process distinct from, but additive to, chronological ageing (92). Repeated sub-erythemal exposure to UVA with or without UVB induces most of the histological changes observed in the epidermis of photo-aged skin (93). Photo-ageing is characterised by deep, coarse wrinkles, mottled hyper-pigmentation and loss of elasticity and skin recoil. The most affected areas are those chronically exposed to UVR – face, hands, arms and nape of the neck (92,94). There are numerous macro- and micro-scopic differences between intrinsic (chronological) ageing and extrinsic (photo-) ageing, summarised in Table 1.3. Recent evidence suggests that longer infra-red wavelengths of light may also contribute to the process of photo-ageing (95).

Table 1.3: Features of intrinsic and extrinsic causes of skin ageing

	Intrinsic Ageing	Extrinsic Ageing
Aetiology	• Chronological age	• UVR exposure • Smoking
Macroscopic Appearance*	• Fine wrinkling • Smooth texture • Clear complexion • Uniform pigmentation • Gradual loss of elasticity • Generalised process	• Coarse wrinkling • Roughened texture • Sallow complexion • Mottled pigmentation • Marked loss of elasticity • Sun-exposed areas affected most
Histological Appearance	• Epidermal and dermal atrophy • Flattened epidermis • Reduced number of mast cells • Reduced fibroblast numbers with less collagen and increased matrix metallo-proteinases	• Epidermis: atrophy & disrupted keratinocyte maturation • Dermis: Hyperplastic disordered elastic fibre network • Loss of fibrillin-rich microfibrils at the dermo-epidermal junction

Adapted from (92)

UVR and the eye

UVR exposure has been causally linked to the development cortical cataracts (96), pterygium (97,98), acute solar retinopathy (99), squamous cell cancer of the cornea and conjunctiva (100), and acute photo-keratitis and photo-conjunctivitis (101,102). Weaker evidence suggests an association between UVR and climatic droplet keratopathy, non-cortical cataracts and ocular melanomas (4).

1.2 Vitamin D

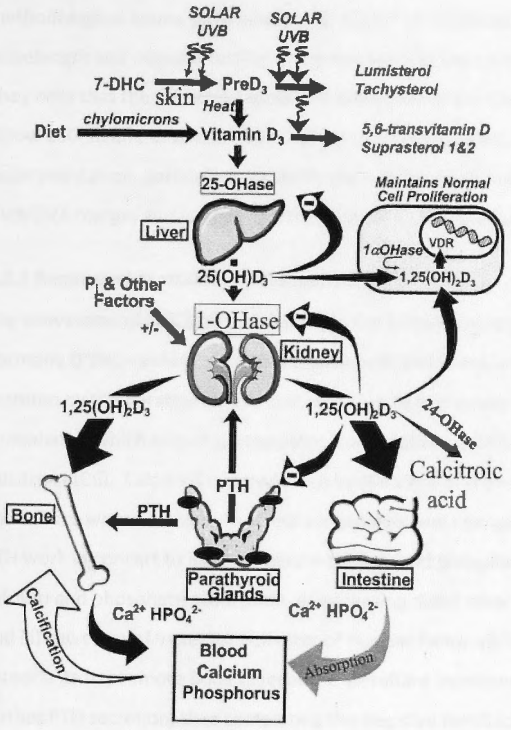
UVB exposure is the major contributor to vitamin D stores in humans, with the remainder coming from dietary sources (103). This section discusses the synthesis of vitamin D, sources and its biological roles.

1.2.1 Endogenous synthesis of vitamin D

The synthesis of vitamin D begins in the skin (Figure 1.8). Provitamin-D3 (7-dehydrocholesterol (7-DHC)) produced by epidermal and dermal cells absorbs UVB, resulting in the formation of pre-vitamin D3 (9,10-secosterol). Pre-vitamin D3 undergoes immediate heat induced transformation into vitamin D3 before entering the venous circulation via the lymphatics where it is tightly bound to vitamin D-binding protein. Within 12 to 24 hours of UVB exposure, maximal vitamin D3 levels are achieved. On passage through the liver, vitamin D3 is converted by 25-hydroxylase into 25-hydroxyvitamin D (25(OH)D) which is the major circulating form of vitamin D and has a half-life of 15 days. Although 25(OH)D is not biologically active, its concentration is used by clinicians to assess an individual's vitamin D stores. 25(OH)D is converted to the biologically active form, calcitriol (1,25-dihydroxyvitamin D ($1,25(OH)_2D$)), in the kidney by the enzyme 1α-hydroxylase (encoded by the gene *CYP27B1*). Of note, UVR exposures less than that required to produce erythema can maximise vitamin D3 synthesis (only a few minutes of summer sun in fair skinned persons). Excess local production of pre-vitamin D3 is itself eliminated by UVB (via conversion to inert photoproducts), so that longer sun exposure does not increase vitamin D3 levels but adds to the risk of erythema and DNA damage (2,3,104–106).

Calcitriol can also be produced extra-renally in many tissues including bone, placenta, keratinocytes, macrophages, T-lymphocytes and dendritic cells. Many of the constitutive cells in these tissues express both 25-hydroxylase and 1α-hydroxylase allowing the synthesis of 25(OH)D and/or $1,25(OH)_2D$. However, only 0.03% of total 25(OH)D is freely available in the tissues with the remainder being tightly bound to D-binding protein in the circulation (2). There is increasing evidence of an autocrine (i.e. an effect on the 1,25(OH)D producing cell itself) and paracrine (i.e. local effect on neighbouring cells) influence of 1,25(OH)D (107). This may be particularly relevant in the skin where many cell types (including keratinocytes, fibroblasts, macrophages, dendritic cells) express the vitamin-D receptor, essential for the genomic effects (including immunological) of vitamin D exposure (104).

Figure 1.8: Production, metabolism, regulation and action of vitamin D



1.2.2 UVR action spectrum for pre-vitamin D_3 synthesis

As described in the previous section, pre-vitamin D_3 is formed following the absorption of UVB photons by pro-vitamin D3 (7-DHC). The UVR action spectrum that is effective for this biological response was first described by MacLaughlin in 1982 following studies involving irradiation of neonatal foreskins (108), and formed the basis for the 'official' CIE action spectrum (109). The effective wavelengths are essentially within the UVB band (280nm – 320nm) with peak effectiveness of UVR at a wavelength of 297nm, (see Figure 1.6). There is an additional minor contribution from shorter wavelength UVA (<1% of total production at wavelengths 320-330nm) (104). Of note, there is significant overlap between the action spectra for erythema and pre-vitamin D_3 synthesis, however with some important differences, including: pre-vitamin D3 is more sensitive to the UVB wavelengths whilst erythema has a higher weighting coefficient in the UVA part of the spectrum (66).

There has been recent debate regarding the validity of the CIE pre-vitamin D_3 action spectrum. Norval et al (110) argue that the original MacLaughlin experiments suffered from methodological issues, particularly with regard to not detailing the doses used at each wavelength and minimal testing at the extremes of the action spectrum (particularly > 315nm). They note that the CIE action spectrum extrapolated the MacLaughlin data at wavelengths below 260nm and between 315 – 330nm using mathematical decay functions. Further experimentation, particularly to clarify the relative contribution of wavelengths at the UVB/UVA margin and further into the UVA band, has been called for.

1.2.3 Regulation of vitamin D metabolism

The conversion of 25(OH)D to calcitriol in the kidney is strictly regulated by plasma parathyroid hormone (PTH), calcium, phosphate, calcitonin and fibroblast growth factor 23 (3,104). PTH is secreted by the parathyroid gland in response to low serum calcium (or very low serum phosphate), which in turn up-regulates transcription of *CYP27B1* to promote production of calcitriol (106). Calcitriol is transported by the vitamin D binding protein to various target cells and tissues where it exerts its effect via genomic and non-genomic pathways. Calcitriol and PTH work in concert to increase serum calcium and phosphate levels by: i) increasing intestinal calcium and phosphate absorption, ii) increasing distal renal tubular reabsorption of calcium, and iii) secretion of 'receptor activator of nuclear factor-κB ligand' (RANKL) which acts on osteoclasts to promote bone resorption. Resultant increases in serum calcium act to suppress further PTH secretion, thus completing the negative feedback loop (106). Calcitriol also self-regulates via stimulation of the enzyme 25-hydroxyvitamin D-24-hydroxylase which catabolises calcitriol and 25(OH)D (3,111).

1.2.4 Effect of skin pigmentation and sunscreen on vitamin D synthesis

Melanin competes with 7-DHC in the epidermis to absorb UVB photons. Therefore darker skinned individuals are less efficient at synthesising vitamin D than fairer skinned individuals for the same UVR dose (74). Persons with Fitzpatrick phototype VI require 10-50 times the amount of solar UVR exposure of a fairer person (Type II-III phototype) to produce equivalent amounts of vitamin D (112).

The latitudinal gradient in population skin pigmentation (i.e. darker skinned populations near the equator, and lighter skinned populations at higher latitudes) has long been attributed to the latitudinal gradient in ambient UVR levels, such that under evolutionary selection pressure, darker skinned persons are 'photo-protected' from intense UVR exposure, whilst fairer skinned persons have adapted to require less UVR to synthesise vitamin D (113). However, doubts regarding the potency of evolutionary selection pressure arising from some adverse effects of

excess UVR exposure (erythema, skin cancer) or low vitamin D levels (apart from rickets) to drive changes in skin pigmentation have led to other theories being proposed (76). One alternative hypothesis is that dark pigmentation evolved in high ambient UVR environments to protect against photolysis of folate in cutaneous blood vessels which has potential implications for foetal abnormalities (such as neural tube defects) and fertility (in males and females) (114).

1.2.5 Sources of vitamin D

The primary sources of vitamin D are exposure to sunlight and diet. For most human populations, sunlight exposure contributes ~90% to vitamin D stores, however the relative contributions can change considerably by season, skin type, diet and culture (103,105,115). The recommended duration of exposure to solar UVR to achieve adequate 25(OH)D levels is the subject of considerable ongoing research. Skin pigmentation, solar altitude (season, latitude, altitude) and sun protection behaviours determine the amount of sun exposure required (105). Local guidelines recommend safe levels of sun exposure that can promote adequate vitamin D stores whilst minimising the risk of erythema. For example, in Australia recommendations for persons with fair skin (Fitzpatrick type II skin) for exposure depending on season, region and time of day have been suggested (see Table 1.4) (116). Due to the low solar altitude in winter months at high latitudes (e.g. > 40°), there is insufficient UVB in sunlight to produce adequate quantities of vitamin D3. The body therefore relies on mobilisation of stores in body fat produced during sunnier seasons, as well as fortified diets or supplementation (74,117).

Table 1.4: Recommended solar UVR exposure for Australian regions

Region	October to March	April to September
	At 12:00	
Northern (Townsville / Cairns)	2 – 5 mins*	3 – 10 mins#
Central (Brisbane / Perth)	2 – 6 mins*	4 – 17 mins#
Southern (Adelaide/ Sydney/Melbourne)	2 – 10 mins*	5 – 34 mins#
	At 10:00 & 15:00	
Northern (Townsville / Cairns)	≤10 mins#	≤16 mins#
Central (Brisbane / Perth)	~ 10 mins#	14 – 44 mins*
Southern (Adelaide/ Sydney/Melbourne)	≤15 mins*	21 mins to ≥1 hr.*
To 15% of the body at 12:00, 10:00 & 15:00, for fair skin people to produce 25(OH)D levels equivalent to current recommended intakes in Australia, if exposure occurs three to four times per week (Adapted from (116)) *With extreme care – sun protection highly recommended # With care – sun protection recommended if times exceeded		

Vitamin D can also be derived from the diet and / or dietary supplements. There are two oral forms: vitamin D_2 (or ergocalciferol) derived from UV irradiation of the yeast sterol, ergosterol; and vitamin D_3 (cholecalciferol) formed through UV irradiation of 7-DHC. Vitamin D_2 and D_3 differ only in a side chain of the sterol skeleton and both forms undergo hydroxylation in the liver to form 25(OH)D. Vitamin D_3 is found in only a few foods, including fatty fish (e.g. salmon, mackerel, sardines), fish liver oil, fish liver and egg yolks. Vitamin D_2 is present in some mushrooms (e.g. shitake) and egg yolk although it has been the predominant form in manufactured supplements (3,118). Recent studies have indicated that vitamin D_3 may be more efficient that vitamin D_2 in increasing vitamin D status (measured as serum 25(OH)D concentration), although there is some conflicting evidence (104,118).

There is ongoing debate as to what constitutes adequate dietary intake of vitamin D (either vitamin D_2 or D_3). It has been estimated that whole body exposure to 1 MED of UVR results in a rise in serum 25(OH)D that is equivalent to 20,000IU of vitamin D_2 supplementation (74). Recent American consensus guidelines report evidence that maintaining adequate 25(OH)D

levels (>50nmol/L) was beneficial for skeletal health, and this could be achieved by oral vitamin D dosing of 600 IU per day for all age groups, except the elderly (> 70 years: 800 IU/day) and infants below 12 months (400 IU/day) in the context of little or no UVR exposure (119). British consensus guidelines recommend that groups that are at increased risk of vitamin D deficiency due to low UVR exposure should fortify or supplement their diet with vitamin D (400 – 800 IU/day) (117).

1.2.6 Biological action of vitamin D

Calcitriol can have biological actions mediated by genomic (i.e. altering gene expression) or non-genomic mechanisms (e.g. opening calcium channels, activation of phospholipase C) (2). Genomic processes occur in cells that express the vitamin D receptor (VDR) (120). When binding with calcitriol in the cell nucleus, the VDR is phosphorylated and combines with the transcription factor 9-cis-retinoid X receptor (RXR). This complex interacts with specific promoter regions of DNA ('Vitamin D response elements') which regulate the transcription of gene products (e.g. cytokines, anti-microbial peptides) (120,121). Genomic effects generally take place over a period of hours, whereas non-genomic actions occur in seconds to minutes (2,104). The range of actions of calcitriol are summarised in Table 1.5.

Table 1.5: Biological actions of calcitriol

Target Organ / Cell	Genomic (G) or non-genomic (NG)	Action
Kidney	**G:**↑expression of Ca^{++} selective channel & calbindin (Ca^{++} binding protein)	↑trans-cellular Ca^{++} transport in distal tubule Negative feedback to ↑ 25-hydroxyvitamin D-24-hydroxylase to catabolise calcitriol
Gut	**G:**↑expression of epithelial Ca^{++} channel & calbindin (Ca^{++} binding protein)	↑intestinal absorption of calcium by 30-40% and phosphate by 80%
Bone	**G:** ↑osteoblast expression of RANKL, inducing preosteoclasts to form osteoclasts	↑Osteoclastic activity which increases serum Ca^{++} and PO_4^{2-}
Parathyroid gland	**G:** ↓transcription of genes encoding PTH & PTHrP	↓PTH / PTHrP synthesis
Miscellaneous cells (keratinocytes, enterocytes, myocytes, osteoblasts, chondrocytes)	**NG:** Activates Ca^{++} influx and release from intracellular stores; adenylate cyclise, phospholipase C, protein kinases C & D modulation; modulates mitogen activated protein & rapidly growing fibrosarcoma kinase pathways	↑Intracellular Ca++ levels; Stimulation of intestinal Ca++ & phosphate transport fluxes; elevation of cGMP levels; Rapid changes in phosphoinositide metabolism

RANKL – receptor activator of nuclear factor-κß ligand
PTHrP – parathyroid hormone related hormone
cGMP - cyclic guanosine monophosphate

Sources: (2,3,104,122)

1.2.7 Health outcomes associated with vitamin D deficiency

Vitamin D deficiency is associated with a number of clinical diseases, as summarised below. Immune system-mediated diseases (e.g. autoimmune disease, infection) that are associated with low vitamin D status, will be discussed in the next chapter.

Musculoskeletal health

Extended periods of vitamin D deficiency can lead to secondary hyperparathyroidism and subsequent decreased calcium and phosphate mineralisation of the skeleton (105,123). When this occurs *in utero* or in childhood, the bone deforming condition 'rickets' can result (124). In adults, this condition is termed 'osteomalacia' (3).

Osteopaenia and osteoporosis are conditions of decreased bone mass often associated with fracture. They are common conditions of multi-factorial aetiology where vitamin D deficiency plays a role (125,126). A number of studies have shown a benefit in terms of reduced fractures in osteoporotic patients in those who take vitamin D and calcium supplementation (rather than either exclusively) (106,119)

Vitamin D deficiency is also associated with skeletal muscle weakness. Studies have shown that muscle strength and speed increases with correction of vitamin D deficiency, and falls risks amongst elderly patients are significantly reduced (127).

Cancer

The genomic influences of calcitriol - including regulation of cell growth and division, differentiation, apoptosis and angiogenesis – can suppress tumour cell genesis and proliferation (3). Many observational studies have demonstrated a favourable association between vitamin D status (measured directly by serum 25(OH)D level or indirectly by duration of sunlight exposure, latitude of residence, dietary intake) and the incidence of colon, breast, prostate and ovarian cancer (128). However the role of oral vitamin D supplementation as a preventative measure or as an intervention in patients with cancer, is presently unclear (106,119).

Other conditions

Vitamin D deficiency has been associated with congestive cardiac failure, increased inflammatory markers associated with cardiovascular disease (e.g. C-reactive protein, IL-10) and hypertension (via modulation of the rennin-angiotensin system) (3,129). Reduced mental health well-being and increased risk of psychiatric illnesses (e.g. schizophrenia, autism and depression) have also been epidemiologically associated with low vitamin D status (3,130,131).

1.3 Summary

Ultraviolet radiation is a component of the electromagnetic radiation spectrum emitted by the sun. Terrestrial UVR levels vary by solar zenith angle (which is determined by time of day, season and latitude), cloud cover and altitude. Extra-terrestrial UVR is differentially filtered by atmospheric gases (primarily ozone), such that ground level UVR comprises mainly longer-wavelength UVA with a smaller (but varying) component of shorter-wavelength UVB. UVA and UVB are biologically active, although UVB possesses disproportionate biological potency because of its inherent higher energy. An individual's personal UVR dose is dependent on their UVR exposure (a function of terrestrial UVR intensity and duration of exposure) and the area of skin exposed (which varies by clothing, sunscreen). Individuals typically are exposed to only a small fraction of terrestrial levels but there is wide variation within a population. Clothing and sunscreen provide varying degrees of protection from UVR exposure. The accepted units of personal UVR dose are the standard erythemal dose (SED) and minimal erythemal dose (MED), which are linked to erythema effective wavelengths of UVR (i.e. erythema action spectrum). The UV Index is a convenient measure of biologically-relevant terrestrial UVR intensity. Skin pigmentation modifies the biological activity of UVR exposure. High or cumulative UVR exposure is associated with a number of adverse health outcomes particularly affecting the eye and skin, including photo-ageing, sunburn, skin cancer and cataracts.

UVR plays a critical role in the local synthesis of vitamin D precursors within the skin and constitutes the major vitamin D source in humans. Dietary intake plays a secondary role in maintaining overall vitamin D stores. UVR catalysed production of pre-vitamin D precursors in the epidermis sets in train a complex multi-organ process that ultimately results in the production of the biologically active form of vitamin D, calcitriol ($1,25(OH)_2D$). The pre-vitamin D3 UVR action spectrum is similar to the erythema action spectrum (i.e. UVB wavelengths are largely responsible). Calcitriol is produced by the kidney, but importantly also by other tissues (including immune cells and keratinocytes). The main function of calcitriol (often acting in concert with parathyroid hormone) is to maintain serum calcium levels within narrow limits, with influences on gut, kidney and bone via genomic and non-genomic actions. Low 25(OH)D levels (the preferred measure of vitamin D stores) are associated with a number of adverse health outcomes including low bone mass, solid organ cancers, autoimmune, cardiovascular and psychiatric disorders.

As adverse health outcomes are related to excessive *and* insufficient UVR exposure, the most appropriate levels of UVR exposure at individual and population levels are unclear. This is the subject of considerable current research activity.

The following chapter will discuss the literature pertaining to the influence of UVR and vitamin D status on human immune function.

Chapter 2: Ultraviolet radiation and immune function

Synopsis

This chapter is presented in three parts. The first section provides a general overview of the immune system, with particular emphasis on the adaptive immune system. The remaining sections review the known and postulated influences of UVR on immune function, both directly, and indirectly via induction of vitamin D synthesis.

2.1 An overview of the human immune system

The immune system defends against microbial infection and plays a significant role in the detection and deletion of cancer cells. Our understanding of the mammalian immune system has become increasingly sophisticated, yet still remains incomplete. Evolution of the immune system within the animal kingdom has occurred over aeons, such that in humans there has developed two parallel but interacting systems: the 'innate' or primitive immune system, components of which are seen even in the simplest organisms; and the 'adaptive' immune system, unique to vertebrates (132).

Innate immunity provides a first line of defence against microbial invaders. It consists of physical barriers (i.e. skin, mucous membranes, gastric acid, salivary enzymes, cough reflex), cellular components (e.g. neutrophils, Natural Killer (NK) cells) and proteins (e.g. complement, Toll-like receptors). These produce a generic, non-specific response that does not change or evolve over an individual's life - that is, there is no 'memory' of previous exposure. When activated, cellular components release chemical signals (e.g. cytokines) which interact with the adaptive immune system, so that the two operate in concert (133).

Adaptive immunity is a more sophisticated response, entailing the ability to recognise, attack and remember specific foreign antigens. It is this capacity for "immune memory" that protects a host from re-infection and which provides the scientific rationale for immunisation. The adaptive immune system is comprised of specialised white blood cells - "B" and "T" – cell lymphocytes. Activation of B cells results in antibody production – either for opsonising (coating) or neutralising targets. T cells are subdivided into T-helper cells (Th or CD4+ cells[1]) and cytotoxic T cells (Tc or CD 8+ cells). Upon presentation of foreign antigen by specialised "antigen presenting cells" (APCs i.e. dendritic cells, macrophages, B cells), T-helper cells further differentiate into effector cells: Th1, Th2, Th17 and regulatory T (Treg) cells (134,135).

[1]CD: Cluster of differentiation

Functionally, the adaptive immune system is again further divided into cell-mediated and humoral immunity, referring to pathogen elimination by either cellular components or antibodies, respectively. Cell-mediated immunity (CMI) is regulated largely by Th1 cells: upon activation, these cells release specific cytokines (i.e. interferon γ, TNF β) that i) activate macrophages; ii) induce B cells to produce opsonising antibodies (i.e. IgG1, IgG3) that coat the invading pathogen for killing by other phagocytic cells; and iii) stimulate cytotoxic T cells. Humoral immunity is principally mediated by Th2 cells, which release cytokines (i.e. IL-4, IL-5, IL-10) that activate B cells to make neutralising antibodies. The adaptive immune response following the first (or primary) exposure to antigen can take several days; however, this stimulates the development of 'memory' B and T cells. These long-lived cells allow humans to develop a rapid immune response on subsequent exposure to the same antigen (133–135).

CMI (Th1) is particularly important for combating intracellular infections (i.e. viruses and certain bacteria such as *Mycobacteria tuberculosis, Listeria sp.*), whilst humoral immunity (T_h2) primarily deals with extracellular infections (i.e. bacteria, parasites). Nevertheless, immune responses against infection generally comprise aspects of innate and adaptive immunity, and cell-mediated and humoral immunity (135).

Disease can occur from both overactivity (up-regulation) of the immune system and immune suppression (down-regulation). Up-regulation of Th2 pathways is implicated in the pathogenesis of allergy (particularly IgE-mediated), whereas up-regulated Th1 responses are implicated in specific autoimmune diseases (such as Type 1 diabetes, scleroderma and multiple sclerosis) (136,137). As the immune system defends against microbial pathogens and aberrant host cells, down-regulation may increase vulnerability to infection and neoplastic disease, and reduce the protective efficacy of immunisation.

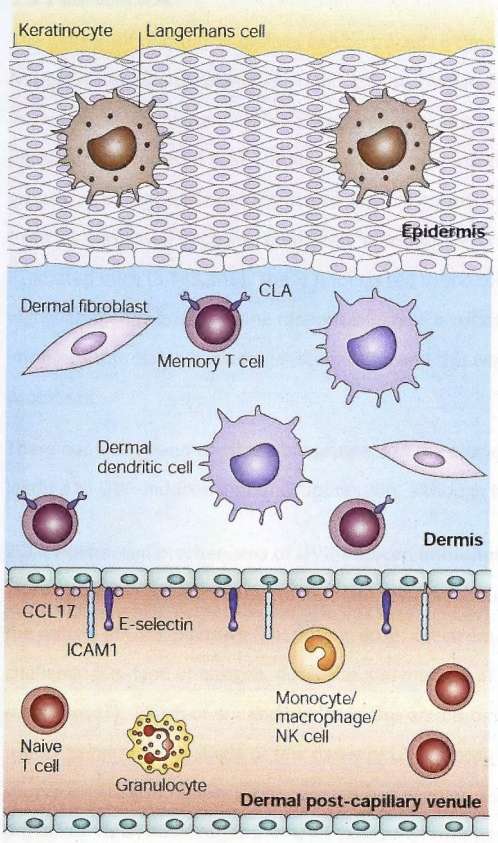
Impairment of specific immune components predisposes to specific types of infection and disease. For example, individuals with very low CD4+ (T-helper) cell counts (e.g. HIV/AIDS patients) are at much higher risk of specific opportunistic infections such as *Pneumocystis jiroveci* pneumonia, mycobacterial infection and toxoplasmosis than those with higher CD4+ T cell counts (138). Primary humoral immunodeficiency - manifest by low or absent levels of circulating antibody (i.e. IgG subclass deficiency, IgA deficiency or common variable immunodeficiency) - is associated with recurrent respiratory and gastrointestinal infections of varying severity (139). Additionally, studies of cancer patients (140) and acutely ill surgical patients (141,142) have shown that impaired cell-mediated immunity (demonstrated by reduced delayed-type hypersensitivity skin reactions) is associated with poorer prognosis and/or higher mortality.

At an individual level, it is these profound impairments of immune function that are associated with significant (i.e. clinically manifest) health consequences. However, at a population level, less profound impairment of specific immune processes occurring with high prevalence may manifest as an increased incidence of infection (e.g. influenza, otitis media or the common cold) (22) and reduced vaccine effectiveness (143). For vulnerable sub-populations – the old, young, ill and in the developing world - these outcomes may be even more pronounced. This thesis postulates that UVR exposure is an environmental agent that can impair aspects of the human immune response, thereby altering the level and distribution of vulnerability within the population.

2.2 Immune response elements of the skin

The skin represents the interface between the human immune system and UVR exposure. Figure 2.1 illustrates the layers of the skin and the immune cells that reside within them in the non-inflamed state. The epidermis is the most superficial layer of the skin and consists primarily of 'keratinocytes', which are epithelial cells able to produce keratin, an extra-cellular protein. Within the epidermis also reside Langerhans cells - which are long-living, immature, bone marrow-derived dendritic cells that act as important 'sentinel' antigen presenting cells; and melanocytes. Below the epidermis, the dermis is comprised mainly of fibroblast-produced, extra-cellular connective tissue, as well as other immune cells, including mast cells, dermal dendritic cells and memory T cells. Keratinocytes and resident immune cells express pattern recognition receptors (such as Toll-like receptors) that recognise specific pathogen components and which can initiate innate immune processes. The dermal post-capillary venule(s) are able to take chemical signals (chemokines and cytokines) produced by immune cells in the skin to the systemic circulation attracting other immune cells to the site of tissue injury or infection. Immune cells such as Langerhans cells, dermal dendritic cells and mast cells can migrate to draining lymph nodes via afferent lymphatic vessels (144).

Figure 2.1 Immune components of the skin



Immune-response elements in non-inflamed skin: Human skin is composed of three distinct compartments relevant to its immune functions. First, the epidermis is composed of keratinized epithelial cells and functions as both a physical barrier and an early warning system. Immune cells resident in the epidermis include specialized dendritic cells (DCs) known as Langerhans cells and intra-epithelial lymphocytes. Second, the dermis is mainly composed of connective tissue produced by dermal fibroblasts. Immune system cells resident in non-inflamed dermis include dermal DCs, mast cells and a small number of cutaneous lymphocyte antigen (CLA)-positive memory T cells. Third, dermal post-capillary venules constitutively express low levels of E-selectin, CC-chemokine ligand 17 (CCL17) and intercellular adhesion molecule 1 (ICAM1). These support the margination and baseline emigration of CLA+ memory T cells into non-inflamed skin. CLA– T cells, including both naive cells and memory/effector cells that are targeted to other tissues, as well as granulocytes (e.g. neutrophils) and other immune cells, lack the appropriate receptors to attach to dermal vessels and emigrate into non-inflamed skin. Text adapted from (144).

Source: (144)

2.3 The influence of UVR on immune function

2.3.1 Introduction

The genesis of photoimmunology can be traced to the seminal experiments conducted by Margaret Kripke and colleagues in the 1970s. They found that skin cancers transplanted into normal, genetically-identical mice were rejected, but grew progressively in immunocompromised hosts. It also became apparent that transplanted skin cancers would grow in mice that had been irradiated with UVR. Furthermore, transfer of T-cell lymphocytes from UVR-exposed mice also conferred 'immunotolerance' to transplanted tumours in non-irradiated mice (6,145,146). These findings led to the following conclusions: skin cancers were highly antigenic; host immune responses played a critical role in tumour control; UVR was immunosuppressive (including systemically) and this was regulated by T-cell mediated processes.

There has since been considerable progress in the elucidation of the underlying mechanisms leading to UVR-induced immunosuppression, although much remains unresolved.

2.3.2 Postulated mechanisms of UVR-induced immunomodulation

The degree and characteristics of immune system modulation vary considerably depending on the nature of UVR exposure (i.e. timing, dose, spectrum, body site exposed) and immune challenge (i.e. type of antigen, dose, site and method of administration, primary or recall response) (7). Much of our knowledge in this area is based on work that has been conducted using rodent models, single or few doses of UVR exposure, erythemal doses of UVR and/or a source emitting non-solar UVR spectrum (e.g. frequently with a high UVB component) (8). The prevailing view, however, is that UVR exposure is immunosuppressive to cell-mediated immune processes in an antigen-specific manner.

The following section will describe the postulated pathways leading from absorption of UVR photons by skin components through to changes in cutaneous cellular and inflammatory mediators, development of a regulatory and tolerogenic immune environment in the draining lymph node and dissemination of local and systemic mediators of immunomodulation.

2.3.3 UVR chromophores

Energy from UVR exposure is part-reflected and part-absorbed by the skin. Absorption occurs via cutaneous chromophores – photon-absorbing molecules that change their structure on exposure to UVR (147). More is currently known about chromophores likely relevant for UVB immunomodulation than for UVA wavelengths (13). UVB chromophores are located superficially in the skin, reflecting the poor cutaneous penetration of UVB (88), and include

DNA, urocanic acid (UCA) and membrane lipid components. DNA within keratinocytes and Langerhans cells (epidermal antigen presenting cells) acts as the major UVB chromophore and DNA damage is a crucial mediator of UVR-induced immunosuppression.

DNA is maximally degraded at 260nm, but UVB wavelengths (290-320nm) are also effective, resulting in photoproducts such as cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone (148,149). UVA also induces DNA damage directly but at much higher doses than for UVB; and indirectly through the generation of reactive oxygen species causing DNA strand damage and mutagenic changes to purines (e.g. 8-oxo-7,8-dihydro-2'-deoxyguanosine) (93). It has been recently shown that oral or topical replacement with nicotinamide (precursor of keratinocyte-produced nicotinamide adenine dinucleotide (NAD), important for cellular metabolic energy) can reduce DNA damage and limit subsequent UVR-induced immunosuppression (150,151). The cytokines IL-12, IL-18 and IL-23 are also important for DNA repair, but UVR reduces the production of these mediators within UVR-damaged Langerhans cells (9).

UCA undergoes UVR-dose dependent isomerisation from *trans*-UCA to *cis*-UCA in the *stratum corneum* (superficial epidermis), with peak transformation occurring at the shorter UVB wavelengths 280 - 310nm, but also into the UVA spectrum (152,153). *cis*-UCA is thought to be an important mediator of cell-mediated immune responses (via TNF-α in particular (154)). For example, higher *cis*-UCA levels have been implicated in lower antigen specific cellular immune responses to immunisation in UVB exposed humans (155). Finally, various cellular membrane components (e.g. phospholipids, surface receptors) are susceptible to changes in redox equilibrium (e.g. lipid peroxidation) following UVB-induced reactive oxygen species synthesis (147,156).

2.3.4 Cellular trafficking and signalling

Keratinocytes, mast cells, macrophages, dendritic cells and sensory nerve cells are induced by UVR-transformed chromophores to synthesise and release a set of chemical signals which mediate local inflammation (i.e. chemotactic signals to attract neutrophils and macrophages to the skin) and set in train subsequent immune modulating processes. Table 2.1 details the main chemical mediators that play a role in this setting.

Table 2.1: Chemical mediators affected by cutaneous UVR-irradiation

Cytokine	Producer cells	Action	UVR effect on mediator
Platelet activating factor	Keratinocytes, CD11b+ macrophage	Activates platelets, monocytes, neutrophils; increases PGE2 production; increases DNA damage; promotes regulatory B cells	Increased
IL-1	Keratinocytes, macrophages	Stimulates inflammation, chemotactic agent, ↑PGE2	Increased
IL-4	T cells, mast cells	Promotes Th2 differentiation; B cell activation; IgE antibody switch	Increased
IL-6	Keratinocytes, T cells, macrophages	B- cell activation	Increased
IL-10	CD11b+ macrophages, mast cells, neutrophils (keratinocytes)	↓LC & DC stimulation of naïve T cells and Th1 cytokine production	Increased
IL-12	Langerhans cells, dendritic cells, macrophages	Promotes Th1 cell proliferation; repairs UVR-induced DNA damage; inhibits Treg cell activity	Reduced
IL-18	Macrophages	Induces IFN-γ by T cells and NK cells; promotes Th1 processes	Reduced
IL-23	Dendritic cells	Reduces DNA damage; induces IFN-γ by T cells; promotes memory T-cells; inhibits Treg cell activity	Reduced
IL-33	Keratinocyte, dermal fibroblasts	Immunosuppressive effect (↓ CHS response); UVB shown to ↑ IL-33	Increased

TNF- α	Keratinocytes, mast cells, activated dendritic cells	Recruits inflammatory cells to injured area, mediates immune effect of cis-UCA, ↑PGE2, promotes migration of dendritic cells to lymph node,	Increased
Histamine	Mast cells	Stimulate prostanoid production, ↑PGE2 (associated with (↓ CHS responses)	Increased

PGE2 = prostaglandin E_2; LC = Langerhans cells; DC = dendritic cells; CHS = contact hypersensitivity; UCA = urocanic acid; Sources: (147,157–160)

Chemical mediators can also be found in the systemic circulation after UVR exposure potentially contributing to systemic immunomodulation. These include histamine, platelet-activating factor, prostaglandins, *cis*-UCA, IL-1, IL-10, IL-4, TNFα, neuropeptides and neuroendocrine hormones (7,156).

In response to cutaneous UVR exposure and release of the aforementioned chemical mediators, several populations of immune cells migrate to and from the skin. Within two hours of UVR-induced epidermal cell damage, macrophages, mast cells and neutrophils are attracted to the site of cell injury; peak infiltration occurs by four to six hours and the inflammatory cell recruitment has largely concluded by 48 hours (64). Macrophages and neutrophils are responsible for phagocytosis of apoptotic cells and also secrete the potent immunosuppressive cytokine IL-10 (161,162).

2.3.5 Influence of UVR on the adaptive immune response

UVB radiation leads to migration of epidermal Langerhans cells and dermal dendritic cells to the draining lymph node, albeit in an impaired fashion (163–165). In addition, UVR affects the ability of dendritic cells to present antigen to T cells in the lymph node by reducing the expression of surface co-stimulatory molecules (e.g. B7, ICAM-1) (166) and MHC class II molecules (147). Furthermore, it appears that UVR-exposed dendritic cells preferentially promote CD4+ T helper cell differentiation into a Th2 pattern rather than Th1 (167,168). Secreted IL-10 (by mast cells that have migrated to the lymph node on stimulation by antigen and CD11b+ macrophages in the skin) is likely to be an important cytokine driving Th2 differentiation (162,169). The contribution of Langerhans cells in promoting a regulatory environment in the draining lymph node, is also receiving increased attention (9,170).

Since Kripke's initial experiments, it has been shown that the immunosuppressive effect of UVR exposure is antigen-specific rather than of a general nature. That is, not only is the immune response to primary cutaneous exposure to antigen affected following UVR exposure (due to the processes mentioned above), but re-exposure to the same antigen at a later time is also impaired, even if exposed at a distant site (systemic immunosuppression) (171). Although initially hypothesised to be the result of 'suppressor' T cells, it is now known that there exists a subset of antigen-specific T-helper cells - regulatory T cells (Treg) that are promoted in the lymph node upon interaction with UVR-affected APC. The phenotype of the UVR-induced Treg lymphocyte is CD4+ $CD25^{hi}$ $CD127^{lo}$ Foxp3+ CTLA-4+ although there are likely to be heterogeneous populations of these cells. They appear to produce IL-10 but not IL-4 or IFN-γ (172). UVR-induced Treg lymphocytes have been shown to be inhibitors of the immune stimulatory cytokine IL-12, inhibit APC function (173) and also switch APCs from a stimulatory to a regulatory (or tolerogenic) phenotype (174).

Other cells have been implicated in the development and maintenance of a local immunosuppressive environment. Following cutaneous UVR exposure, increased local IL-10 concentrations have been shown to induce 'regulatory B cells' which act to inhibit the ability of dendritic cells to activate (particularly Th1) cell-mediated processes within the lymph node (175,176). A population of IL-4 producing Natural Killer T (NKT) cells are also thought to down-regulate immune responses following interaction with UVR exposed Langerhans cells that have migrated to the lymph node (177).

Given that Th1 cell-mediated processes are primarily affected by UVR exposure, assessment of the T-dependent antibody response would be informative. However, the limited number of studies examining this specific question have demonstrated conflicting results (178,179). Nevertheless, a series of recent experiments in mice has demonstrated that UVR exposure following immunisation suppressed the antigen-specific antibody response to T-dependent antigens but not T-independent antigen, and suppression was related to the generation of IL-10 producing regulatory T cells (178,180,181). T follicular helper (Tfh) cells, a specialised subset of CD4+ T cells, are important in providing an environment for B cells to produce antibody in the germinal centre of lymph nodes. Recent evidence suggests that IL-10 producing mast cells that have migrated from UVR-irradiated skin to lymph nodes (169) can suppress T follicular helper cells, formation of germinal centres and subsequent antibody formation (182).

2.3.6 UVR and *in vivo* cell-mediated immune response testing

Contact hypersensitivity (CHS) or delayed-type hypersensitivity (DTH) responses are functional *in vivo* assays of cell-mediated immune function. They have been utilised in a number of controlled studies in humans to assess the local and systemic immunomodulatory influence of UVR exposure (183). Both the CHS and the DTH responses are mediated by antigen-specific effector T cells and require: i) sensitisation of naïve CD4+ T helper cells with antigen (i.e. by immunisation or natural exposure) and ii) elicitation of a quantifiable inflammatory response by re-exposure to antigen (usually several weeks after sensitisation). The elicitation phase results in a delayed inflammatory response (which is primarily made up of macrophages, phagocytes and plasma) that can be measured usually 24-72 hours following antigen re-exposure (134). Exposing volunteers to UVR *before* antigen sensitisation assesses suppression of the induction arm. Assessment of the elicitation arm is made by exposing participants to UVR sometime *after* initial sensitization, then re-challenging with the relevant antigens or contact allergen (184). Local immunosuppression is detected when there is reduced response to antigen applied to UVR exposed skin, and systemic (or distal) immunosuppression occurs when there is reduced response following antigen exposure to a site remote from the UVR exposure (184). Differences between CHS and DTH responses are summarised in Table 2.2 and further details regarding the mechanisms of DTH response are given in Section 3.1.6.

Table 2.2 Features of contact hypersensitivity and delayed type hypersensitivity responses

Immune response	Site of antigen exposure	Major antigen presenting cell involved	Example of antigens	Description of typical skin response
Contact hypersensitivity	Epicutaneous (i.e. topically applied to skin)	Langerhans cell	Nickel, chromate, Poison ivy	Local epidermal reaction: redness, swelling, cellular infiltrate, vesicles
Delayed type hypersensitivity	Intra-cutaneous (i.e. intra-dermal injection)	Dermal dendritic cell	Tuberculin, lepromin, insect venom, Candida, Trichophyton	Local skin induration: redness, swelling, cellular infiltrate

Sources: (134,183)

UVR exposure has been shown to be immunosuppressive in humans using various experimental designs. Studies utilising DTH responses have used tuberculin antigen (185,186), lepromin antigen (187), and the Multitest kit (containing seven commonly encountered proteins) (15,16,188). Antigens used for CHS testing have included nickel (189,190) and dinitrochlorobenzene (DNCB) (191–193). Experimental design has varied according to the DTH/CHS phase being investigated (i.e. sensitisation *vs.* elicitation); source of UVR exposure (i.e. UVR lamp *vs.* natural sun exposure); UVR spectrum (i.e. UVA *vs.* UVB *vs.* solar UVR (+/- simulated)); dose (erythemal *vs.* sub-erythemal) and timing of UVR exposure; site of testing (i.e. buttock *vs.* back *vs.* arm); and use of sunscreen (reviewed in: 61,62). Some examples of these studies are shown in Table 2.3.

These studies, taken in total, have consistently shown that UVR is immunosuppressive to antigen-specific *in vivo* cell-mediated immune responses, and have been the basis for the ascertainment of the immunosuppression UVR action spectrum (Section 2.3.7) and the immune-protective properties of sunscreen. Some important findings from these studies are outlined below:

UVR wavelength – Immunosuppression was induced in studies using UVB (190,194,195), UVA (188,190,196,197) and solar simulated (SS)-UVR wavelengths (15,185,192) but there were differences in the respective time courses of suppression. (See section 2.3.7)

UVR exposure dose – UVR influenced immune function at even low levels of exposure. A single sub-erythemal dose of SS- UVR at 0.25 – 0.5 MED was sufficient to suppress the induction phase of the CHS response to DNCB by 50 – 80% (192). Sub-erythemal doses of UVR (~0.6 MED; equivalent to six minutes midday summer sun exposure) significantly suppressed nickel CHS elicitation responses in participants after a single dose of SS-UVR and UVA, and after two doses with UVB (190).

Local *vs.* systemic response – There was strong evidence for UVR-induced local immunosuppression, but also evidence of a systemic effect. DTH elicitation responses to Multitest kit antigens were reduced at the site of UVA, SS-UVR and/or natural UVR exposure and distally in two studies assessing the immune-protective efficacy of sunscreen (15,16). In another study, participants irradiated with a single dose of SS-UVR (3 MED) to a small area of skin, demonstrated both local (12/12) and systemic (10/12) immunosuppression using CHS assays (198). Sleijffers et al (19) demonstrated that five days of 1 x MED UVB was sufficient to cause a slight but significant reduction in the concentration of circulating NK cells, compared with a non-irradiated control group.

Table 2.3 Examples of *in vivo* cell-mediated immunity testing: Assessing the immunomodulatory effects of UVR exposure in humans

First Author (Year)	Participant characteristics	UVR spectrum	Doses	*In vivo* immune parameter	Antigen	Outcome
Moyal D (2008)	Fitzpatrick skin type II-III; aged 18-40yrs	UVA; SS-UVR; natural UVR	Variable dosing – sub-erythemal and erythemal doses, both with sunscreen	DTH – elicitation phase	Multitest kit*	Reduction in local & systemic DTH response in all groups. Sunscreen protective if UVA range included
Damian D (1998)	Fitzpatrick skin type I-V; aged 21-53 yrs	SS-UVR	Sub-erythemal and erythemal doses; 5 day protocol; 4-5 week protocol	DTH – elicitation phase	Tuberculin	Dose response UVR-immunosuppression. Weak evidence of adaptive response to chronic UVR
Damian D (1999)	Fitzpatrick skin type I-V; aged 18-62 yrs.	SS-UVR; UVA; UVB	Sub-erythemal doses; Short (<5 days) and long (4 week) protocols	CHS – elicitation phase	Nickel	SS-UVR, UVB, UVA immunosuppressive. UVB & UVA maximal effects at different times. UVA transient effect
Kelly D (2000)	Fitzpatrick skin type I-IV; aged 18 -35 yrs.	SS-UVR	0.25 – 3 MED	CHS – sensitisation phase	DNCB	Low dose SS-UVR immunosuppressive. Differential response by skin type –Type I/II skin > Type III/IV

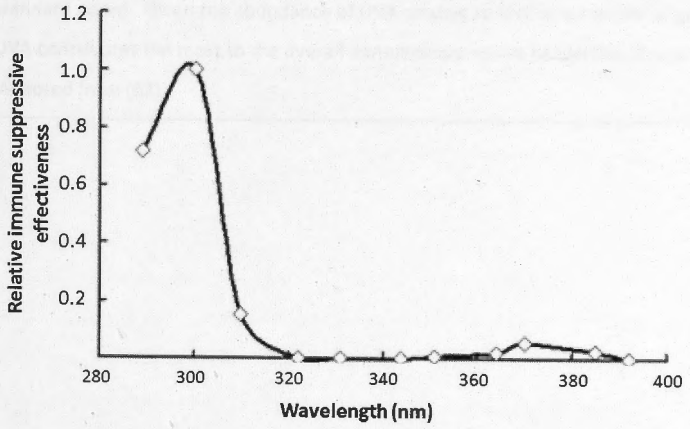
* Multitest kit (Pasteur / Merieux) – containing tetanus toxoid, diphtheria toxoid, *Proteus mirabilis, Trichophyton mentagrophytes, Candida albicans*, streptococcus, tuberculin SS-UVR: simulated solar UVR; DTH: delayed type hypersensitivity; CHS: contact hypersensitivity; DNCB: dinitrochlorobenzene

2.3.7 UVR-induced immunosuppression action spectrum

Recent research has delineated the immunosuppressive potency of UVR at different wavelengths by assessing the effect of narrow-band UVR irradiation on the nickel CHS response (67). UVB radiation is immunosuppressive in a linear dose-dependent manner, with maximal suppression occurring at a wavelength of 300nm (195). By contrast, the immunosuppressive effect of UVA is more complex, with maximal suppression occurring at 370nm and not being apparent following exposures of ~23 minutes or greater of midday summer sunlight (196,199). The postulated action spectrum for UVR-induced immunosuppression is shown in Figure 2.2a.

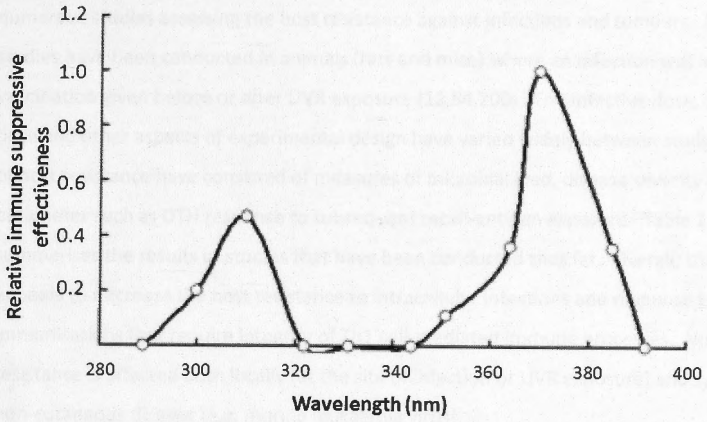
Given that the majority of ground level UVR from sunlight is comprised of UVA, and that there is ~90 times the level of UVA at 370nm than UVB at 300nm in sunlight, UVA has a far greater immunosuppressive effect than UVB at incidental doses of sunlight (i.e. less than ~ 20 minutes) (67,199). The immunosuppressive potency of UVR from sunlight is shown in Figure 2.2b.

Figure 2.2a UVR action spectrum for immunosuppression



This graph was generated following experiments assessing nickel contact hypersensitivity responses following narrowband UVR exposure at 11 wavebands from 289nm to 392nm and comparing with responses in adjacent unirradiated skin. Peak immunosuppression occurs in the UVB wavelengths (300nm), with a much smaller peak occuring in the UVA waveband (370nm). Adapted from (67).

Figure 2.2b Solar UVR action spectrum for immunosuppression



This graph is derived by multiplying the wavelength dependency for UVR-induced immunosuppression at each waveband by the irradiance of each wavelength in solar UVR (at standard noon). Given the abundance of UVA relative to UVB in solar UVR at ground level, UVA contributes the most to the overall immunosuppressive properties of solar UVR. Adapted from (67).

2.3.8 UVR and host resistance to infections and tumours

Host resistance studies in animals

Evidence of the clinical significance of UVR induced immunosuppression has come from numerous studies assessing the host resistance against infections and tumours. Controlled studies have been conducted in animals (rats and mice) where an infection was induced or live vaccination given before or after UVR exposure (12,64,200). The infective dose, UVR exposure dose and other aspects of experimental design have varied widely between studies. Measures of host resistance have consisted of measures of microbial load, disease severity or an immune parameter such as DTH response to subsequent recall-antigen exposure. Table 2.4 summarises the results of studies that have been conducted thus far. Overall, UVR exposure appears to decrease the host resistance to intracellular infections and response to immunisations that require integrity of Th1 cell-mediated immune processes. Notably, host resistance is affected both locally (at the site of infection or UVR exposure) and systemically for non-cutaneous disease (e.g. murine leukaemia virus).

Observational studies in humans

Conducting host resistance studies in humans is considerably more ethically and logistically challenging than in animals, and therefore evidence of the influence of UVR exposure has largely been derived from non-controlled, observational studies. Surrogates of UVR exposure such as latitude (i.e. low latitude/tropical region *vs.* high latitude/temperate region) or season (i.e. winter *vs.* summer) have often been used, and the prevalence or incidence of an infection or disease has been used as a measure of host resistance.

In a large Japanese study that assessed the role of various precipitants of recurrent Herpes simplex virus (HSV)-1 infection (responsible for oral 'cold sores'), sunlight exposure was the third most important precipitating factor after fatigue and upper respiratory tract infection (201). Incidence of varicella zoster virus (VZV) clinical infection (shingles) has also been shown to have a seasonal variation in two recent epidemiological studies – with lowest rates occurring in the winter (correlating with lowest ambient UVR) (202,203). There was also a report of higher recurrence rate of HSV and shingles in an immunosuppressed population (renal transplant recipients) (204). UVR is postulated to affect latent viral skin infections (e.g. HSV and VZV) in the following ways: i) indirectly, via impairment of critical virus-controlling cell-mediated immune processes, and ii) directly, via effects on the viral genome affecting latency within nerve endings (205).

Table 2.4: Host resistance studies in animal models with UVR exposure

Microorganism	Model	Effect
Mycobacterium bovis	Mouse	DTH:↓/no effect; microbial load ↑
Mycobacterium leprae	Mouse	↓DTH; ↑microbial load; ↑symptoms
Listeria monocytogenes	Mouse	↓DTH response; microbial load ↑
Candida albicans	Mouse	DTH:↓/no effect; ↑mortality
Murine leukaemia virus	Mouse	↑liver damage; ↑splenic pathology
Rat cytomegalovirus	Rat	↑viral load, inflammation and tissue necrosis
Herpes simplex virus	Mouse / Rat	↓DTH; ↑severity of lesions; viral load ↑
Borrelia burgdorferi	Mouse	↓DTH; ↑symptoms
Trichinella spiralis	Rat	↑parasite load; ↓T-cell immunity to parasite; ↓IgE
Leishmania major	Mouse	↓DTH; ↓symptoms
Schistosoma mansoni	Mouse	No effect
Plasmodium chabaudi	Mouse	↑mortality
Influenza	Mouse	↑mortality; ↑IFN-γ in lungs; ↑thymic atrophy
Sources: (7,12,64,200,205)		

A number of studies have also examined the association between UVR exposure and efficacy of vaccination at a population level (see Table 2.5). These studies have varied by population (e.g. paediatric *vs.* general), geographic region, type of vaccine and time points for measuring immune end points (e.g. antibody response, disease incidence). Despite these differences in study design, again the overall picture is one of lower vaccine efficacy in regions or seasons associated with high ambient UVR exposure (e.g. summer season, low latitude/tropical regions).

Taken in total, these studies indicate that populations potentially exposed to high ambient UVR levels are at risk of decreased vaccine effectiveness and increased incidence of certain infections. However, the influence of potentially important modifying or confounding factors – such as demographic differences (e.g. gender and age profile), environmental conditions (e.g. temperature), socioeconomic variables (e.g. sanitation, poverty, malnutrition, overcrowding) and other prevailing infections – were not measured, and therefore firm conclusions are not possible.

Table 2.5: Observational studies – the effect of latitude, season and UVR exposure on vaccination response

Vaccine	Effect of UV Radiation	Reference	Region / Year of publication
Poliovirus	Antibody response higher in temperate *vs.* tropical area	(206)	Tropics (1972)
Poliovirus	Antibody response higher if vaccinated in winter *vs.* summer	(207)	Subtropics (1972)
Influenza virus	Immunogenicity higher if vaccinated in winter *vs.* summer	(208)	Russia (1987)
Hepatitis B	Initial antibody titre higher if vaccinated in winter *vs.* summer. No difference after final vaccination.	(209)	Netherlands (1999)
Rubella virus	Antibody response higher if vaccinated in winter *vs.* summer	(17)	Israel (2009)
Bacillus Calmette-Guérin	Vaccine offered greater protection against tuberculosis at higher latitudes	(210)	Tropics/subtropics (1994)
Measles	Immunity wanes with higher solar UVR exposure	(211)	India (2004)

2.3.9 Photoadaptation and photoprotection

"Photoadaptation" refers to adaptive changes within the skin that follow repeated doses of UVR exposure, such that a given cutaneous response diminishes with subsequent equivalent UVR doses (212). An obvious example of this would be skin erythema – the skin adapts to repeated solar UVR exposure by tanning and this diminishes the erythemal response to subsequent equivalent doses of UVR. "Photoprotection" refers to photoadaptive processes allowing the skin to nullify a subsequent single high dose of UVR (8).

The literature has recently been reviewed by various authors (8,93) to assess whether photoadaptation and photoprotection responses were also evident with regard to UVR-induced down-regulation of immune function. They concluded that repeated (or cumulative) UVR exposure did not lead to significant photoadaptation or protection for the majority of immune parameters tested, with epidermal DNA damage and macrophage phagocytosis (in animal studies) the notable exceptions to this.

2.3.10 Influence of skin colour on UVR-induced immunosuppression

There has been conflicting evidence from studies regarding the effect of skin colour on UVR-induced immunomodulation. An earlier study (213) demonstrated that in people with deeply pigmented skin (either via genetics or sun tanning), a single low dose of UVB radiation was able to deplete epidermal Langerhans cells. Additionally, CHS elicitation responses were reduced when DNCB hapten was applied to UVR exposed skin. The investigators concluded that melanisation did not protect against the immunosuppressive properties of UVB.

By contrast, a study conducted with participants of Fitzpatrick skin phototypes I to IV (192), demonstrated that skin types I/II experienced more severe SS-UVR induced immunosuppression (as measured by CHS sensitisation response to DNCB exposure) compared with those with skin types III/IV (although all groups experienced some degree of CHS response reduction).

Matsuoka et al (1999) administered whole body, low dose UVB irradiation to heavily and lightly pigmented participants, and evaluated peripherally circulating immune cells. NK cell activity was found to be significantly higher in the heavily compared with lightly pigmented group compared, whilst all other parameters were similar (214).

Given that skin pigmentation and photo-type are under polygenic control (76), it is likely that the susceptibility to UVR is also influenced by the prevalence of genes encoding for release of soluble mediators by the skin cells (i.e. IL-10, TNF- α) within different ethnic groups (192).

2.4 Vitamin D and immune function

2.4.1 Introduction

Despite the potential negative health consequences of solar UVR exposure, it performs a vital role in catalysing endogenous vitamin D production. The biologically active form of vitamin D, calcitriol ($1,25(OH)_2D$)), is an essential steroid hormone required by the body for optimum bone and muscle function (through calcium regulation), photo-protection (reducing UVR-induced cellular DNA damage) and possible immune system regulation (see Section 1.2).

2.4.2 Mechanisms of vitamin D-associated immunomodulation

The 'non-classical' immune modulating action of vitamin D is mediated through the vitamin D receptor (VDR) and results in altered gene expression. The VDR is a member of the nuclear receptor family of transcription factors and is produced by many immune cells (e.g. monocytes, activated macrophages, DC, NK cells, B- and T-cell lymphocytes) (3,104,120,121).

Central to the influence of vitamin D on immune function is the capacity of extra-renal tissues (including keratinocytes, macrophages, T-cell lymphocytes and dendritic cells) to up-regulate the 1α-hydroxylase gene (*CYP27B1*), so that the complete pathway of vitamin D synthesis (from UVB-induced vitamin D precursors to calcitriol can be generated by keratinocytes within the skin *in vivo* (215). Although cutaneous production of calcitriol is unlikely to be a significant contributor to circulating levels of the active hormone, autocrine and paracrine effects may be of importance to immune function in the skin.

2.4.3 Vitamin D and the innate immune system

The innate immune response against *Mycobacteria tuberculosis (M.tb)* is a striking example of the influence of vitamin D on immunity. It has been recently shown that activation of certain pathogen recognition receptors for Gram-positive bacteria and *M.tb* (e.g. Toll like receptors (TLR2/1)) leads to up-regulation of the *VDR* and *CYP27B1* genes in monocytes (216). Increased local production of calcitriol acts in an autocrine fashion on the VDR, resulting in increased expression of the anti-microbial peptide, cathelicidin (LL37), which has activity against *M.tb*. Furthermore, the level of cathelicidin expression appears to be directly associated with the vitamin D status of the host – indicating that local conversion to calcitriol may be an important step (217,218). Many immune cells have now been shown to produce antimicrobial peptides (e.g. LL37, β-defensin 2) under the influence of calcitriol including monocytes, macrophages, neutrophils and epithelial cells (219). There appear to be numerous other innate immune modulating effects of calcitriol including enhanced microbial killing via alteration of pathogen cell membranes, enhanced macrophage phagocytic activity, increased chemotaxis of inflammatory cells and down-regulation of pattern-recognition receptors (219,220).

2.4.4 Vitamin D and the adaptive immune system

Calcitriol influences the adaptive immune system in numerous ways including effects on the proliferation and differentiation of the main constitutive cells (i.e. antigen presenting cells, T and B-cell lymphocytes) (12). As a general rule, evidence suggests that calcitriol exerts an overall inhibitory or regulatory action on the adaptive immune system (221).

Experiments have shown that calcitriol inhibits the maturation of monocyte-derived dendritic cells, impairing their ability to process and present antigen (222,223). Calcitriol modulated dendritic cells also appear to induce Treg cells (CD25+ FoxP3+ IL-10 secreting) (224) and to down-regulate the production of co-stimulatory molecules (HLA-DR, CD40, CD80, CD86) that activate T cells (225). The results of these critical influences are to promote a tolerogenic or regulatory phenotypic role for dendritic cells (226).

Calcitriol appears to have wide ranging effects on T-cell lymphocytes. Expression of VDR is increased upon T-cell antigenic activation. Calcitriol acts subsequently to reduce T-cell proliferation (via ↓IL-2) and influences the differentiation into effector T cell populations (via ↓IL-12) (220). The bulk of evidence suggests that calcitriol preferentially suppresses Th1 immune processes and promotes Th2 processes (227,228). However, there is evidence suggesting that Th2 responses may also be suppressed (229). Additionally, development and proliferation of Th17 cells, important for neutrophil function and inflammation, are suppressed by calcitriol via reduced IL-23 and IL-6 production (221,230). As previously mentioned, Treg cells (that can secrete the immunosuppressive cytokine IL-10) are promoted by $1,25(OH)_2D$-induced tolerogenic dendritic cells. In addition, calcitriol can also directly induce Treg differentiation (231). Finally, calcitriol appears to influence the migration of T-cell lymphocytes, with increased homing to areas of inflammation in the skin (via up-regulation of chemokine receptors on keratinocytes (e.g. CCR10)) (232) but decreased migration to lymph nodes (233).

Further evidence suggests that calcitriol influences B-cell lymphocyte proliferation and activity, working in an autocrine or paracrine manner (107). Early work suggested that B cell function, including antibody production, may be indirectly influenced by T-cell mediated effects of calcitriol (i.e. T cell dependent antibody response) (234). More recently, Chen et al., (2007) found that there were direct calcitriol effects on B-cell proliferation, differentiation (including inhibition of plasma cells and post-class switching memory cell production) and antibody production (235).

2.4.5 Epidemiological evidence of the immunomodulatory effect of vitamin D

A number of immune system-mediated diseases have been epidemiologically linked to host vitamin D status – primarily to deficiency in circulating 25(OH)D. In particular, the demonstration of calcitriol-induced down-regulation of cell-mediated (Th1 and Th17) immune processes gave weight to evidence suggesting a link between vitamin D deficiency and increasing incidence of so-called 'Th1- mediated' autoimmune diseases such as multiple sclerosis. The positive effect of calcitriol on the innate immune system (i.e. for antimicrobial peptide production), also suggests an association with reactivation of particular infections (e.g. *M.tb*) in the setting of vitamin D deficiency (3,106,236).

Vitamin D status and *Mycobacterium tuberculosis*

The beneficial effects of UVR for *M.tb* infection have been known for over a century (237). Numerous studies have shown that vitamin D deficiency is associated with reactivation of *M.tb* clinical disease (238). However, recent observations that calcitriol promotes *M.tb*-induced cathelicidin production (218) has prompted research into whether the relationship is causal and if vitamin D supplementation could reduce the risk of relapse or severity of infection (220). One study has shown that vitamin D supplementation with standard therapy reduced the time for sputum clearance of organisms (239). However, a randomised, placebo- controlled study conducted in Africa did not show any benefit of vitamin D treatment above that of standard therapy when assessing clinical end points, including mortality at 12 months. It should be noted, however, that vitamin D status was not improved by supplementation (two high doses 100,000IU over 8 months) in the intervention arm of this study (240).

Other clinical infections linked to reduced antimicrobial peptide production associated with vitamin D deficiency include *Bordetella bronchiseptica*, *Pseudomonas aeruginosa* and *Helicobacter pylori* (121).

Vitamin D status and autoimmune disease

An inverse association between occurrence of multiple sclerosis (MS) (an autoimmune demyelinating condition affecting the central nervous system) and latitude has been known for many decades (241–244). However the potential link to host vitamin D status has only more recently been explored (245). Studies using an experimental model of MS in mice (experimental autoimmune encephalomyelitis) have shown that treatment with calcitriol (resulting in hypercalcaemia) suppressed the symptoms of disease (246,247). Various studies have now shown that higher levels of circulating 25(OH)D are associated with a lower incidence of MS (245) and also a lower rate of clinical relapse or new lesions in existing cases (248). Recent and ongoing research has focussed on whether vitamin D supplementation can

alter the epidemiology and clinical course of MS, with conflicting provisional results (236,249,250).

The incidences of other autoimmune diseases that have also been shown to be inversely associated with vitamin D status include rheumatoid arthritis , inflammatory bowel disease and Type 1 diabetes (106,251–253).

2.5 Summary

The human immune system protects the host from infection and from proliferation of aberrant cells, and comprises innate (non-specific) and adaptive (antigen-specific) components. The skin acts as a crucial interface between the immune system and solar UVR exposure. UVR is absorbed by chromophores (i.e. DNA, urocanic acid and cellular membrane components) within epidermal cells that then initiate a complex cascade of immune processes. The overall effect is one of down-regulation of cell-mediated (Th1) processes and promotion of a regulatory environment within draining lymph nodes. Contact hypersensitivity and delayed hypersensitivity testing - *in vivo* assays of cell-mediated immunity - have shown that UVR exposure (at various doses, wavelength bands and anatomical sites) leads to antigen-specific local and systemic immunosuppression. The action spectrum for UVR-induced immunosuppression indicates that there are two immunosuppressive peaks for solar UVR – centred around 300nm (UVB) and 370nm (UVA). Host resistance and observational studies indicate that UVR-associated immunosuppression may have clinical relevance, with UVR exposure leading to an increased burden of infectious diseases in animal and human models and decreased vaccine efficacy.

Calcitriol ($1,25(OH)_2D$) has immunomodulatory properties that are mediated by the vitamin D receptor (VDR) found in the nucleus of many immune cells. Calcitriol can be derived from the systemic circulation or be produced locally by keratinocytes or by immune cells (e.g. macrophages, T-cell lymphocytes). Calcitriol can have autocrine, paracrine or endocrine effects on immunological processes. Calcitriol can stimulate the production of anti-microbial peptides (e.g. cathelicidin), that are important components of the innate immune response. With regard to adaptive immune responses (and similar to the overall direct effect of UVR exposure), calcitriol acts to promote a tolerogenic / regulatory T-cell environment. Adding clinical weight to these findings, growing epidemiological evidence suggests an association between decreased 25(OH)D levels and the incidence or prevalence of so-called "Th1-mediated" autoimmune diseases (e.g. multiple sclerosis).

UVR exposure appears to modulate immune responses, either directly or indirectly via the action of calcitriol. However, much remains unknown, including the effect of solar UVR on immune function at doses relevant to activities of daily living.

The following chapter will discuss the field of immunotoxicology, whose principles and methods are relevant for conducting studies aiming to assess the influence of environmental exposures (such as UVR) on immune function.

Chapter 3: Immunotoxicology

Synopsis

The principles underpinning the field of immunotoxicology can inform an assessment of the effect of personal solar UVR exposure on human immune function. This chapter is divided into the following sections: i) overview and principles of immunotoxicology; ii) conducting an epidemiological study assessing the potential for UVR-mediated immunotoxicity; and iii) management of other immunomodulatory factors.

3.1 An overview of immunotoxicology

The field of immunotoxicology relates to the study of adverse influences of 'agents' on immune function and the resultant risks posed to health (20). Agents can be chemicals (including pharmaceuticals and pollutants), biological materials, medical devices or physical factors (e.g. UVR) (254,255). The adverse influences on immune function can manifest in diverse ways, from immunosuppression predisposing to infection and cancers, over-stimulation leading to allergy or chronic inflammatory states, or impaired tolerance predisposing to the development of autoimmune disease (21).

It is important to emphasise that robust homeostatic immune mechanisms are at play when the host immune system is challenged. The influence of putative immunotoxic agents may not necessarily result in "pathological deviation from a homeostatic norm for host resistance" (256) (pp. 363), as there are generous redundancies in immune processes and multiple immune pathways that assist return to homeostasis. Changes in immune function that lead to illness or disease result from more significant perturbations of an immune system unable to return to a homeostatic state, for example, following a more sustained or intense exposure to an immunotoxic agent or more minor changes in a susceptible host. Figure 3.1 illustrates the common pathways that maintain homeostasis and that can also lead to immune-mediated disease.

Figure 3.1: The possible pathways to toxin-mediated immunomodulation (Source: 256)

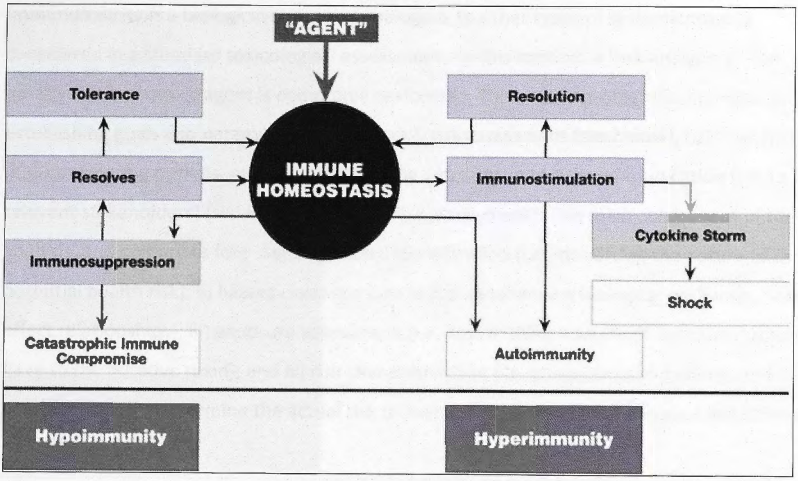


Table 3.1 lists examples of agents that have been implicated in immune-mediated manifestations. Historically, most research has focussed on immunosuppressive agents such as the halogenated aromatic hydrocarbon group (which includes dioxins and polychlorinated biphenyls (PCBs)) or agents causing contact allergy (e.g. latex) (254). More recently, research has diversified into developmental immunotoxicology, autoimmunity, and refinement of molecular techniques to determine patterns of genetic susceptibility (257).

Table 3.1: Examples of immunotoxic agents and their main immunomodulatory effect

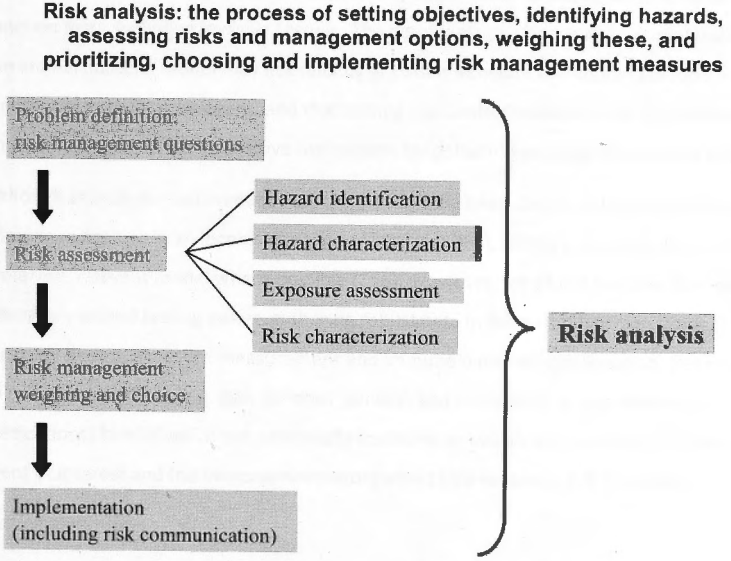
Immune outcome	Examples of implicated immunotoxic agent
Immunosuppression	Polyhalogenated aromatic hydrocarbons (i.e. polychlorinated biphenyls), pharmaceuticals (e.g. cyclosporine, anti-neoplastic agents), metals (e.g. lead, cadmium, mercury), radiation (e.g. UVR, ionizing), pesticides (e.g. chlordane, parathion)
Allergy	Metal salts (e.g. chromium, nickel), anhydrides (e.g. phthalic), proteins (e.g. latex), dyes (e.g. rifafix yellow), wood dusts (e.g. western red cedar), epoxy resin, mercaptobenzathiole
Autoimmunity	Organic solvents (e.g. polyvinyl chloride), antibiotics (e.g. penicillins, sulphonamides), anti-convulsants (e.g. phenytoin)
Chronic inflammation	Beryllium, silicosis, organic dusts containing dairy or grain products

Adapted from (254)

3.1.1 Approach to immunotoxicological studies

Immunotoxicity is a biological end-point analogous to other types of systemic toxicity considered in a standard toxicological assessment. In this context, a 'risk analysis' of the putative immunotoxic agent is commonly performed. This entails problem formulation (e.g. establishing goals and parameters of the study), risk assessment (see below), risk management (e.g. considering options, making and actioning decisions) and risk communication (i.e. to relevant stakeholders) (see Figure 3.2) (21). Risk assessment is the main component of the risk analysis, and comprises four steps: i) hazard identification (i.e. identifying the nature of the potential health risk); ii) hazard characterisation (i.e. determining biological pathways, dose-effect relationships); iii) exposure assessment (i.e. determining how much exposure humans have to the putative toxin); and iv) risk characterisation (i.e. combining the findings of the previous steps to determine the actual risk to humans at varying levels of exposure) (258).

Figure 3.2: Steps involved in undertaking an immunotoxicological risk analysis



Source (21)

Hazard identification and characterisation of ultraviolet radiation as an immunotoxic agent have been made via extensive studies in animals and humans as outlined in Chapter 2. This thesis is primarily concerned with the latter components of risk assessment - exposure assessment (i.e. what levels of UVR exposure are healthy adults routinely exposed to?) and risk characterisation (i.e. given the level of UVR exposure and known immunosuppressive pathways, what is the extent of immunosuppression in humans from routine UVR exposure?).

3.1.2 Testing strategies in immunotoxicology

Animal versus human data

The vast majority of immunotoxicological studies performed thus far have been conducted using animal models, primarily rodents (259–261). Apart from the logistic, time and cost benefits of conducting laboratory animal rather than human research, the following advantages are afforded: i) control / exclusion of potential confounding variables; ii) precise measurement of exposure variables; iii) opportunity to test a wide range of exposure doses; iv) use of genetically homogeneous animals; and v) opportunity to use an array of immune function tests, including invasive testing. The disadvantage of laboratory animal testing is that the animal immune model may not reliably or closely simulate the human immune response to a potential immunotoxic agent, and that testing in a controlled-laboratory (unstimulated) environment may be an insensitive test system for detecting immune dysfunction (21,262).

Although animal-derived immunotoxicology data have been shown to be predictive for human immune outcomes in an increasing range of settings (263), primary human data are the preferred, relevant model where possible (256). However, for all the reasons that make laboratory animal testing easier, gathering robust data in humans poses significant challenges for study design, exposure measurement and immune parameter delineation. Humans differ by numerous intrinsic (e.g. age, genetics, gender) and extrinsic (e.g. diet, behaviour, medications) factors which can potentially confound or modify the association between the agent of interest and the immunomodulatory effect (see section 3.2.3) (256,264).

Testing algorithms

As the influence of immunotoxic agents may be subtle, intermittent, and affect various components of the immune system differentially, the choice of immune parameters to be tested in immunotoxicological studies is of paramount importance. The general aims of immunotoxicological testing are to:

i) Choose sensitive tests that can detect subtle immunological changes

ii) Assess the specific component/s of the immune system most likely to be affected, based on current knowledge (i.e. innate vs. adaptive; humoral vs. cell-mediated) and at the most appropriate site (e.g. based on the site of exposure interface)

iii) Use a combination of tests that can confirm a pattern of immunomodulation (i.e. changes in values of parameters in a single test may not reflect true immunomodulation)

iv) Choose tests that can assess immune parameters at the appropriate time points for suspected immunomodulation (particularly if changes are transient)

Tier testing

In the 1980s, groups in the Netherlands and United States (in particular), performed much of the early work in establishing screening and functional assays in mice and rat studies involving chemical immunotoxicity (265,266). As part of the US National Toxicology Program (NTP), Luster et al (266) tested over 50 putative chemical immunotoxins and developed a comprehensive battery of tests that were divided into two 'tiers' (see Table 3.2) – Tier I tests acted as a screen to detect evidence of immunotoxicity resulting from an agent, and Tier II tests were more comprehensive tests designed to further define the immunotoxic effect. Whilst no individual test was found to be completely predictive for immunotoxicity, later validation studies demonstrated that combinations of two or three immune tests increased the sensitivity of predicting immunotoxicity of tested chemicals in mice to over 90% (and up to 100% using specific combinations (267)). Quantification of the T cell dependent antibody response (i.e. to sheep red blood cell immunisation) (78% concordance) and/or quantification of lymphocytes by analysis of cell surface markers (83% concordance) had very high predictive properties, whilst some Tier I screening tests (i.e. white cell count and organ weights) were quite insensitive. Further modelling studies examined the relationship between the above immune function tests and 'hard' functional end-points such as host-resistance tests (i.e. following exposure to an immunotoxic agent, the laboratory animal was challenged with infection or tumour cell transplantation at a dose sufficient to cause morbidity in 20% of animals) (268). These studies showed a 'good' correlation between immune function assays and host resistance end-points, with high sensitivity for detecting diminished host resistance, but with low specificity. However, no individual assay or combination of assays was particularly predictive of diminished host resistance.

The development of numerous national and International guidelines on immunotoxicology testing (21,264,269,270) has been informed by the seminal work conducted by the NTP and others, and is reflected in recommendations which have changed by iteration over the years (reviewed in: 20).

Table 3.2 Tiered approach to immunotoxicology testing in animals

Tier	Immune function tests
Tier I (Screening)	• Immunopathology (e.g. spleen, lymph node &/or thymus histology) • Haematology (e.g. white cell count and differential) • Total body and organ weights (e.g. spleen, thymus, kidney) • Functional immune testing (e.g. quantification of IgM antibody plaque-forming cells following T-cell dependent antigen immunisation; lymphocyte proliferation assays using non-specific mitogens; and NK cell activity).
Tier II (Comprehensive)	• Quantification of splenic B & T lymphocytes (cell surface markers) • Cytotoxic T-lymphocyte assays • Quantification of IgG antibody response following T-cell dependent immunisation • Host resistance challenge – testing host resistance to infection (e.g. bacterial, viral, parasite) or tumour cell transplantation

Source: (267)

3.1.3 Immunotoxicology testing in studies involving humans

The tier approach to immunotoxicology testing can also be applied to human research (264), however data validating its use are limited (271) and there are considerable logistic and ethical issues with obtaining appropriate samples (e.g. tissue for histology or weight) or conducting host resistance testing.

A comprehensive report resulting from a World Health Organisation (WHO)-sponsored symposium (23) synthesised the views of International experts in the field of clinical immunology, epidemiology and immunotoxicology and concluded that the "antibody response to a primary immunisation is recommended as the most convenient and comprehensive measure of immunocompetence" and the "primary immune response is also among the most predictive assays for immunotoxicity in laboratory studies" (pp. 141). Delayed-type hypersensitivity testing, NK cell assays and phenotypic analysis of lymphocyte surface markers

by flow cytometry have also been suggested by various authors (23,256,264). The postulated or known mechanism of immunomodulation has a large influence on the types and range of immunology tests chosen.

3.1.4 T-cell dependent antibody response

Quantification of a primary antibody response to immunisation with a T-cell dependent (TD) antigen has been promoted as a sensitive tool for assessing immunotoxicity in epidemiological studies (22,23,272). In the literature, the immune response to immunisation with a TD antigen is commonly referred to as T-cell dependent antibody response or 'TDAR'.

Protein antigens (e.g. sheep red blood cells, keyhole limpet haemocyanin, tetanus toxoid, diphtheria toxin, poliovirus, hepatitis B surface antigen) are examples of TD antigens (274–276). Antigens are presented to naïve T-cells by APCs (e.g. Langerhans cells) in the form of a peptide:MHC class II complex. This interaction leads to the generation of antigen-specific T-helper cells (CD4+) (See section 2.1.1). A humoral response from naïve B cells cannot be generated against TD antigens without T-helper cell stimulation. Naïve B cells detect TD antigen which binds to surface immunoglobulin and internalised, processed and re-displayed on the cell surface as a peptide:MHC class II complex which can be recognised by a primed T-helper cell. The bound T-helper cell up-regulates the B-cell stimulatory molecule, CD40 ligand, leading to T cell production of cytokines (e.g. IL-4, IL-5, IL-6) that stimulate B cells to proliferate and differentiate into antibody secreting plasma cells and resting memory B cells (134). As described above, the generation of a primary TDAR requires all pathways of the adaptive immune system to be functioning and therefore an immunotoxic agent-induced deficit of any component will be reflected by reduced antigen specific antibody production (261). Primary immune responses to TD antigens are considered a more sensitive immunotoxicological tool compared with secondary responses, as the latter does not necessarily require operation of the full range of adaptive immune processes (264).

In animal immunotoxicity research, TDAR using sheep erythrocytes or keyhole-limpet haemocyanin (KLH) antigen has become the functional primary immune assay of choice (275,277,278). For human epidemiological immunotoxicology studies, opportunistic monitoring of the responses to routine childhood vaccinations (e.g. tetanus, diphtheria, pertussis) has been advocated (23,272).

Determining what is an ideal vaccine candidate for generating TDAR

The properties for an ideal TD antigen vaccine have been previously described (23,264,279). Ideally, such antigens should:

i) Be a pure homogeneous substance available as a clinical grade product;

ii) Be harmless in humans, if not beneficial, to the recipient (e.g. commercially available vaccination where protection from a commonly encountered infectious agent is conferred);

iii) Be highly immunogenic for the whole population without any genetic restriction;

iv) Have no natural or cross-reacting antibody;

v) Elicit predictable primary antibody responses and/or a delayed hypersensitivity response (without need for an adjuvant) following a single administration;

vi) Produce a measurable antibody response which can differentiate subtle changes in immunomodulation (i.e. have high sensitivity to detect change);

vii) Have a quantifiable humoral response that can be measured using validated tests (e.g. enzyme-linked immunosorbent assay).

Table 3.3 shows the commonly used TD antigen vaccines in animal and human research and how they fulfil the criteria listed above. Commercially available vaccines (e.g. hepatitis B, influenza, tetanus) have the advantages of already passing strict safety regulatory processes, providing a protective benefit for study participants and being available in a clinically appropriate form. The main disadvantages are that in a non-paediatric population, many participants will have already been exposed to antigen from wild-type infection or previous vaccination; and commercial vaccines produce a very robust immune response thereby potentially overwhelming the assay's ability to detect subtle changes in immune response. As mentioned, keyhole limpet haemocyanin is often used in animal immunotoxicological research and has many of the qualities of an 'ideal' vaccine candidate (279) and will be discussed in more detail in Chapter 4.

Table 3.3: Candidate thymus-dependent antigen vaccines for immunotoxicology studies

TD-antigen Vaccine candidate	Regulatory safety testing in humans	Provides protection against infection?	Previous exposure to antigen (wild-type infection or immunisation)	Natural or cross-reacting antibody if previously naive?	Reliable antibody response after single administration?	Antibody level cut-off correlating with clinical protection?	Sensitive enough to detect immunomodulation	Studies using vaccine in human immunotoxicological studies
Sheep erythrocytes	No	No	No		Yes	N/A		No
Keyhole limpet haemocyanin	Yes	No	No	*Schistosoma mansoni* #	Yes	No	Yes, depending on dose	Yes (280–282)
Measles	Yes	Yes	Possible/ probable	No	No**	Yes		Yes (283)
Influenza	Yes	Yes	Possible	Possible*	Yes**	No		Yes (284,285)
Hepatitis B	Yes	Yes	Possible	No	No**	Yes	May overwhelm immune-modulatory signal	Yes (19,286)
Tetanus	Yes	Yes	Possible	No	No**	Yes		Yes (287)

*Antibodies to non-vaccine strains of pathogen # Reference (288) **Source: (289)

3.1.5 Use of TDAR in immunotoxicological epidemiology studies

The next section gives examples of immunotoxicological studies in human populations where TD antigen immunisations have been used.

Commercially available hepatitis B vaccine has been used in two recently reported (and related) studies assessing the effect of pesticide exposure on the vaccine response (286,290). In both studies, hepatitis B antibody was quantified approximately two weeks following the second vaccination. Neither study found a significant difference in hepatitis B antibody titres between exposed and non-exposed groups, which, when combined with the results of other immune parameter testing (e.g. total immunoglobulin, complement levels, lymphocyte subpopulations and natural killer cell levels), lent weight to the conclusion that pesticide exposure in these study settings did not influence immune function in a way that posed a health hazard.

Weisglas-Kuperus et al (283) followed a Dutch cohort of 207 Caucasian mother-infant pairs from birth to 42 months and investigated whether the immunotoxic effects of prenatal exposure to polychlorinated biphenyl (PCB) and dioxin persisted into infancy. As part of the Dutch immunisation program, infants received primary vaccination to measles, mumps and rubella at approximately 14 months of age. At 42 months, blood was assayed for antibodies against the vaccine constituents, and in a subgroup, lymphocyte subpopulations. The authors found that prenatal PCB exposure was associated with significantly lower antibody levels to mumps and measles at 42 months, as well as altered T-cell subsets.

Two observational studies have used primary vaccination to assess the influence of season of vaccination on subsequent vaccine-induced antibody response. Season of vaccination was used as a surrogate of ambient UVR, the putative immunotoxic agent. In a recently reported Israeli study (17) rubella antibody titres were assayed three to four years after primary rubella vaccination in 203 infants of similar ethnicity and skin complexion attending kindergartens. The researchers found statistically higher rubella antibody titres in infants vaccinated in winter compared with summer months (mean geometric titre 73.0 IU *vs.* 47.6 IU; $p<0.05$), and a similar trend with regard to proportions of infants adequately vaccinated (i.e. rubella titre >15 IU).

Similarly, a cohort of young Dutch adults vaccinated with hepatitis B vaccine had lower vaccine specific antibodies after the first and second vaccinations (administered at 0 and one month respectively) when these were given in summer compared to winter (209). However, soon after the third vaccination (at six months), the difference in antibody levels between groups was no longer apparent.

In the only reported controlled study aiming to assess the influence of UVR exposure on vaccination response, half of a group of 191 healthy young Dutch volunteers were randomised to receive five days of one (personal) MED of whole-body UVB, whilst the other half remained unexposed controls (19). Both groups were vaccinated with a commercial hepatitis B vaccine preparation (at 0, 1 and 6 months). At various time points post-vaccination, blood was taken for hepatitis B-specific antibody titres, lymphocyte proliferation assays and NK cell assays. Although there was evidence of local immunosuppression at the site of UVB exposure (evidenced by reduced CHS responses), there were no differences in antibody levels or T-cell proliferation assays between exposure groups. This study will be discussed in more detail in Chapter 8.

3.1.6 Delayed-type hypersensitivity response

The DTH response is an *in vivo* assessment of cell-mediated immunity and has long been used as a measurement assay in immunotoxicological research. The NTP analysis of the predictive effectiveness of assays highlighted DTH testing as having high concordance with immunotoxicity particularly when used as a component of a multi-functional evaluation (267). In clinical practice the DTH response to purified protein derivative (PPD) has a long history of use for determining past exposure to *M.tb* (i.e. the tuberculin skin test or 'Mantoux test') (291).

The DTH response (otherwise classically known as a Type IV hypersensitivity reaction) manifests as a small area of indurated (i.e. raised, oedematous, often erythematous) skin. It develops on re-inoculation of antigen into the dermal layer of skin when the host has been pre-sensitised to the antigen (i.e. had prior exposure naturally or via vaccination). DTH inducing antigens can be proteins, *Mycobacteria*, haptens or grafted tissue. The DTH response classically takes 24 – 72 hours to develop, although there is some variation depending on the antigen used (292).

The immunopathogenesis of DTH responses has largely been elucidated. The first step is antigen-specific priming (sensitization) of naïve T-helper cells, which can occur through natural exposure to antigen from infection or via immunisation (e.g. prior Bacillus Calmette-Guérin (BCG) vaccine in the case of tuberculin skin testing). Antigen presenting cells display antigen in the form of antigen:MHC class II complexes and release IL-12 and IL-18, promoting differentiation of naïve T helper cells to Th1 lymphocytes (293). The 'elicitation' phase on re-exposure to the antigen usually occurs in the dermal layer of the skin, where immune responses can be easily monitored. Following re-exposure to antigen, a number of cell types are recruited to the site of challenge including neutrophils, dendritic cells, macrophages and memory T-helper (particularly Th1) lymphocytes, which are present in differing concentrations

at varying time-points (293). Numerous cytokines and chemokines are released, promoting the inflammatory response and include: IL-8, monocyte chemoattractant protein-1, macrophage inflammatory protein 1-alpha, TNF-α and granulocyte-macrophage colony-stimulating factor (294). P- and L- selectins are also very important in attraction and formation of the multicellular infiltrate, and particularly for binding of memory Th1 cells (295). Regulatory T cells can also influence the development of the DTH response (296,297).

Essentially, the DTH response is a Th1-driven cell-mediated immune process which can be disrupted by problems with initial T-cell sensitisation, interference with cellular trafficking and chemotaxis to the site of re-exposure or regulatory T-cell influences (294).

The DTH response is seen as a valuable functional *in vivo* test of cell-mediated immunity. There are a number of practical issues however. Although tuberculin skin testing has been standardised in terms of preparation of antigen, dosing, administration and interpretation of the result, the same is not true for other antigens (23). Furthermore, even with long established tuberculin skin testing, subjectivity with reading of responses is a significant ongoing challenge (298).

3.1.7 Use of DTH in immunotoxicological epidemiology studies

There have been numerous studies that have used DTH testing to assess the influence of potential immunotoxins on the immune response. The recall antigen(s) in these studies have usually been either tuberculin antigen (in those previously immunised with BCG) or the 'Multitest CMI' (a commercial preparation of seven commonly encountered antigens). The latter battery of antigens has been well validated and standardised in DTH testing (299).

A Taiwanese investigation into the immunological effects of accidental PCB ingestion (300) demonstrated that DTH responses to tuberculin and a streptokinase/streptodornase combination were significantly lower in the exposed group compared with controls, even many years after exposure. The percentage of exposed participants demonstrating a DTH response, as well as the size of the response, reduced with increasing markers of initial PCB exposure.

Several studies have assessed the immunotoxic properties of UVR by measuring the DTH response using either the Multitest CMI (16) or tuberculin antigen (183,185) (reviewed in Section 2.3.6).

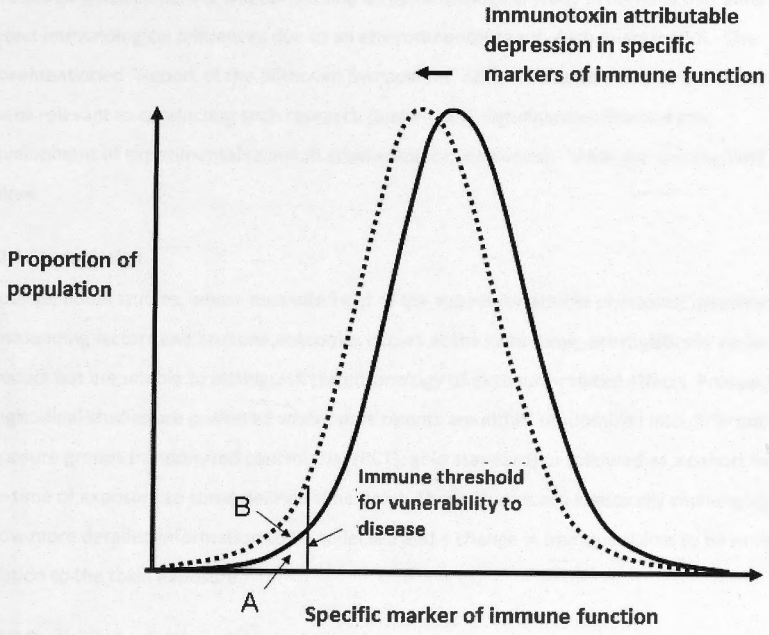
3.1.8 Clinical significance of testing

The purpose of conducting immunotoxicity testing is to establish changes in immune parameters (or 'biomarkers') that also reflect some real change in the susceptibility of the host to disease. Host resistance testing is one way to assess the predictive ability of changes in a given immune parameter – although this is more easily done in an experimental setting using animal models. Using various animal infectious disease and tumour models, immune tests were found to be very sensitive (though not specific) for detecting immunotoxic changes of clinical relevance (i.e. worsening infectious or tumour burden) (268). To determine whether a threshold level of immunotoxicity existed, the same researchers studied the effects of increasing doses of cyclophosphamide (a potent immunosuppressant medication) on animals as a host-resistance model. They found an essentially linear relationship between change in the immune parameter and host-resistance (albeit acknowledging the results were derived from a limited data set).

If the measured change in immune parameter is profoundly abnormal, such as very low lymphocyte counts in people with HIV infection (138) or primary immunoglobulin deficiency (302), individuals are at high risk of developing disease. In general though, using immune parameters as a surrogate for increased disease risk in the host is problematic, given the aforementioned functional immune reserve capacity (20). Therefore, a mild to moderate alteration to TDAR or DTH response in an individual following an exposure to an immunotoxin does not necessarily mean that an otherwise healthy individual is at risk of disease.

As previously noted however, if there is a linear relationship between change in an immune parameter and disease outcome, then at a population level (which would include vulnerable people – the aged, very young, ill), less profound impairment of specific immune processes occurring with high prevalence may manifest as an increased incidence of infection (e.g. influenza, otitis media or the common cold) (22) and reduced vaccine effectiveness (143) (Figure 3.3). Consistent with this view, Selgrade (303) stated that "it is reasonable in hazard identification to consider any statistically significant and consistent suppression on an immune function test as a potential risk to a population (pp.62)"

Figure 3.3: Changes in population susceptibility to disease as a result of immunotoxin exposure – a concept diagram



The proportion of a population susceptible to immune-mediated disease is represented by area 'A'. Exposure to an immunotoxic agent (e.g. ultraviolet radiation) is postulated to shift the population curve, such that a greater proportion of the population (Area 'A' + 'B') is susceptible to immune-mediated disease.

3.2 Conducting an epidemiological study assessing immunotoxicity of ultraviolet radiation

Apart from immunotoxicological parameters, careful consideration of numerous other factors is required when designing and conducting an epidemiological study in humans that aims to detect immunological influences due to an environmental agent, such as solar UVR. The aforementioned "Report of the Bilthoven Symposium" (23), canvassed many of the important issues relevant to conducting such research (and indeed, significantly informed the development of experimental research conducted for this thesis). These are summarised below.

3.2.1 Study design

Cross-sectional studies, where measurement of the exposure variable of interest, possible confounding factors and immune outcomes occurs at the same time, are logistically easier to conduct but are unable to distinguish the chronology of exposure-related effects. Prospective, longitudinal studies are preferred where participants are either randomized into different exposure groups (randomized control trial (RCT); gold standard) or followed as a cohort from the time of exposure to some defined time point. These studies are logistically challenging but allow more detailed information to be collected and a change in immune status to be noted in relation to the toxin exposure.

3.2.2 Participant selection and sample size

Based on previous research or pilot study data, calculations regarding an adequate participant sample size should be determined. This calculation will be based on the expected difference between exposure groups (either absolute difference or effect size), study power and alpha level. Decisions regarding participant selection will depend on: i) demographic of interest (e.g. children versus adults); ii) potential recruitment population (i.e. from the general population versus some select sub-population); iii) strategies to reduce confounding variables (e.g. well defined inclusion and exclusion criteria such as age or gender restriction); and iv) logistic and cost issues (e.g. research budget, research staff, time to completion).

3.2.3 Measurement of exposures and potential confounders

Using validated instruments to measure exposures and potential confounding variables is important. Direct measures are preferred for exposures rather than questionnaires or diary data which can be prone to recall error and/or bias. Environmental measures of exposure (i.e. ambient levels of UVR) or surrogates of UVR exposure (e.g. using latitude of residence) do not necessarily reflect an individual's exposure and risk false inferences about the exposure-outcome relationship (the so-called "ecological fallacy") (304). Measurement/s at an

individual level are therefore preferred (305). Keeping in mind the aforementioned limitations, questionnaires and diaries are convenient, low cost and frequently used in epidemiological studies. Using validated questionnaires (i.e. that have had internal consistency checks and been tested in reference populations) is critical to maximise their reliability (23). They are most useful when recording contemporaneous information rather than retrospective data. Questionnaires are also reliable for obtaining demographic information and other 'set' information (e.g. family history, medications) and information difficult to measure (e.g. clothing worn, sunscreen use, other sun protection behaviour).

3.2.4 Potential confounding variables and effect modifiers

In a study of UVR and immune function, there are many potential confounding variables including age, gender, smoking status, nutrition, vitamin D status, ethnicity, psychological state, physical fitness, co-existing infections, medications and comorbid conditions. It is critical to also take validated, detailed measures of potential confounding variables and effect modifiers where possible (as important as measuring the exposure variable), otherwise important associations may be mis-estimated, and/or misinterpreted. Some confounding can be avoided in the way participants are chosen (i.e. only females, restricted age or ethnic group, or other definable characteristic) which is important particularly in the non-RCT context. Section 3.3 will expand on the issues relevant to confounding variables.

3.2.5 Participant burden

A balance needs to be found where enough information is obtained from participants (i.e. from direct measurements, blood tests, questionnaires, diaries) to be able to assess associations between exposures and outcomes taking account of potential confounding factors, but without overburdening participants such that they do not accurately complete or fulfil study requests or attend study visits, or become distressed by their ongoing participation. The boundary between these outcomes is often not clear and maybe highly individualised; however this needs to be considered when designing and conducting the study.

3.3 Potential confounding variables

Numerous intrinsic and extrinsic conditions and exposures have the potential to influence the immune response to antigenic challenge. Those factors that *also* vary with UVR exposure could therefore be confounding variables. It is important to measure these variables, test them as possible confounders and adjust for them where necessary, in order to properly assess the effect of UVR exposure on immune function (306). This section will detail the evidence for relevant immunomodulatory variables and discuss in particular, how they may influence the response to immunisation.

3.3.1 Age

At both extremes of life, the mammalian immune system operates sub-optimally (307). During infancy, there is ongoing maturation of the immune system such that the immune response to polysaccharide antigen (in particular) is not robust until after the age of 2 years. In later life, the process of 'immunosenescence' or diminution of the immune system occurs with numerous elements of immune pathways being affected (See Table 3.4). Immunosenescence is a gradual process – indeed some structural changes begin even in childhood (e.g. thymic involution) but the functional and clinical manifestations do not manifest until later in life (308,309). The main clinical implications of immunosenescence are decreased response to immunisations, increased incidence of autoimmune disease and increased morbidity from infections (310).

Numerous studies have shown that post-vaccination antibody titres are lower in the elderly (or 'older' age groups), including for influenza (311), hepatitis B (312) and tetanus vaccines (313). For example, the proportion of antibody sero-conversion (to a level which corresponded with clinical protection) following hepatitis B vaccine was assessed in a group of adult Pakistani health care workers (n=652) (314). The proportion of serological non-conversion increased with increasing age, such that the odds of non-conversion in the >50 years group was 17.8 times higher than the <25 years group ($p<0.001$). Hainz et al (315) measured anti-tetanus toxoid antibody and tick-borne encephalitis (TBE) antibodies in vaccinated Austrians aged between 18 – 93 years. Post-vaccination antibody titres declined with age, in a linear manner throughout life for TBE antibodies, and from the age of 40 years for tetanus.

Table 3.4 Immunological changes with aging

Component of the Immune system	Summary of changes occurring with aging
T Lymphocytes - $CD4^+$ T cells	• ↓ T-cell receptor (TCR) signalling intensity • ↓ Expansion in response to TCR stimulation • ↓Th1 and Th2 effector differentiation • ↓Cognate helper function
- $CD8^+$ T cells	• ↓TCR repertoire diversity • ↓Anti-tumour responses
- Regulatory T cells	• ↑ Number • Retain/gain function with age • Down-regulation of anti-tumour responses • May contribute to skewing towards Th17 response
B Lymphocytes	• ↓Diversity of B cell repertoire • ↓Expression of co-stimulatory molecules (CD27 CD40) • ↓Antibody affinity • ↓Antibody class switching
Innate immunity	• Neutrophils: ↓oxidative burst, phagocytic capacity, bactericidal activity • Macrophages: ↓oxidative burst, phagocytic capacity • Natural Killer cells: ↓Proliferative response to IL-2, cytotoxicity; • ↑ Number of NK cells • Dendritic cells: ↓Capacity to stimulate T cells, lymph node homing • Cytokines & chemokines: ↑IL-6, IL-1β, TNFα serum level

Source: (308,316,317)

3.3.2 Sex

Human immune function is influenced by sex, largely due to differing levels of sex hormones but also due to behavioural, dietary and psychological differences. Oestrogens generally enhance immune function, particularly for humoral responses, whereas androgens (such as testosterone) are generally immunosuppressive to cell-mediated and humoral processes (318,319). In general, women have higher levels of circulating immunoglobulins and generate more vigorous humoral responses to exogenous antigens than males (320). Additionally, the prevalence of numerous autoimmune disease states is higher amongst females than in males. Examples include MS (321), Grave's disease (322) and autoimmune rheumatic disease (323).

Green et al (324) assessed the antibody response to live attenuated measles vaccine in 223 males and 66 females aged 18 -20 years in Israel. Females had higher geometric mean titres (~50% higher) at both 2 and 4 weeks post-vaccination compared with males. In the previously described study of Pakistani healthcare workers (314), young males demonstrated more than twice the rate of serological non-response to hepatitis B vaccination compared with young females (18% vs. 8%; $p<0.001$), consistent with the findings of other research (325).

A recent systematic review (326) of studies specifically examining the differences by sex in the humoral response to vaccines, demonstrated higher antibody responses in adult females compared with males for influenza (elderly adults, young adults with acute stress and eccentric exercise), hepatitis A, hepatitis B, rubella, measles, tetanus and *Brucella*. Male adults had higher vaccine-associated antibody responses than females for pneumococcal polysaccharide (healthy and alcoholic adults), yellow fever and meningococcal A and C. Clinical vaccine effectiveness was consistent with the above findings (i.e. females had less clinical disease post-vaccine than males for influenza, hepatitis A and B; males had less pneumococcal disease). All the studies assessed, regardless of vaccine type and mechanism for promoting immune response, showed differences in humoral response by sex. The author therefore concluded "it (be) mandatory that vaccine clinical trialists recruit a representative sample of females and males to be able to assess sex-differences which may have clinical implications" (pp. 3555).

3.3.3 Genetics and ethnicity

One of the most challenging issues in conducting non-experimental epidemiological studies is accounting for the heterogeneous genetic makeup of human participants, unlike the controlled, genetically homogeneous conditions of inbred animal research. Much of the important genetic variation pertaining to human immune function relates to genes (human leucocyte antigens (HLA)) encoding the MHC class I and II molecules that are critical for presenting antigen to T lymphocytes. There are over 400 alleles (variant HLA genes) encoding for MHC class I and II molecules (134). This variation or 'polymorphism' affects antigen

recognition by T cells by influencing peptide binding and the interaction between T-cell receptor and MHC molecule (134). Variations in responses to influenza, measles and hepatitis B vaccines have been noted in individuals with certain HLA combinations (haplotypes) (Reviewed in 86).

Non-HLA genetic polymorphisms have also been associated with vaccine responses. Sleijffers et al (328) found that participants in their hepatitis B randomised controlled study that were homozygous for a minor variant (polymorphism) of the IL-1β gene, demonstrated significantly reduced antibody response following UVB exposure compared with non-irradiated homozygous counterparts. This research suggested that genetic determinants of critical immune mediators can modulate the effect of UVR exposure differentially. In another study, a number of single nucleotide polymorphisms (SNPs) within cytokine receptor genes (regulating Th1, Th2 and innate immune functions) were associated with variations in the humoral response to measles vaccination in a Somali population (329).

Ethnic variation in general immune parameters is also well known (330). For example, on average, compared with Caucasian populations, African Americans have serum IgG levels 10-15% higher, neutrophil counts 10-15% lower and higher circulating B-lymphocyte counts (264). The clinical significance of these ethnic differences is not certain however.

Taken together, these studies support the contention that individual genetic variation is important in the response to immunisation.

3.3.4 Psychological state

Researchers in the field of psychoneuroimmunology over the past four decades have demonstrated that an individual's psychological state can influence human immune function in diverse ways. A meta-analysis on this topic, which reviewed over 300 empirical studies (331), utilized a useful taxonomy for categorizing psychological stressors which distinguished between two important dimensions of stress – duration and course (continuous or discrete). *Acute time-limited stressors* (e.g. mental arithmetic or public speaking, lasting between 5 to 100 minutes) increased immune parameters particularly associated with innate immunity (e.g. NK and neutrophil numbers in peripheral blood). *Brief naturalistic stressors* (short-term challenges such as academic examinations, spaceflight) tended to depress cell-mediated immunity (Th1) as evidenced by decreased T cell proliferation, and elevation of Th2-associated cytokines and antibodies to latent viruses (e.g. Epstein Barr virus (EBV)) (332,333). *Chronic stressors* (e.g. unemployment or living with a physical disability) were associated with deterioration of aspects of both innate and adaptive immunity.

The use of immunisation for examining the influence of various psychosocial factors on immune function *in vivo* has been a common and informative research model (334–341).

Immunisation with keyhole limpet haemocyanin (KLH) has been used to assess the effect of distress on the immune response in two studies involving medical students. In the first study (Smith, Vollmer-Conna, et al., 2004), 45 students were immunized with KLH during either an exam period (stressful environment) or an exam-free period (non-stressful environment). The Profile of Mood Scores (POMS) questionnaire was used to measure psychological distress at the time of immunisation. The authors found that the likelihood of developing an *in vivo* antigen-specific cell-mediated immune response (DTH response) was significantly reduced in the most distressed students. In a second study of 166 medical students immunised with KLH, distress levels at baseline, and increased levels of alcohol consumption at baseline and at 3-week follow-up, were associated with reduced likelihood of development of an antigen-specific DTH response (342).

A recent meta-analysis (341) summarised the results of 13 clinical studies that examined the influence of psychological stress on the antibody response following influenza vaccination. In five studies, the psychological stress was related to providing care to ill persons (usually the elderly or children). In the remaining studies, data were collected regarding self-reported stress related life events or perceived stress. The authors concluded that there was a significant negative association between psychological stress (of all types measured) and the antibody response to influenza vaccination (effects size correlation coefficient (ESR) -0.18; $p<0.0001$). The association was present both in older and younger aged persons, although the effect size was non-significantly higher in the older group (ESR -0.25 vs. -0.17; $p=0.32$).

The psychological states that have been associated with altered immune response to vaccination include: distress (335,342), stressful life events (343), chronic stress (344) and perceived stress (345).

3.3.5 Under-nutrition

Under-nutrition is the commonest cause of immune suppression world-wide and may result from inadequate intake of macro-nutrients (carbohydrates, protein, fat) and/or of micronutrients (essential vitamins, minerals) (346,347). Macro-nutrients provide the energy required to carry out daily activities and to build and repair tissues. Micronutrients are required for the optimal functioning of cellular processes and metabolic pathways.

Macro-nutrient deficiency or "protein-energy malnutrition" (PEM) results in a generalized depression of immune function, particularly in young children (348–350). Within the innate immune system, complement activation, phagocytosis and cytokine production are all

depressed (346,347,349,351,352). Lymphatic organs (spleen, thymus and lymph nodes) undergo atrophy (346,353). There is a reduction in both T-helper cells (CD4+) and cytotoxic T cells (CD8+), as well as a reduction in the CD4+:CD8+ ratio – thought to be an important correlate of susceptibility to infection (346). Function of the adaptive immune system, as evidenced by DTH skin test responses, is impaired and antibody secretion and affinity to antigen is reduced (347). This has implications for the effectiveness of vaccinations (e.g. tetanus) (350).

Micro-nutrient under-nutrition often accompanies PEM, but can occur as an isolated deficiency (i.e. of iron, vitamin A or zinc). The extent and nature of immune dysfunction depends on the specific micro-nutrient involved. A deficit of zinc, for example, is associated with lymphoid atrophy and decreased DTH skin test responses and has been also associated with increased mortality and morbidity from infection in animal models (*Enterovirus*, coxsackie B, *Listeria monocytogenes*) (346). Of the micro-nutrients, deficiencies of vitamins (A, C, E, B6), selenium, zinc, copper, iron and folic acid are associated with impaired immune function and/or increased rates of infection in humans (346,350).

3.3.6 Physical activity

There is a vast amount of literature which describes the effect of exercise and physical activity on innate and adaptive immunity (see comprehensive synthesis: (354)). The current prevailing hypothesis is that there is an 'inverted J' association between exercise and immune function, such that little or extreme physical activity is detrimental to immune function, and moderate exercise is immune system enhancing (355). For example, moderate physical activity (i.e. 1 hour of endurance exercise at 50% maximal oxygen intake) enhances immune function, whereas intense activity (i.e. 2 hours of concentric exercise at 65 – 75% maximal oxygen intake or shorter periods of more intense activity) diminishes immune function for between 2 and 24 hours (356). Many components of the immune system are influenced by exercise as summarised in Table 3.5. Following an exercise session, acute changes in circulating leukocyte numbers and function typically return to pre-exercise values within 3–24 hours (357).

Table 3.5: Effects of exercise on human immune function

Component of the immune system	Acute effects
Stress hormones	↑Adrenaline, cortisol, growth hormone, prolactin
Cytokines	↑IL-6, IL-10,IL-1 receptor antagonist; inhibition of TNF-α
NK cells	↑numbers during exercise followed by ↓post-exercise for 2 hours; normal by 24 hours; NK cell activity ↓post-exercise (transient)
T-cell lymphocytes	↓proliferative response to mitogen; ↓numbers (strenuous exercise)
B-cell lymphocytes	↓immunoglobulin production (following prolonged, strenuous exercise)
Neutrophils	↑circulating neutrophils
Antigen presenting cells	↓macrophage MHC-class II expression
DTH response	↓after strenuous, prolonged exercise

Source: (357)

Several studies have assessed the effect of physical activity on the immune response to immunisation. Smith et al (358) assessed the influence of cardiovascular fitness and age on the humoral response to KLH immunisation. They recruited healthy men of specific age groups and physical activity status – younger (aged 20–35 years); older (aged 65–79 years), sedentary; and physically active categories. The sedentary men had performed no regular exercise for two years prior to study entry, and the physically active men had performed regular aerobic physical activity at least three times per week for two years. The researchers found that the physically active older group had significantly higher anti-KLH IgM, IgG, IgG1, and DTH skin responses, but not IgG2, three weeks post-immunisation, when compared with the sedentary older group. Antibody levels were generally higher in the physically active younger age group compared with sedentary individuals, but the difference was not statistically significant (albeit with low study numbers).

Other studies have assessed the effect of short term activity on the immune response to immunisation. Edwards et al (359) recruited 160 healthy young adults and randomised them

into a control (no activity) group and three activity groups (who exercised at 60, 85 or 110% of a pre-determined concentric exercise maximum (e.g. bicep curl, lateral raise movement). All participants were vaccinated immediately with a reduced dose (50%) influenza vaccine. Compared with the controls, participants in the exercise groups demonstrated an enhanced antibody titre to the least immunogenic influenza strain (with lesser effects on the remaining strains). Exercise intensity was not related to rise in antibody titre.

3.3.7 Cigarette Smoking

The various components within cigarette smoke affect the immune system in diverse ways – changes that are immunosuppressive and/or pro-inflammatory, local and/or systemic, involving adaptive and/or innate immune processes and caused by active and/or passive smoking. An excellent recent review is provided by Stämpfli & Anderson (360). Table 3.6 summarises some of the more consistent research findings.

Many studies have studied the effect of cigarette smoking on vaccine responsiveness, with conflicting results. Winter et al (361) vaccinated 115 health care workers with hepatitis B vaccine on either a rapid (0, 1, 2 and 12 month) or routine (0, 1 and 6 month) schedule. They found that smokers were significantly less likely to seroconvert at the end of the study regardless of vaccination schedule. Shaw et al (362) demonstrated that smoking status, increasing age and increasing skin-fold thickness were each related to a decreased likelihood of successfully seroconverting following hepatitis B vaccination if vaccination was administered to the buttock rather than the upper arm in healthy adults.

Cruijff et al (285) assessed the effect of smoking on influenza vaccination efficacy (i.e. ability to protect against influenza) and the antibody response to vaccination in a large group of Dutch adults aged 60 years and older. They concluded that smoking was associated with higher post-vaccination antibody titres against two of four vaccine-included strains, and there was no relation between smoking and the development of influenza post-vaccination (i.e. indicating no effect on vaccine efficacy compared with non-smokers). Another study showed no significant difference in post-influenza vaccination antibody titres between smokers and non-smokers (363). However, older studies (284,364) demonstrated lower influenza specific antibodies post-vaccination after one year in smokers compared with non-smokers, a delayed time period not tested in more recent studies.

Table 3.6 Immunomodulatory effects of different components of cigarette smoke

Cigarette smoke component	Cellular target/s	Immunological effect
Nicotine	Endothelial and epithelial cells, lymphocytes, macrophages	Suppression of effector function by macrophage skewing towards Th2 response
Hydrocarbons	Endothelial and epithelial cells, lymphocytes, macrophages	Gene regulation mediated by basic loop-helix-loop proteins; adaptive up-regulation of metabolic and bio-transforming enzymes
Carbon monoxide	Epithelial cells, lymphocytes and macrophages	Suppression of effector function and reduced proliferation
Oxidants and reactive nitrogen species	Epithelial cells, lymphocytes , NK cells, macrophages, endothelial cells of micro-vessels	DNA damage, cell senescence, lipid peroxidation, chemical modification of intracellular signalling
Acrolein	Macrophages and T cells	Suppression of effector function and reduced proliferation
NKK (nitrosamine 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone)	Cytotoxic T cells, NK cells	Suppression of killing

Adapted from (360)

3.3.8 Associations between UVR exposure and potential confounding variables

To be classed as a confounder, a factor must fulfil three criteria: i) be correlated (within the study data-set) with the exposure variable (i.e. UVR exposure); ii) be an independent risk factor for the outcome (i.e. immune function parameter) and iii) not be an intermediate step between the exposure and outcome variables (365). The preceding sections discussed the second criteria for the putative confounders (apart from vitamin D status; reviewed in Chapter 2). Table 3.7 shows evidence demonstrating an association between potential confounding variables and UVR exposure (or its surrogates, sun exposure, vitamin D status).

Table 3.7: Association between potential confounders and UVR exposure

Potential confounder	Association with UVR / Sun exposure
Age	• Annual personal UVR ambient (percentage of total annual ambient UVR) varies by age group (38)
Sex	• On average, males have substantially higher acute UVR exposure than females (40) • Males have higher cumulative sun exposure than females, based on measures of actinic sun damage (306,366)
Psychological state	• Low sun exposure and/or low vitamin D (25(OH)D) status been associated with prevalence of seasonal affective disorder (367), depression (368) and schizophrenia (131). Note that the evidence for these associations is weak.
Ethnicity	• United States – ethnic differences in sun protection practices involving clothing and behaviour between Hispanics and non-Hispanics (369) • Low sun exposure amongst south Asian women living in New Zealand (370)
Physical activity	• High levels of sun exposure amongst cyclists and triathletes; sunburn in young adults undertaking physical activity (371–373) • Adult outdoor workers have higher personal UVR ambient exposures than indoor workers (~10% *vs.* ~3%; with wide variation) (38,40) • Higher grades of actinic skin damage in outdoor workers (374)
Vitamin D status	• Incidences of a number of autoimmune conditions (e.g. MS, rheumatoid arthritis) correlate positively with latitude of residence and/or inversely with vitamin D status (12)

Whilst cigarette smoking and nutritional status do not have good evidence for co-varying with an individual's UVR exposure, they are important factors to measure as they could potentially act as confounders (or effect modifiers) in an epidemiological study.

3.4 Summary

The field of immunotoxicology examines the influence of agents (e.g. UVR) on immune function and their health sequelae. An immunotoxicological assessment typically follows a 'risk analysis' structure. This thesis focuses mainly on the risk assessment component of a risk analysis, and specifically on exposure assessment and risk characterisation.

Whilst animal testing affords logistic, cost and study design advantages over human immunotoxicological research, human-derived data are preferred where robust, valid methods are possible. The choice of immune function assays is critical in an immunotoxicological study aiming to detect and quantify possible subtle and varied changes to immune processes. Based on extensive animal-based studies, a number of immune function assays have been found to be sensitive in detecting immunomodulation, particularly when performed in combination. In human epidemiological research, quantification of the response (humoral and cell-mediated) to primary vaccination with a T-cell dependent antigen has been advocated to assess the immunomodulatory effect of a putative immunotoxic agent.

Immunotoxin-mediated immunosuppression appears to have a linear dose-dependent relationship. At an individual level, it is only severe depression of immune competence that is likely to lead to disease (e.g. high risk of opportunistic infection in patients with advanced HIV infection). There is evidence however, that low to moderate immunosuppression occurring at high prevalence at a population level can lead to increased rates of common infections and/or reduced vaccine effectiveness.

Numerous factors need to be considered when planning an epidemiological study to detect possible immunotoxic effects of UVR, including: study type (e.g. cross-sectional, longitudinal), study length, participant selection and sample size, measurement of UVR exposure and possible confounding factors, and participant burden. To avoid the ecological fallacy, measures of UVR exposure and other immune-modulatory measures taken at an individual level are preferred over aggregate data.

Apart from UVR, other immunomodulatory factors (which might act as confounding variables of effect modifiers) include age, gender, ethnicity, physical activity, psychological state, nutritional state, smoking and vitamin D status. These need also to be carefully measured.

Chapter 4 Development of immunisation and immunoassay protocols

Synopsis

Primary immunisation with a protein antigen has been promoted as a valid and effective method to assess the influence of extrinsic (e.g. environmental exposure) or intrinsic (e.g. psychological distress) factors on the human immune system. Keyhole limpet haemocyanin (KLH) is such an immunogenic protein which is often used in animal immunotoxicological studies and has found numerous applications in the human context. This chapter discusses the relevant properties of KLH, its use in epidemiological and clinical studies and the development of appropriate assays to assess the immune response to vaccination. Finally, in the first experimental work conducted for this thesis, the development of a pilot study that assessed methods potentially usable in a larger study is discussed.

4.1 Keyhole limpet haemocyanin

4.1.1 Structure and properties

Keyhole limpet haemocyanin (KLH) is derived from the haemolymph of the inedible marine mollusk, *Megathura crenulata*, native to the Pacific coastal waters of California and Mexico (375). Hemocyanins are cylindrical, copper-containing molecules that act as oxygen transporting proteins for many mollusk species (288). KLH is an extremely large molecule (>8,000kDa[2]) comprising a variable number of sub-units (KLH1 (390kDa) and KLH2 (350kDa)) (376,377). The remarkable immuno-stimulatory properties of KLH result from high antigenicity derived from numerous carbohydrate and peptide epitopes (288,375).

4.1.2 Biological uses for KLH

The potent immunogenicity of KLH has been known for over 40 years (378–380) and in that time KLH has been used extensively in animal and human research to delineate cellular and humoral immune responses, as a carrier protein for cancer vaccines and as bladder cancer immunotherapy (278, 375, 382). KLH appears to have anti-proliferative action against certain tumour cell lines, including breast, pancreatic and oesophageal cancer (383,384). KLH is currently listed in 29 US National Institutes of Health human clinical studies that are actively recruiting participants (www.clinicaltrials.gov; accessed 3 August 2012).

As KLH exposure promotes a strong T-dependent antibody response (TDAR), and given that humans are naïve to this antigen, KLH immunisation permits assessment of the complex

[2] kDa = kilo Daltons, unit measure of molecular weight

primary immune response involving antigen presentation, priming and collaboration of T and B lymphocytes, antibody production and cytokine-dependent antibody class switching (278). In recent years, use of KLH immunisation for immunotoxicological investigations in animal models has been promoted (385).

4.1.3 Previous studies using KLH as a test of immune function in humans

KLH immunisation has been used to assess the influence of various exposures on immune function in a number of clinical studies. Exposures have included psychological stress (335,338,342,386), cardiovascular exercise (358,387), cancer and/or chemotherapy (279,380,388), immunodeficiency states (389,390) and atopy and asthma (391,392). Table 4.1 details the human clinical trials conducted since 1970 where a trans-cutaneous KLH immunisation schedule has been used for the purpose of assessing immune function and where KLH-specific immune parameters are the primary immunological end-point. The studies have varied by the choice of KLH dose, formulation, mode of administration (route and site) and measured KLH-specific immune parameters. Of note, the two papers from Snyder et al (338,386), report results from different components of the same study (i.e. humoral and cell-mediated primary immune responses).

Dose

Previous studies have used KLH doses ranging from 10μg to 5000μg, although the most common dose used in human clinical studies was 100μg. All studies were able to show an antigen-specific response at doses given for that study, although Grant et al (387) failed to demonstrate a DTH response at 3 weeks post-immunisation with 125μg KLH (with a 5μg intra-dermal skin test). In the only published study to assess the effect of different doses of the same KLH formulation, Curtis et al (380) reported no significant difference in the kinetics or magnitude of the immune response amongst participants immunised with 10μg, 100μg or 5000μg of high molecular weight KLH.

Formulation

KLH for clinical use comes in two forms – high molecular weight (HMW) and sub-unit preparations (375). Both preparations are available in a clinical grade formulation that is sterile and endotoxin and pyrogen free. Sub-unit KLH (~400kDa) is often used as a vaccine carrier protein that is coupled to a carbohydrate or other non-immunogenic molecule to boost T-cell priming (e.g. novel anti-cancer vaccines (393)). HMW KLH (or "native" KLH) preserves the weight of the larger molecule, although manufacturers have found quality control issues to be more challenging (377).

HMW-KLH has greater immunogenicity compared with sub-unit KLH, as evidenced by the study conducted by Miller et al (388). Here, three forms of 1000μg KLH were administered to healthy participants: HMW KLH, sub-unit KLH or sub-unit KLH with mineral oil adjuvant (Montanide ISA-51). A similar and potent immune response was seen in participants immunised with the HMW-KLH and sub-unit KLH with adjuvant, but not in those immunised with sub-unit KLH alone. It was postulated that the lack of response was not due to a lack of important immunogenic epitopes in sub-unit-KLH but, instead, was due to an adjuvant property of HMW KLH that was successfully substituted by use of the mineral oil adjuvant.

An adjuvant is simply an agent which enhances the immunogenicity of antigens (134). Common adjuvants, such as 'alum' (aluminium phosphate and aluminium hydroxide) and oil-water emulsions (e.g. Freund's adjuvant, mineral oil adjuvant), work by sequestration and slow release of associated antigen to surrounding immune cells (394).

Mode of administration (route and site)

A measureable, robust antigen-specific immune response can be generated following administration of KLH by a number of routes – intra-dermal (380), sub-cutaneous (338), intra-muscular (387) and inhalational (392). Studies which administered KLH intra-muscularly used the deltoid muscle. Site of sub-cutaneous administration was not stated in any study, whilst intra-dermal immunisation in one study was described as occurring in the 'upper arm' (389). In those studies assessing the DTH response, intra-dermal KLH was administered to the volar aspect of the forearm (342) or upper arm (279).

4.1.4 Safety profile

KLH appears to have an excellent safety profile which has been noted by several authors (342,382). In their comprehensive review, Harris & Markl (375) stated, "Importantly, KLH is considered to be an extremely safe substance for *in vivo* use in man, as a direct antigenic stimulus and immunotherapeutic agent" (*pp.* 614). KLH immunotherapy for bladder cancer has received European regulatory approval (377). There was no report of significant adverse events related to the use of KLH in any human clinical study reviewed for this thesis.

However, there are potential adverse effects associated with vaccine adjuvants. The use of alum for example, has been associated with local erythema, subcutaneous nodules, granulomas, and macrophagic myofasciitis (394). Oil-water adjuvants (e.g. Freund's complete and incomplete adjuvants) are potent immune-stimulants but have been associated with local skin necrosis and inflammation which has affected their tolerability in the human setting (394). Newer mineral oil adjuvants (e.g. MF59, Montanide ISA-51) have better safety profiles and have been used in vaccines in human trials (395).

4.1.5 Summary

KLH is a potent immuno-stimulatory antigen that has been used in a number of human clinical research settings. A robust immune response can be attained from immunisation with a single dose of KLH via a number of routes and doses. Sub-unit KLH needs to be combined with an adjuvant to match the immunogenicity of HMW KLH. KLH itself has an excellent safety profile in humans, although the use of adjuvant agents has been associated with local adverse effects.

Table 4.1 Comparison of human clinical studies that have used primary KLH immunisation to assess immune function (1990 – 2010)

Study and aim	KLH formulation, dose, route & site of administration	Antibody assessment: ELISA type, target immunoglobulin(s), serum titre, sampling	Cell-mediated immunity (*Ex vivo* and *in vivo* testing)
Spazierer (2009) (391) Establish immunisation protocol to induce *de novo* Th2 response using primary KLH vaccine	Sub-unit KLH 100µg KLH with alum (dose not specified); IM; site not specified; 3 doses: baseline, d 15 & d 29	Indirect and sandwich ELISA Anti-KLH IgG1, IgG4, IgE, IgM Serum titre not specified; Baseline, day 15, 29, 43 & 57	***Ex vivo***: Lymphocyte proliferation assays ***In vivo***: Not evaluated
Grant et al (2008) (387) Effect of aerobic exercise in sedentary older adults on primary KLH vaccine immune response	HMW-KLH 125µg IM Deltoid muscle	Indirect ELISA Anti-KLH IgG1 IgG2 IgM 1:64; Baseline, 2, 3 and 6 weeks post-immunisation	***Ex vivo*:** Not evaluated ***In vivo*:** 5µg KLH ID 3-weeks post-immunisation; Date of reading not specified
Miller et al (2005) (388) Comparison of responses to KLH vaccines in healthy adults; Immunisation response in immunosuppressed patients (cancer & bone marrow transplant)	1. Sub-unit KLH 1000 µg SC 2. HMW-KLH 1000µg SC 3. Sub-unit KLH 1000µg with Montanide-ISA-51 adjuvant (0.6mL)	Indirect & sandwich ELISA anti-KLH IgG1 IgG2 1:50, 1:100, 1:200, 1:400 & 1:800 Anti-KLH IgM 1:25000, 1:50000, 1: 10000; Baseline and 4 weeks post immunisation	***Ex vivo*:** Lymphocyte proliferation assays; Enzyme-linked immunosorbent spot (ELISPOT) assay for cellular responses to KLH ***In vivo*:** Not evaluated

Study and aim	KLH formulation, dose, route & site of administration	Antibody assessment: ELISA type, target immunoglobulin(s), serum titre, sampling	Cell-mediated immunity (*ex vivo* and *in vivo* testing)
Smith A et al (2004) (335) Effect of distress on primary KLH vaccine response in young adults	Sub-unit KLH 100µg adsorbed to 0.9mg alum IM Deltoid muscle	Indirect ELISA; Anti-KLH IgG 1:100, 1:1000 Baseline, 3 weeks post-immunisation	***Ex vivo***: Lymphocyte proliferation assays ***In vivo***: 1 µg ID 3 weeks post-immunisation; Volar aspect of arm; Read at 48 hours
Smith A et al (2004) (342) Effect of distress on DTH response following primary KLH immunisation in young adults	Sub-unit KLH 100µg adsorbed to 0.9mg alum IM Deltoid muscle	Not tested	***Ex vivo***: Not evaluated ***In vivo***: 1 µg ID 3 weeks post-immunisation; Volar aspect of arm; Read at 48 hours
Smith TP et al (2004) (358) Effect of age and physical activity on primary KLH vaccine immune response	Sub-unit KLH 100µg adsorbed to 0.9mg alum IM Deltoid muscle	Indirect ELISA Anti-KLH IgG, IgG1, IgG2 & IgM; 1:100 (IgM); 1:200 (IgG); 1:2 (IgG1); 1:1 (IgG2) Baseline, 1, 2, 3 & 4 weeks post-immunisation	***Ex vivo***: Not evaluated ***In vivo***: 1 µg ID 21 days post-immunisation; Volar aspect of arm; Read 24, 48, 72, 96 & 120 hours
Kondratenko (1997) (389) Evaluate immune response to primary KLH immunisation in patients with immunodeficiency state	HMW KLH 200 µg ID Upper arm	Indirect ELISA Anti-KLH IgG, IgM Serum dilutions not stated; Baseline, 2 & 4 weeks post-immunisation	***Ex vivo:*** Lymphocyte proliferation assay ***In vivo***: Not evaluated
Snyder et al (1993)# (386) Effect of stressful events on primary KLH vaccine cell-mediated immune response	HMW KLH 100µg SC Site not specified	Not evaluated	***Ex vivo:*** Lymphocyte proliferation assays ***In vivo:*** Not evaluated

Study and aim	KLH formulation, dose, route & site of administration	Antibody assessment: ELISA type, target immunoglobulin(s), serum titre, sampling	Cell-mediated immunity (*Ex vivo* and *in vivo* testing)
Amlot et al (1986) (279) Comparing primary immune response to KLH immunisation in healthy controls & cyclosporine-treated bone marrow transplant patients	HMW KLH 200µg ID Upper arm	Enzyme radio-immunoassay Anti-KLH IgG, IgM Serum titre: Starting at 1:20, final dilution not stated. Baseline, 1, 2 & 3 weeks post-immunisation	***Ex vivo:*** Not evaluated ***In vivo:*** Three doses (0.1, 1 & 10µg) KLH ID 7 and 14 days post-immunisation; Same site as primary immunisation Read at 24 and 48 hours post-skin test
Curtis et al (1970) (380) Primary immune response to KLH immunisation in healthy adults & patients with solid tumours	HMW KLH 5000µg SC over deltoid region or 100µg ID* or 10µg ID* * site not specified	Haemagglutinin assays using sheep red blood cells. Serial dilution titres – result reported as highest sera dilution with haemagglutination; Baseline, 5, 7, 14, 21, 28, 56, 84 & 112 days post-immunisation (not all subjects on all days)	***Ex vivo***: Lymphocyte proliferation assays ***In vivo***: Variable testing (100µg+/-10µg+/-1µg) KLH ID; Volar aspect of arm; Variable day of skin test post-immunisation; Read at 24 and 48 hours post skin test

Legend: IM: Intra-muscular; SC: sub-cutaneous; ID: Intra-dermal HMW: High molecular weight DTH: Delayed type hypersensitivity

These studies report different components of the same study sample

4.2 Immunological assays

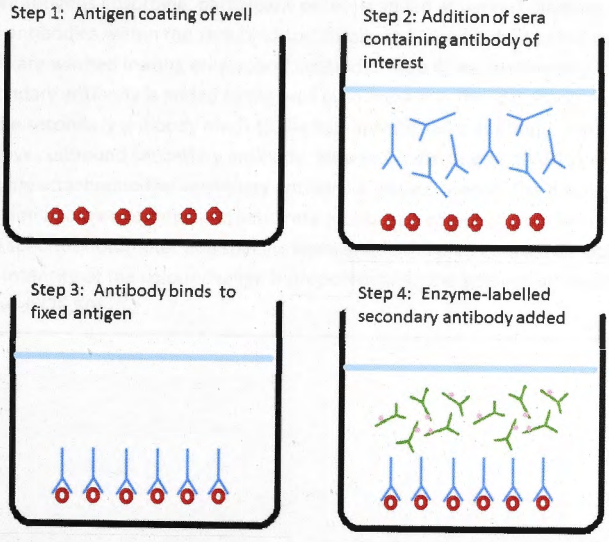
Reliable and sensitive immunological assays to measure changes in KLH-specific immune parameters following vaccination are essential for this research. Humoral immunity is commonly assessed by measurement of antibody titres, whilst cell-mediated immunity can be assessed *ex vivo* using several varieties of lymphocyte assays or *in vivo* via CHS testing or DTH testing. The following sections will discuss these assays in more detail.

4.2.1 Quantification of antigen-specific antibodies: ELISA

Identification of antibody type and quantification of response has been made considerably easier with the development of assays which measure the direct binding of antibody to its antigen - enzyme linked immunosorbent assay (ELISA) being a prime example (396).

There are several variations of ELISA assays (e.g. direct, indirect, 'sandwich') but the underlying principle is that an enzyme-labelled antibody binds with a specific ligand (antigen or antibody of interest) which can be detected by addition of a substrate that causes a coloured reaction product (134). For detection of an unknown concentration of antibodies in sera, the indirect ELISA assay is preferred. The basic steps to detect anti-KLH antibodies in participant serum are outlined below (Figure 4.1).

Figure 4.1 Steps performed in the indirect ELISA assay to detect anti-KLH antibody



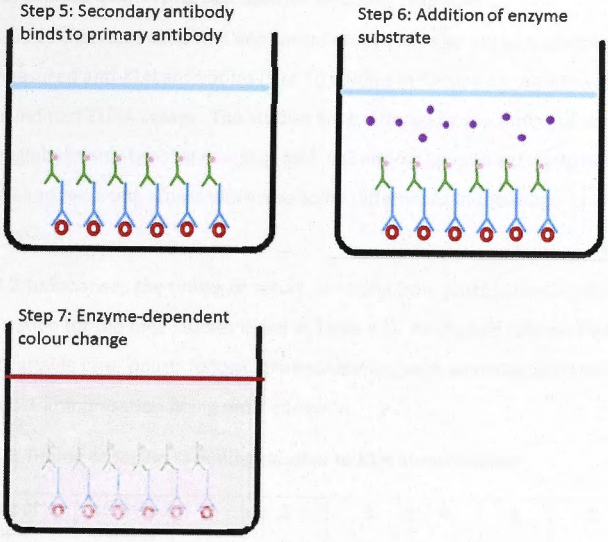


Figure 4.1 illustrates the basic necessary steps in performing an indirect ELISA assay to detect anti-KLH antibody. **Step 1:** KLH antigen (e.g. in lyophilised (powdered) form) is passively adsorbed to the bottom of a well within a 96-well microtitre plate (causing antigen to become fixed). **Step 2**: After washing (e.g. with mild detergent such as phosphate buffer solution (PBS)) and blocking (e.g. with skim milk) the well to prevent other antigens adsorbing, participant serum is added at various dilutions. **Step 3**: Anti-KLH antibodies within the sera bind specifically to KLH adsorbed to the well base. The wells are washed leaving only bound antibody. **Step 4**: An enzyme (e.g. biotin) -labelled secondary antibody is added to the well (also known as the 'detection antibody'); **Step 5**: The secondary antibody binds to the KLH-specific fixed antibody. Further washing removes unbound secondary antibody. **Step 6:** A substrate (e.g. ABTS) specific for the enzyme attached to the secondary antibody is added; **Step 7**: The product of the reaction between enzyme and substrate produces a colour change which can be read by a spectrophotometer at a specific wavelength of light (reported as 'optical density'). The intensity of the colour change is proportional to the amount of specific antibody in the well (26,50).

Previous studies quantifying KLH-specific antibody response

Most studies that have used KLH immunisation to assess an antigen-specific immune response have measured anti-KLH antibodies (9 of 10 studies in Table 4.1), with the more recent studies utilising indirect ELISA assays. The studies have differed by the timing of serum sampling, immunoglobulin sub-type targets (e.g. IgM, IgG and/or IgG sub-sets) and how the result was analysed and reported. There were also some differences in the reagents and protocols used for the assays.

Table 4.2 summarises the timing of serum sampling from participants relative to KLH immunisation for the nine studies listed in Table 4.1. All studies collected serum at baseline, then at variable time-points following immunisation, with sampling at baseline, 2, 3 and 4 weeks post-immunisation being most common.

Table 4.2 Timing of serum sampling relative to KLH immunisation

Timing of Sample (weeks post-immunisation)	0 (baseline)	1	2	3	4	6	8	12	16
Number of studies (% of studies from Table 4.1)	9 (100%)	3 (33%)	6 (67%)	7 (78%)	5 (56%)	2 (22%)	3 (33%)	1 (11%)	1 (11%)

Anti-KLH IgG (or IgG sub-set) was assayed in 9 (100%) studies and anti-KLH IgM in 7 (78%) studies. Anti-KLH IgE was assayed in only one study (391), which aimed to investigate the pathogenesis of allergic disorders.

The studies also differed by how anti-KLH antibody titre was read and presented. Two studies generated standard curves using sera with known concentrations of anti-KLH antibody, and from these interpolated the concentration of anti-KLH antibodies in subjects' samples (388,391). Three studies compared the optical density (measure of colour change/light absorbance in wells) of sample sera at defined dilutions with the positive and negative control sera on the same plate which also allowed adjustment for inter-plate variation in absorbance readings (335,358,387). The older studies gauged anti-KLH concentration by comparing the highest dilution of serum at which antibody was still detectable (279,338,380).

4.2.2 Cell-mediated immunity assays

There is a diverse range of *ex vivo / in vitro* assays available for examining cell-mediated immune processes, largely aiming to identify and enumerate lymphocyte sub-populations and/or determine lymphocyte effector function. In this section, discussion is limited to assays involving peripheral blood lymphocytes, acknowledging that, whilst easiest to obtain, they are not necessarily representative of the lymphoid system at large or of lymphocytes involved at the site of antigen processing (134). Isolation of lymphocytes is most commonly achieved by allowing peripheral blood to separate by density centrifugation over a step-wise gradient consisting of the carbohydrate polymer, Ficoll, and an iodine-containing compound (e.g. metrizamide, diatrizoate), in a test tube. Differential migration of the varying cell types following centrifugation allows the layer of lower density peripheral blood mononuclear cells (PBMCs) – consisting of lymphocytes and monocytes – to be separated from the higher density granulocytes and erythrocytes (134,397). Isolated PBMCs can be used fresh following isolation, or after careful freezing and thawing, for further analysis.

Flow cytometry, in combination with a fluorescence-activated cell sorter (FACS), allows detailed assessment of lymphocyte populations (398). PBMCs are first tagged with a fluorescent-dyed monoclonal antibody against specific cell surface markers (e.g. CD3, CD4) or intracellular cytokines (e.g. IL-4, IL-17, IFN-γ) and passed in a droplet stream of single cells through a laser beam. Sensors are able to detect the resultant scattered light which gives information on the granularity and size of cells. Additionally, the monoclonal antibodies fluoresce differentially upon excitation by the laser, further characterising the identity and functional role of lymphocytes (e.g. Th1 *vs.* Th2 *vs.* Th17) (134,399). Improving technology now allows for the assessment of multiple cell types from a single lymphocyte sample, as the number of fluorescent dyes that can be analysed simultaneously has increased (398).

Other methods used to detect cytokine production by lymphocytes include ELISA, and its variation, the enzyme-linked immunosorbent spot (ELISPOT) assay (134). These assays employ cytokine-specific antibodies to bind to secreted extra-cellular cytokines following lymphocyte stimulation. The ELISPOT assay in particular is a very sensitive test, and can detect cytokine production at a single lymphocyte level. It is therefore useful for determining the frequency of cytokine-secreting cells within a population and requires approximately ten-fold less cells than flow cytometry methods (400). However, it does not provide information about the proportions of different sub-populations within a sample (134).

An emerging technology, convenient for separating lymphocyte sub-populations and identifying the cytokines produced, makes use of antibody-coated magnetic beads. When these beads are mixed with a heterogeneous cell population of interest, they bind to their

specific ligand (e.g. cell surface marker or cytokine target). The magnetic bead-bound targets are held suspended when a magnetic field is applied, whilst the unbound fraction is washed away. In this fashion, and similar to the FACS method, multiple targets can be assessed simultaneously (134,401).

Assessment of antigen-specific cellular immune responses is more problematic than for humoral immune responses (where quantification of antigen-specific antibody response is straightforward). There are multiple reasons for this, including the low frequency of circulating memory T-helper cells and that T-cell receptors require presentation of antigen by specific MHC-class molecules (134,402). Notwithstanding, antigen-specific T-cell responses have been achieved by incubating lymphocytes with the recall antigen-of-interest (e.g. KLH) or a 'polyclonal mitogen' to which many or all lymphocytes of varying types and specificities will respond (e.g. phytohaemaglutinin, pokeweed mitogen), and then measuring a particular parameter (e.g. proportion of cytokine secreting cells by ELISPOT, sub-population identification and enumeration by FACS). The 'lymphocyte proliferation assay' (LPA) involves incubating PBMCs in cell culture for a pre-defined time (usually several days) in the presence of polyclonal mitogen or recall antigen, followed by measurement of the incorporation of ^{3}H-thymidine into DNA; this correlates with T-cell proliferation (403). LPA has been perhaps the most common assay for assessing post-vaccine T-cell response but does not elucidate the identity or function of the cell subpopulation involved (134).

Previous studies using cell-mediated immunity assays

Several studies have assessed cell-mediated immunity *ex vivo* following KLH immunisation (335,380,386,388,389,391) (See Table 4.1). The majority of these studies used conventional LPA, with the main difference between them being the PBMC incubation times with KLH, ranging from 4 days (386) to 7 days (388).

Cytokine production by stimulated PBMCs following KLH immunisation was assessed in two more recent studies. Interferon-γ production was determined by ELISPOT assay after thawed PBMCs were incubated with KLH for 20 hours (388). Spazierer et al (391) incubated cells with KLH for 40 hours then tested the supernatant for IL-4, IL-5, IL-10, IL-13 and IFN-γ using antibody-coated magnetic bead assays.

For all the studies in Table 4.1, PBMC isolation was undertaken using Ficoll density centrifugation methods as outlined previously.

4.2.3 Delayed-type hypersensitivity responses

As discussed in Section 3.2.2.4, delayed-type hypersensitivity (DTH) testing is a validated *in vivo* test of antigen-specific cell-mediated immunity. This section will discuss its use in previous studies, methods for administration and reading of the test.

Previous studies utilising DTH tests

A number of human clinical studies have utilised DTH tests to assess *in vivo* KLH-specific cell-mediated immunity (279,335,358,380,387). The studies have varied by the initial immunisation KLH dose, as well as formulation and subsequent skin test dose (see Table 4.1). There are conflicting data regarding the minimum skin test dose required to induce a reliable DTH response. Grant et al (387) were unable to elicit a DTH response after administration of a 5μg intra-dermal skin test dose 3 weeks following immunisation with 125μg HMW-KLH administered intra-muscularly. Other researchers found that the DTH response was independent of initial sensitising immunisation dose (i.e. 5000μg / 100μg / 1μg were equivalent). However, higher skin test dose yielded a higher proportion and magnitude of positive DTH responses in a dose dependent manner (i.e. 100μg > 10μg > 1μg) (380). Skin testing with simultaneous doses of 0.1, 1 and 10μg KLH at day 7 and day 14 post-immunisation (with 200μg HMW KLH) achieved a DTH response rate of 68% amongst healthy control patients (although the results at each skin test dose were not provided) (279).

The upper arm (where primary immunisation occurred) (279) and the volar aspect of the forearm (335,358) have been the only sites reported for KLH skin testing.

Administering skin test and interpreting result

The assessment of a DTH response secondary to tuberculin skin testing (TST or 'Mantoux testing') has long been used by clinicians to determine past exposure to *Mycobacterium tuberculosis* in humans (404). The TST method has been adopted for DTH testing using other antigens (294). The established method for administering the skin test is to inject antigen (to which the host has been pre-sensitised) intra-dermally such that a small raised area of skin ('bleb') 5-8 mm in diameter becomes apparent (405). The subsequent DTH response takes a number of hours to develop, but in most studies the result is read at 48 to 72 hours post-skin test (183,291). The DTH reaction is manifest by raised (indurated) and erythematous skin over the site of the skin test. It is the indurated area that correlates with the cell-mediated immune response rather than the area of erythema. The 'palpation method' has been the traditional method for assessing the area of induration, although this requires considerable training and experience to ensure reliable readings (406). More recently, the 'pen method' has been shown to have greater sensitivity and reproducibility than the palpation method, whilst also

being easier to learn and administer (298,406–408). Essentially, this technique involves using a ball point pen to run along the skin towards the area of induration until it meets resistance. The process is repeated on the opposite side and the distance between pen markings is measured to obtain a diameter of induration (in millimetres) (405). Some studies have averaged two readings taken at right angles (342,380).

4.3 Pilot study

This section describes the initial experimental component of this thesis research.

4.3.1 Background

The objective of this pilot study is to develop protocols for the use of KLH immunisation to quantify the influence of solar ultraviolet radiation exposure on human immune function. As described in previous sections, KLH has attributes that make it an ideal candidate for testing the immune system. KLH is a potent immunogen resulting in measurable immune responses after only single administration, elicits a reliable *in vivo* cell-mediated DTH response, can be administered via various routes, and is safe and well tolerated.

The dose, site and formulation of KLH used in this pilot study were determined based on the results of previous studies examined in the early part of this Chapter. High molecular weight KLH was the formulation of choice for this study. However, due to commercial scarcity, sub-unit KLH with adjuvant (Montanide ISA-51) was used instead, though at a lower dose than that used by Miller et al (388).

4.3.2 Aims

The pilot study was a non-blinded randomised controlled study designed to assess the effect of sub-erythemal solar-simulated UVR on the humoral and cell-mediated immune responses to immunisation with the novel protein, KLH.

This aims of the pilot study were to:

i) Determine the feasibility and appropriateness of using primary KLH immunisation as a tool to assess the influence of UVR exposure on immune responses

ii) Determine whether a 125μg KLH dose was appropriate for this purpose (i.e. stimulate responses in participants with enough variability to show an influence at different levels of UVR exposure)

iii) Develop reliable antigen (KLH)-specific antibody assay protocols

iv) Develop a protocol for *in vivo* testing of KLH-specific cell-mediated immune function (DTH test)

4.3.3 Methods

This study was conducted after approval by the Human Research Ethics Committee of the Australian National University. Informed written consent was obtained from all participants on enrolment into the study. This study was also registered with the Australian Therapeutic

Goods Administration under the Clinical Trial Notification scheme and the Australian and New Zealand Clinical Trials Registry (ACTRN 12609000380291).

Study setting

This study was conducted in the winter months (June and July) of 2009 at the Australian National University, Canberra, Australia. The average ambient clear-sky solar noon UV Index in Canberra during these months ranges between 2 (low-intensity) and 3 (moderate-intensity) (Source: Bureau of Meteorology: www.bom.gov.au).

Recruitment

Volunteers were recruited from advertisements placed in university departments, university email lists and by word-of-mouth. Participant inclusion criteria were: i) aged between 18 and 40 years, inclusive; ii) self-reported as 'healthy', and iii) Fitzpatrick skin phototype II-III skin (i.e. "fair" complexion). This last criterion was to standardise the dose of solar-simulated UVR administered to all subjects without the need to determine a MED for each individual. Exclusion criteria were: i) known allergy to shellfish; ii) infection or immunisation within 30 days of study entry; iii) pre-existing immunodeficiency state (including diabetes, HIV, chronic renal or liver disease, recurrent infections, solid organ transplantation); iv) use of immune-suppressive agents within 30 days (systemic) or 7 days (topical) prior to study entry; and v) pregnancy or currently breastfeeding.

Randomisation & UVR exposure

Upon recruitment, subjects were randomly assigned to a control group (i.e. no UVR exposure) or UVR-exposure group using a computer randomisation algorithm. Subjects were informed of their assigned group prior to the first visit. It was planned that thirty participants be enrolled, with fifteen randomised into each group. The study protocol is summarised in Figure 4.2.

Control Group: The control group were instructed to take UVR-protective measures (e.g. when outside to wear long sleeve shirts and trousers, UVA/UVB blocking sunscreen (provided) and sunglasses) from 5 days prior to immunisation.

UVR-Exposure Group: Subjects in the UVR-exposure group were irradiated with a sub-erythemal erythemally-weighted dose of solar-simulated UVR (via a calibrated UVR lamp). The spectral output of UVR by the lamp approximated solar UVR in the UVA range, but there was a lower proportion of lamp output in the shorter wavelength UVB range than would be expected in solar UVR (Figure 4.3) (although, given the high solar zenith angle present for much of the day in winter at mid-latitudes, the proportion of UVB in ambient UVR would be low).

Figure 4.2: Pilot study algorithm

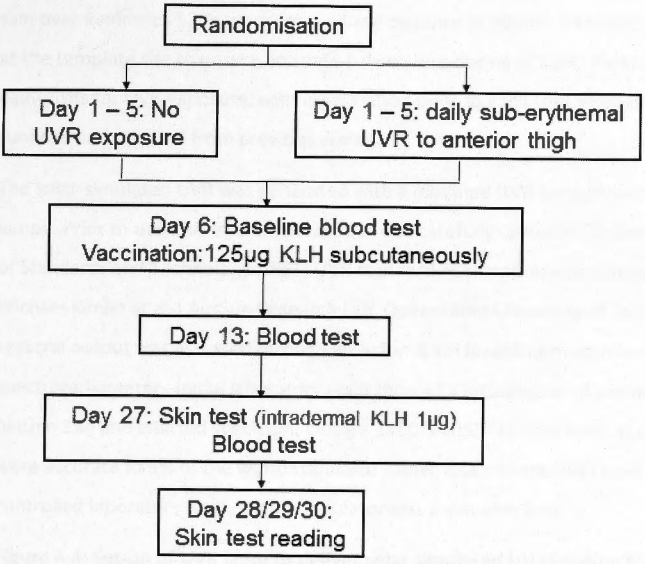
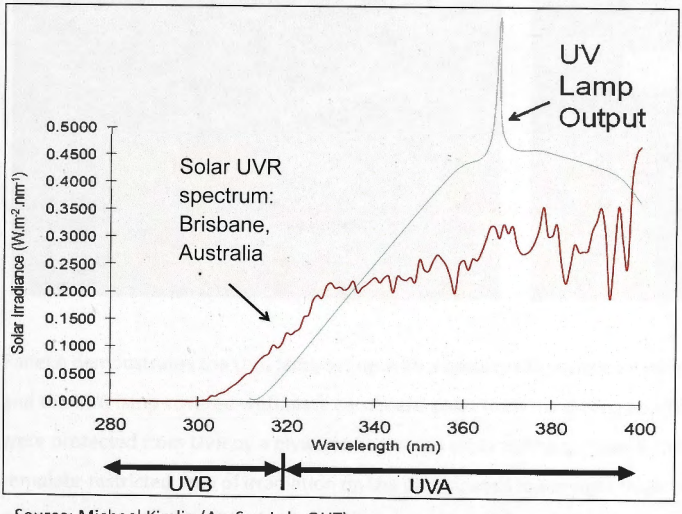


Figure 4.3 Spectral output of UVR lamp compared with solar UVR spectrum



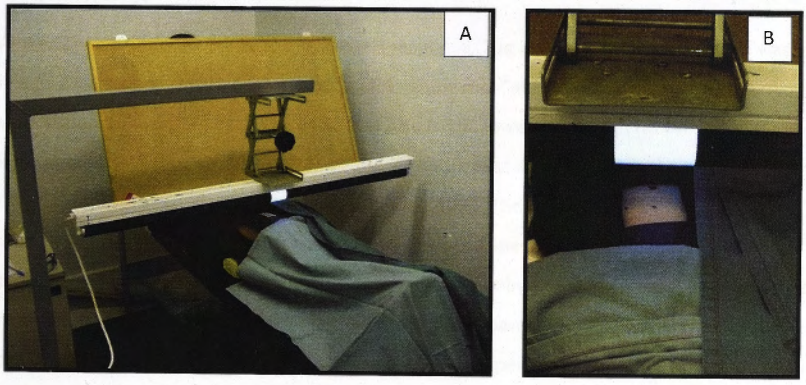
Source: Michael Kimlin (AusSun Lab, QUT)

The UVR dose was delivered to a 5cm x 5cm area on the right lower thigh using a cardboard template (See Figure 4.4). The position for irradiation was located by anatomical landmarks (lower margin of irradiation was 5cm above the superior margin of the patella when the knee was in an extended position). The pre-determined dose of UVB (~ 20mJ/cm^2, equivalent to 0.8

of an MED for Fitzpatrick Type II-III skin) was delivered by a UVR-emitting lamp exposing the skin over 4 minutes 51 seconds from a fixed distance of 50mm. Indelible ink marks were made at the template site to ensure accurate subsequent dosing of UVR. Participants attended for 5 daily visits for UVR exposure, with examination prior to each UVR irradiation to ensure that no sunburn had resulted from previous dosing.

The solar-simulated UVR was generated with a modified UVR lamp (Phillips Medical UVB TL11 lamp). Prior to use, this instrument output was carefully calibrated to the US National Institute of Standards and Technology (NIST) UVR standard. Calibration was conducted by Professor Michael Kimlin at the AusSun Research Lab, Queensland University of Technology. The spectral output was assessed through an Acton 0.5m focal length double monochromator spectroradiometer. Initial laboratory tests showed a high degree of reproducibility of the dose (within 3%) and emitted spectrum. Using a 1000W NIST FEL UVR lamp as a standard, doses were accurate to 3% of the world standard. Other researchers (409) have suggested that for controlled laboratory work, such a simulator was a valuable tool.

Figure 4.4: Set-up of UVR lamp to deliver solar simulated UV radiation to participants



Panel A demonstrates the UVR lamp set up with a participant positioned with leg extended and the UVR lamp covered with black cardboard apart from the dosing aperture. Participants were protected from UVR by a plywood board and other clothing. Panel B shows the 5 x 5cm template-restricted area of irradiation on the participant's lower right thigh. Indelible ink marks were made to enable accurate daily positioning of the template.

Immunisation

On study day 6, all subjects underwent immunisation with 125µg sub-unit KLH (Stellar Biotechnologies, USA) emulsified with 0.6mL Montanide-51 mineral oil adjuvant (Seppic, France) as per the manufacturer's instructions. Immunisation was by the subcutaneous route at a standardised site on the right lower thigh (at the same site chosen for UVR exposure).

Blood sample collection

Venous blood was collected at baseline (pre-immunisation), and at 7 days and 21 days post-immunisation. At baseline, blood was tested for full blood count, kidney and liver function, C-reactive protein and random blood glucose level (ACT Pathology laboratory, Canberra Hospital). At each visit, serum was separated from clotted blood by centrifugation and stored for later analysis in 1ml aliquots at -20°C. At the end of the study, serum 25(OH) D level was analysed at baseline by high performance liquid chromatography – tandem mass spectrometry (LC-MS/MS) performed at the Royal Melbourne Institute of Technology Drug Discovery Technologies laboratory (see Section 5.5.6).

Delayed type-hypersensitivity response

At day 21 post-immunisation, to elicit a DTH response, 1µg KLH (in 10µL sterile water) was injected intra-dermally via a 29-gauge insulin needle (BD Catalogue no. 591823) to the mid-volar aspect (underside) of the forearm and also to the lower right thigh (at the site of immunisation +/- irradiation). At 24, 48 and 72 hours post KLH-challenge, the resultant skin induration was measured by the 'pen technique' (406). The diameter of induration was measured in millimetres in the horizontal and vertical planes by a single (non-blinded) observer. The measurement at 48 hours was undertaken by the study investigator, whereas measurements at 24 and 72 hours were taken by participants after instruction. Measurement of the DTH response by participants has previously been performed in other similar studies (342) although the reliability of self-reported measurement has been questioned (291). A positive DTH response was defined as a reaction to KLH skin test of 2mm or greater (140,342).

Anti-KLH IgG response

Anti-KLH IgG, IgG1 and IgG2 concentrations were determined by indirect ELISA assay, the protocol for which was developed based on published studies, using standard methods (335,358,387). A summary of the assay protocol is provided here – a detailed explanation, including ELISA optimisation steps, is provided in Appendix A. To control for intra- and inter-plate variation, sera were tested in duplicate for all three time-points of blood collection (i.e. baseline, day 7 and day 21 post-immunisation), along with a negative and positive control, on a single ELISA plate.

Ninety-six well ELISA plates (Nunc Maxisorb, Sigma-Aldrich) were coated with 1 μg/mL KLH (Sigma-Aldrich) in a carbonate coating buffer, then refrigerated overnight. The plates were washed with PBS-Tween 20 (Phosphate Buffered Saline with Tween 20, Sigma-Aldrich), blocked with 1% dry skim milk, incubated for 2 hours at room temperature (or for 45 minutes at 37°C), then washed again. Based on previous titration studies, participant serum (1 in 50 concentration) was pipetted into wells diluted with 1% dry skim milk and made up to 100μl per well. Following further incubation and washing steps, an enzyme-labelled secondary antibody – IgGFc (full complement) (Monoclonal anti-human IgG Biotin Conjugate; Sigma-Aldrich), IgG1 (Monoclonal anti-human IgG1 Biotin Conjugate; Sigma-Aldrich) or IgG2 (Monoclonal anti-human IgG2 Biotin Conjugate; Sigma-Aldrich) - was added at dilutions of 1/50,000, 1/2000 or 1/25,000, respectively, in skim milk. Following further incubation and washing steps, 100μl of avidin peroxidise (Sigma-Aldrich) in skim milk was added. To each well, 100μl ABTS (3,3,5,5 tetramethylbenzidine liquid substrate system, Sigma-Aldrich) solution was added to catalyse the colorimetric enzyme reaction. After 15 minutes, 1M phosphoric acid in distilled water was added to stop the reaction. Colour change within the well was measured by an optical density reading performed by a microplate spectrophotometer (Thermo Labsystem Multiskan Ascent) at wavelength 450nm.

Due to relatively high inter-plate coefficients of variation for positive control sera (IgG ~33%; IgG1 ~ 15%; IgG2 ~ 22%), anti- KLH antibody values are presented as "corrected" optical density (OD) by dividing raw absorbance by the standard positive control sample that was run on each plate in each assay (387). The laboratory technician was blinded to the UVR exposure status of the participant.

Statistical analysis

The results were described using descriptive statistics (i.e. continuous data: mean/median, range; categorical data: proportions). Comparisons involving continuous variables used parametric tests where the variable was normally distributed or could be transformed into a normal distribution, and non-parametric tests otherwise. Comparison of proportions was performed using χ^2 or Fisher's exact tests as appropriate. Two-tailed statistics were used throughout.

4.3.4 Results

Participant characteristics

Twenty one participants were recruited and randomised: intervention (n=9); control (n=12) over the winter months (June and July) of 2009. Participants attended all visits and completed all tasks. The groups were well matched at baseline in relation to gender, age, vitamin D (25(OH)D) level and body mass index (see Table 4.3). Although the participant target sample size was set arbitrarily at thirty, further recruitment was suspended because a minority of participants (7/21; 33%) experienced delayed and transient redness and discomfort at the site of injection. This was thought to be secondary to the vaccine's mineral oil adjuvant component.

Table 4.3 Pilot study participant characteristics

	Control (n = 12)	**UVR Exposure (n = 9)**	**p value**
Age (mean; range)	26.6 (22 – 35)	28.0 (24 – 31)	0.80*
Male (n; %)	7 (58%)	4 (44%)	0.67#
Baseline 25(OH)D level (nmol/L)	67.8	55.4	0.14*
BMI	25.4	24.4	0.62*

* Unpaired t-test # Fisher's Exact Test

Immune function assays

The DTH responses and anti-KLH IgG, IgG1 and IgG2 results for all participants are shown in Table 4.4.

Table 4.4: Pilot study immune outcomes

		DTH response at 48 hours (mm)		Antibody Results for anti-KLH IgGFc /IgG1 / IgG2 [$#] (Baseline, Day 7, Day 21 post-immunisation)		
Study No	UVR Exposure group	Arm	Thigh	IgG_{Fc}	IgG_1	IgG_2
1	Control	8.5	4.5	1.36 / 1.20 /1.20	0.45 / 0.47 / 0.90	0.51 / 0.48 / 0.60
2	Control	7	0	1.04 / 1.02 / 1.34	1.27 / 1.14 / 1.13	0.30 / 0.29 / 1.22
3	Control	10	16	0.84 /0.88 / 0.88	0.56 / 0.49 / 0.80	0.39 / 0.34 / 0.41
4	Control	3	9	0.84 / 0.82 / 0.88	0.43 / 0.28 / 0.58	0.50 / 0.51 / 0.70
5	Control	10	7.5	0.23 / 0.27 / 0.63	0.62 / 0.79 / 1.11	0.35 / 0.30 / 0.51
6	Control	11.5	9.5	0.66 / 0.62 /0.11	0.50 / 0.53 / 0.76	0.30 / 0.29 / 0.35
7	Control	4.5	6.5	0.56 / 0.64 / 1.04	0.57 / 0.61 / 0.67	0.26 / 0.21 / 0.74
8	Control	9	11	0.83 / 0.77 / 0.13	0.59 / 0.57 / 0.81	0.55 / 0.53 / 0.72
9	Control	5	0	1.09 / 1.07 / 0.96	0.61 /0.58 / 0.86	0.49 / 0.53 / 0.92
10	Control	9	9	0.67 / 1.04 / 2.41	0.59 / 0.51 / 0.96	0.42 / 0.42 / 0.64
11	UVR exposed	7.5	0	0.79 / 0.95 / 0.46	0.63 / 0.45 / 0.85	0.52 / 0.48 / 0.77
12	UVR exposed	0	0	0.27 / 0.37 / 0.56	0.46 / 0.60 / 1.27	0.40 / 0.37 / 1.5
13	UVR exposed	9	6	* / * / *	0.57 / 0.55 / 1.01	0.35 / 0.29 / 0.69
14	UVR exposed	6	5	0.82 / 0.85 / 0.28	* / 0.53 / 0.73	* / 0.62 / 0.66
15	Control	9	0	0.99 / 0.82 / 0.11	0.48 / 0.42 / 0.94	0.38 / 0.40 / 3.07
16	Control	4	0	* / * / *	0.55 / 0.38 / 0.65	0.37 / 0.38 / 0.46
17	UVR exposed	8	8	0.09 / 0.06 /0.08	0.60 / 0.63 / 0.68	0.36 / 0.38 / 0.59
18	UVR exposed	9	0	0.48 / 0.47 /0.87	0.52 / 0.48 / 1.12	0.30 / 0.29 / 0.35
19	UVR exposed	0	0	0.88 / 0.88 / 1.02	0.67 / 0.32 / 1.66	0.35 / 0.28 / 0.36
20	UVR exposed	6.5	0	0.73 / 0.74 / 0.84	0.30 /0.39 / 1.18	0.35 / 0.30 / 0.48
21	UVR exposed	4	5	0.63 / 0.67 / 0.56	0.46 / 0.54 / 0.95	0.48 / 0.37 / 0.52

[$] Corrected optical density – relative to the optical density reading of the positive plate control to adjust for plate-to-plate variation; IgGFc = IgG full complement (including all IgG subsets)
* Missing result;
Results are reported for serum concentrations of 1:50

Delayed-Type Hypersensitivity Responses

Table 4.5 shows data illustrating the DTH response rate by anatomical site. Overall, DTH responses above 2mm diameter were elicited from 31 of 42 skin tests (73.8%) administered. However, 19 of 21 participants (90.5%) developed a DTH response at either the arm or the thigh suggesting that true anergy to the antigen was uncommon. There was a statistically significantly higher number of DTH responses following forearm skin tests compared with tests at the thigh (90.5% *vs.* 57.1%; p=0.014).

Table 4.5: Participants with positive DTH responses by anatomical site

	Site of skin test administration	
	Thigh	**Forearm**
DTH response, n (%)	12 (57.1%)	19 (90.5%)
DTH non-response, n (%)	9 (42.9%)	2 (9.5%)
Total (n)	21	21

The median magnitude of DTH response peaked at 24 hours at both the thigh and forearm, although a number of DTH responses were not taken by participants at the 24 hour and/or 72 hour time-points (see Table 4.6).

Table 4.6: Delayed type hypersensitivity reaction at various time points following skin test

	Skin test result (median*, range) (mm)		
Site	**24 hours (n=17)**	**48 hours (n=21)**	**72 hours (n=12)**
Thigh (mm)	6.5 (0 – 20)	5 (0 – 16)	0 (0 – 4.5)
Forearm (mm)	8.5 (0 – 17.5)	7.5 (0 – 11.5)	0 (0 – 9)

*Average of two diameters taken at right angles

Table 4.7 shows data comparing the magnitude of the DTH response according to exposure group and anatomical site. The UVR exposed group demonstrated a reduced (but not statistically significant) median DTH response to KLH challenge compared with the control group, with the largest effect occurring at the site of irradiation (7 *vs.* 0 mm; p=0.10). Reduction in the median DTH response distal to the site of irradiation (forearm) was less pronounced (8 *vs.* 6.5 mm; p=0.41). Two participants in the UVR group were non-responsive to KLH at either the arm or thigh (true anergy).

Table 4.7 Comparison of DTH responses by UVR exposure group at different anatomical sites

	Groups (median*; range (mm))			
Intra-dermal skin test site	**Control (n=12)**	**UVR exposed (n=9)**	**p value****	**Overall (n=21)**
Thigh	7 (0 – 16)	0 (0 – 8)	0.10	5 (0 – 16)
Forearm	8 (0 – 11.5)	6.5 (0 – 9)	0.41	7.5 (0 – 11.5)

*Average of two diameters taken at right angles, 48 hours post intra-dermal challenge

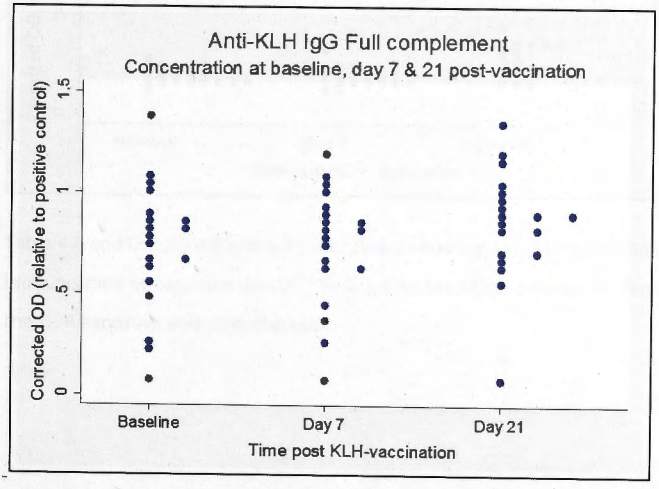
** Statistical test: Wilcoxon-Mann-Whitney test for non-parametric data

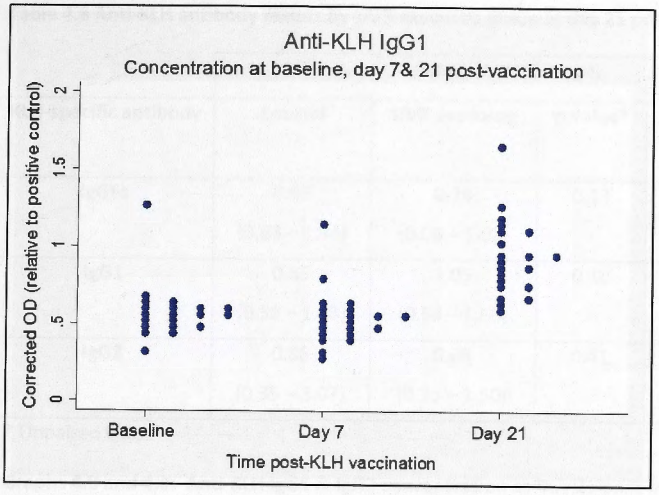
KLH-specific antibody titres

Table 4.4 and Graphs 4.5 – 4.7 show overall data for anti-KLH IgG, IgG1 and IgG2 at the various time points relative to immunisation for all participants. The data demonstrate minimal IgG antibody detection at baseline and 1 week post immunisation, then a strong rise in antibody titre by week 3 post-immunisation.

For participant 8, as there was no serum available for IgG1 and IgG2 testing at baseline, the negative control value was substituted.

Graphs 4.5, 4.6 & 4.7: Distribution of serum anti-KLH IgGFc, IgG1 and IgG2 titres relative to positive plate control - Combined UVR exposure and control groups





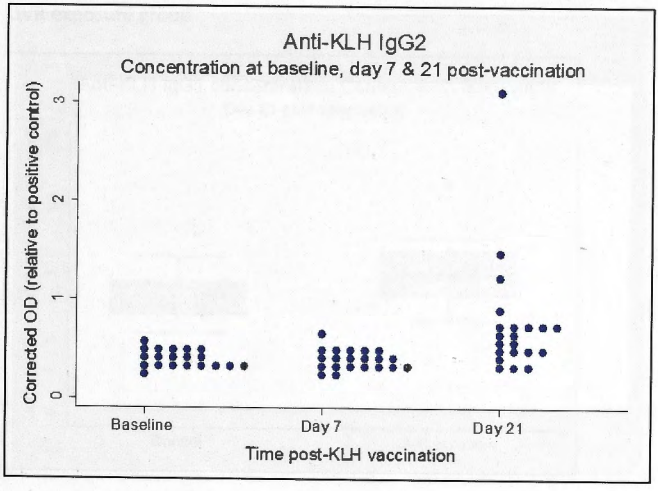


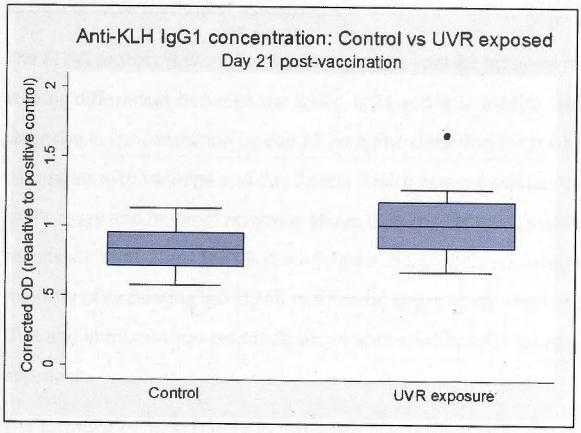
Table 4.8 and Graphs 4.8 and 4.9 show data comparing anti-KLH IgG1 and IgG2 at day 21 post-immunisation by exposure group. There are no statistical differences demonstrated between the UVR exposure and control groups.

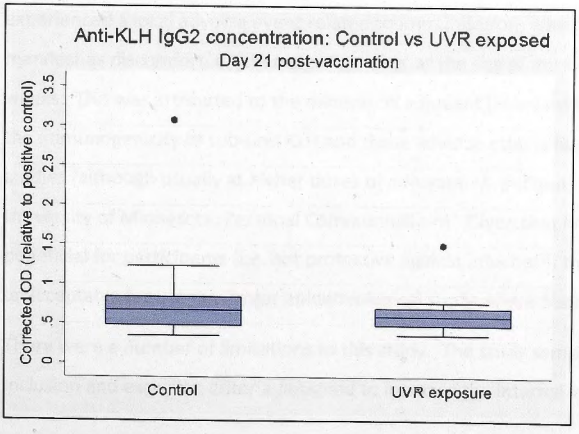
Table 4.8 Anti-KLH antibody results by UVR exposure group at day 21 post-immunisation

	Group (mean; range)			
KLH-specific antibody	**Control**	**UVR exposure**	**p value***	**Overall**
IgGFc	0.93 (0.63 – 1.34)	0.74 (0.08 – 1.02)	0.13	0.85 (0.08 – 1.34)
IgG1	0.85 (0.58 – 1.13)	1.05 (0.68 – 1.66)	0.10	0.94 (0.58 – 1.66)
IgG2	0.86 (0.35 – 3.07)	0.66 (0.35 – 1.50)	0.41	0.77 (0.35 – 3.07)

* Unpaired t-test

Graphs 4.8 and 4.9: Anti-KLH IgG1 & IgG2 concentration at Day 21 post-immunisation, by UVR exposure group





4.3.5 Discussion

This pilot study was successful in achieving a number of its stated aims. It showed that primary immunisation with KLH was a sensitive tool to assess the influence of UVR exposure on antigen-specific humoral and cell-mediated immune function. An antigen-specific immune response was generated in the majority of participants, which supported the dose and route of KLH administration and also the type and sensitivity of the immune assays performed.

Although this small study was unable to disprove the null hypothesis (that solar UVR does not influence the antigen-specific immune response to primary immunisation), the findings were consistent with similar research in this area (See Section 2.3.6).

Following sub-cutaneous immunisation with 125μg KLH, ~ 90% of participants developed a measureable DTH response in at least one anatomical site. The lower rate of DTH response at the thigh compared with the arm may be related to the anatomical site itself or a greater effect of UVR on the local immune response (185). Self-report of DTH response did not appear to be a reliable method of assessment.

The ELISA protocols demonstrated an antigen-specific humoral response; however there were striking differences between the IgGFc, IgG1 and IgG2 assays. Anti-KLH IgG1 assays showed a clear rise in concentration by day 21-post immunisation for the majority of participants compared with baseline and day 7 sera. There was considerable background 'noise' for the IgGFc assay and minimal response above baseline for IgG2, making interpretation of these results difficult. Since IgG1 is considered a Th1-associated antibody and also makes up the majority of circulating IgG (134), in a future, larger study assessing the association between UVR and immunisation response, an antigen-specific IgG1 assay would be a reasonable first approach.

The formulation of KLH used in this study was problematic. One third of participants experienced a local adverse event related to immunisation, albeit minor and temporary, manifest as discomfort, erythema or 'bruising' at the site of immunisation and lasting days to weeks. This was attributed to the mineral oil adjuvant (Montanide ISA-51) used to promote the immunogenicity of sub-unit KLH and these adverse effects have been reported in other studies (although usually at higher doses of adjuvant +/- antigen) (410); Jeffrey Miller, University of Minnesota, Personal Communication). Given that immunisation is not otherwise beneficial for participants (i.e. not protective against infection), this side-effect profile is unacceptable for use in a larger epidemiological study where healthy participants are enrolled.

There were a number of limitations to this study. The study sample was small with multiple inclusion and exclusion criteria designed to increase the internal validity of the study. The

spectral output of the UVR lamp approximated solar UVR in the UVA range but was limited in the solar UVB range. Also, the energy output of the lamp was designed to approximate 0.8 of an MED for participants with Fitzpatrick skin phototypes II & III, although this was by necessity a crude estimation. The lamp was calibrated initially against an accepted standard, however it was not rechecked following the initial calibration, leaving open the possibility for variation in spectral output through the study (183).

4.4 Summary

KLH is an ideal antigen for use in immunisation studies to assess influences on immune function. KLH immunisation produces a reliable and measureable immune response after single administration via the subcutaneous or intra-dermal route (particularly relevant to cutaneous UVR immunomodulation). Measurement of antibody and DTH responses at baseline and three weeks post-immunisation appears to be a relevant, convenient and common assessment time point. Testing for antibody level at one week post-immunisation does not appear to be useful for assessing change in IgG concentration, although it provides a useful check of baseline levels.

Safety of immunisation is paramount. The combination of sub-unit KLH with mineral oil or alum adjuvant confers an unacceptable risk of minor, local reactions. Therefore, for any future study, clinical grade HMW-KLH is the clear preference.

In this pilot study, participants in the UVR exposure group were required to attend nine visits over a 31 day period which represented a high participation burden that would be difficult to maintain in a larger study. However, participants were comfortable with attending five visits over that time period with the attendant blood tests, immunisation and skin test.

To advance this research, a larger well-designed study would need to be conducted that measured personal solar UVR exposure, assessed and adjusted for potential confounding factors and performed the array of *ex vivo* and *in vivo* immune function testing required. Chapter 5 will discuss the preparation and methods used for the larger Australian Ultraviolet Radiation and Immunity (AusUVI) study in more detail.

Acknowledgments

Professor Michael Kimlin (Aus5un Lab, Queensland University of Technology), provided and calibrated the UVR lamp used in the pilot study. Tanelle Dun and Umang Srivastava contributed to the development and conducted the anti-KLH IgG ELISA assays on pilot study participant sera.

Chapter 5: AusUVI Study Methods

Synopsis

The Australian Ultraviolet Radiation and Immunity (AusUVI) Study aimed to determine the influence of solar UVR exposure, at doses relevant to day-to-day activity, on the immune response to primary immunisation in a healthy population of young adults. To conduct this type of epidemiological study, careful consideration needed to be given to obtaining an appropriate participant sample (i.e. demographics, location, sample size) and using validated, robust tools to measure UVR exposure (i.e. acute and cumulative individual exposure), potential confounding variables (e.g. age, gender, physical fitness, psychological state) and immune function outcomes (cell-mediated and humoral). The previous chapter discussed the development of the immunisation protocol and immune function assays in the context of the pilot study. In two sections, this chapter addresses the following issues – Section I: Rationale and evidence for the methods chosen for this research; and Section II: The AusUVI Study design, protocol and detailed methodology for the chosen measurements.

SECTION I Rationale and evidence for the methods chosen for this research

5.1 Measuring ultraviolet radiation exposure

Chapter 2 discussed the current evidence supporting the immunomodulatory influences of UVR exposure in humans. It appears that both short-term and cumulative-UVR exposure may play a role, although more is known about the immunomodulatory mechanisms associated with the former where controlled experiments have been conducted (e.g. using CHS and DTH testing). For the latter, there has been reliance on epidemiological data (e.g. comparing occurrence rates of immune-mediated diseases in different ambient UVR environments).

For the AusUVI Study, where the immune response to immunisation (at an individual level) was the primary outcome of interest, emphasis was placed on obtaining detailed and accurate measurement of short-term personal UVR exposure in the periods (days) either side of KLH immunisation. As discussed in Section 3.2.3, direct measures of (UVR) exposure were preferred over data acquired by recall or by use of surrogate exposure markers (e.g. ambient UVR, latitude of residence). However, to obtain a complete data set looking at various aspects of UVR exposure (and sun protective behaviours), it was important to use a number of methods concurrently.

Measurement of cumulative UVR exposure was also undertaken, though acknowledging at the outset that this would be of lower resolution than for acute exposure data and would represent varying periods of past sun exposure.

The advantages and disadvantages of different UVR measurement tools (See Table 5.1) will be discussed in the following sections. Reflectance spectrophotometry can be used for objectively assessing changes in skin pigmentation over time (i.e. UVR-induced tanning) and for constitutive pigmentation. This method is discussed separately in Section 5.2.5. Similarly, changes in serum 25(OH)D level largely reflect recent UVR exposures (or changes in diet) – but will be discussed separately in Section 5.2.4.

Apart from accuracy and reliability, cost-effectiveness and participant acceptability were other important considerations when deciding on appropriate methods for assessing UVR exposure.

Table 5.1: Methods for measuring acute and cumulative UVR

	Possible measurement tools
Short-term (days to weeks)	• Direct measurement – personal UVR dosimeter • Reflectance spectrophotometry – change in pigmentation of sun-exposed skin over time • Sun exposure diary • Recent history of sunburn • Changes in vitamin D status over time (serum 25(OH)D level) • Ambient UVR monitoring – outdoor UVR monitor
Cumulative (months, years, life-course)	• Microtopography – silicone skin casts • Sun exposure questionnaires

5.1.1 Measuring short term UVR exposure

UVR personal dosimeters

There are a number of UVR dosimeter types (i.e. chemical, biological, electronic), but the basic requirement is that their response function approximates the UVR action spectrum of the biological outcome of interest (e.g. erythema) (411). Studies using personal dosimeters began in the mid-1970s with the development of chemical dosimeters (412). These used chemical substrates (e.g. polysulphone, allyl diglycol carbonate, nalidixic acid, 8-methoxypsoralen) that underwent photo-transformation on exposure to biologically-relevant UVR wavelengths (413). Most clinical studies have used the polymer polysulphone for which the spectral response to UVR closely approximates the erythemal action spectrum (39,413). Polysulphone is usually mounted on a cardboard holder with a central aperture allowing direct UVR exposure. The

change in optical absorbance at 330nm is read after UVR exposure and compared with a standardised UVR curve generated by a spectroradiometer, to derive an erythemally weighted dose (i.e. SED) (43,414). Polysulphone badges are small, light and can be pinned to clothing or worn as a wrist band. The main disadvantages of chemical dosimeters are that they are dose saturable and therefore need changing regularly if undertaking a longer period of observation, indicate only a cumulative dose, and require a lengthy data extraction process (414).

Biological dosimeters rely on UVR-induced DNA damage of a biofilm containing bacteria, spores or bacteriophages (415). Most interest has centred on the bacterium *Bacillus subtilis,* that has a spectral response in the UVB wavelengths that closely approximates the erythemal action spectrum (411). A small number of published studies have utilised biological dosimeters, although a number of logistical issues limit their widespread use.

Electronic dosimeters are the latest iteration of personal UVR dosimeters. They have several important advantages over chemical and biological dosimeters, namely a non-saturable response, short sampling intervals, 'time-stamped' data, re-usability and ease of data transfer (416). Disadvantages include potential for software and hardware malfunction, and expense. Electronic dosimeters are comprised of a sensor, amplifier, data logger and cell battery which are typically housed in a plastic casing that can be placed on a wrist band (i.e. worn as a watch) or worn as a badge (39,416,417).

A recent well-designed study compared the accuracy of polysulphone dosimeters and electronic dosimeters against a reference spectroradiometer under natural daylight conditions (414). They found that polysulphone dosimeters had an increased average and maximal deviation from reference data of 26% and 44%, compared with 15% and 33% for electronic dosimeters, respectively. They concluded that electronic dosimeters used in the study were more reliable than polysulphone versions. Table 5.2 summarises the advantages and disadvantages of the various dosimeter types.

The wrist has been found to be a reliable site for personal UVR measurement and receives around 50% of the dose received at the top of the head (which is the best approximation of ambient UVR) (418,419). Downs and Parisi (420) recently reported that the dorsum of the hands received a similar fraction of UVR even at varying solar zenith angles.

Table 5.2 Comparison of UVR dosimeters

Type of dosimeter	Advantages	Disadvantages	Studies utilising technology
Chemical (e.g. polysulfone)	• Response function approximates the erythemal action spectrum • Reliable • Experience with use • Light, small • Ease of preparation, use, calibration • Thermal stability	• Saturable response • Only cumulative dose • Replaced frequently • Laborious data extraction	(43,44,421–424)
Biological *(e.g. Bacillus subtilis)*	• Response function approximates the erythemal action spectrum • Higher saturation ceiling compared with chemical dosimeters • Commercial dosimeters available (e.g. Viospor)	• Complicated preparation and analysis • Saturable response	(418,425–428)
Electronic	• No upper SED limit • Monitor intra-daily doses • Re-usable for weeks • Readings at programmable intervals (e.g. 8 seconds, 1 minute) • Instant read-outs • Long battery life • Small, light, portable	• Expensive & laborious to produce • Issues with static, battery life, circuitry, uploading data • Software issues	(25,42,414,416,429–432)

Sun diaries

Sun diaries where participants are asked to log the time spent outdoors or "in the sun" for a period of time, usually days to weeks, have been utilised by numerous researchers (25,42,423,433,434). For example, Glanz et al (423) asked lifeguards, parents and children to log their sun exposure in a diary for four consecutive days by marking each hour that they were outside. Correlation with polysulphone dosimeter data showed fair to good correlation with diary data on weekdays and weekends for children ($r=0.18$ and 0.34; $p<0.05$ and $p<0.001$), parents ($r= 0.29$ and 0.28, $p<0.01$) and lifeguards ($r=0.38$ and 0.57; $p<0.001$). Sun diary data are straightforward to analyse; however there is an increased participant burden which may be an issue over longer periods of observation. Using a similar study design, Chodick et al (42) also found significant correlation between recorded diary data of "time spent outdoors" and UVR dosimeter data. Notably, better correlation occurred at around noon compared with the extremes of the day. An additional benefit of sun diaries is that detail regarding concomitant use of clothing, sunscreen and other sun protection behaviours can be elicited.

Assessments of recent sun exposure by self-report have also commonly asked about frequency of sunburn (i.e. over the previous 12 months) (435,436) and time spent outside on weekends and weekdays (423,437,438). Chodick et al (438) looked at the agreement between contemporaneously recorded diary data and subsequently recalled data of time spent outdoors on weekends and weekdays six months later in a group of American indoor workers. They concluded that recalled data for weekdays was more reliable than for weekends, possibly due to the lower number and less variation in spread of hours spent outdoors on weekdays.

5.1.2 Measuring cumulative UVR exposure

Sun exposure questionnaires

Recall of past sun exposure has been commonly used in epidemiological studies assessing personal UVR exposure. There is no 'gold standard' approach to survey design and as such studies have varied by type of question asked, period of time (or age) recalled and by objective measures used to validate questionnaire responses (439). Researchers commonly ask participants to recall the pattern of sun exposure from some previous defined time period (i.e. on vacation, during childhood, pregnancy) (440–442). This type of historical recall has shown reasonable correlation with objective measures of cumulative sun exposure such as silicone skin casts (440). The advantages of using a questionnaire-based approach are convenience, participant acceptability, low cost, acquisition of data covering a wide range of other variables (e.g. sun protection strategies, demographic information, and dietary history) and time

periods, and utility within a large study population The obvious disadvantages of this method are that significant recall bias may be introduced particularly if participants have an inclination as to how sun exposure affects the outcome measure of interest (particularly relevant for case-control studies), poor reliability of data particularly when using data from a very remote period (i.e. early childhood) and low data resolution (i.e. can only obtain estimated averages of sun exposure over the period of interest).

Using silicone casts to assess microtopography of skin

Measuring actinic damage from chronic or cumulative solar UVR exposure with the use of skin casts (impressions) has been used in a number of studies since the 1980s. Photo-ageing is a pathological process distinct from chronological ageing, with characteristic macroscopic features (see Section 1.1.7).

Beagley & Gibson (443) proposed a six-grade system to classify actinic damage based on skin surface characteristics of silicone rubber skin casts observed under low power light microscopy (microtopography). The Beagley-Gibson (BG) system uses deviations from features of non-photo-aged skin in its grading descriptions (see Table 5.3). Undamaged skin (Grade 1) is described as having a series of diagonal and transverse *primary lines* which intersect to form quadrilaterals and triangles. *Secondary lines* are defined as smaller lines within primary line formations which form star like patterns at the points where they intersect (444). At higher skin damage grades, there is progressive flattening of secondary lines, loss of distinctive triangle formation, macroscopic deterioration in cast appearance, and deepening and increasing separation of primary lines.

Table 5.3 Beagley-Gibson system of grading skin microtopography

Grade	Features
1	Primary lines are all of the same depth. Secondary lines are all clearly visible, are nearly the same depth as the primaries, and often meet to form an apex of triangles ('star formation')
2	Some flattening and loss of clarity of the secondary lines. Star formations are still present, but often one or more of the secondary lines making up the configuration is/are unclear.
3	Unevenness of the primary lines. Noticeable flattening of the secondary lines with little or no star formation.
4	Macroscopic deterioration in texture. Coarse, deep primary lines. Distortion and loss of secondary lines.
5	Noticeable flat skin between the primary lines. Few or no secondary lines.
6	Large, deep and widely spaced primary lines

Source: (444)

Uses and validation of the Beagley and Gibson classification system

Numerous epidemiological studies have utilised silicone skin casts and the BG classification system to assess cumulative UVR exposure (25,366,440,444,445). Most studies have taken skin casts from the dorsum of the hand as this represents a sun exposed area that is not likely to be covered by clothing and is easily accessible (420).

The test characteristics of the BG system have also been assessed in several studies. Holman et al (444) graded the skin casts (dorsum of left hand, two independent graders) of 1216 participants aged 16 to 86 years, and compared the results with questionnaire data on prior treatment for non-melanocytic skin cancer (NMSC) and a dermatological examination of sun exposed areas for solar ("actinic") keratoses. They found a significant increasing trend in age-adjusted odds ratio between higher microtopographic grade and presence of solar keratosis ($p<0.0001$) or past treatment of NMSC ($p=0.004$). There was 64% agreement of cast scores by graders, with a kappa statistic of 0.54 (moderate agreement). Battistutta et al (374) compared the microtopographic score of the dorsum of the left hand of 195 Australians (aged 18 – 79 years) with the histological findings of a 2mm punch biopsy at the same site. Higher grade casts had 3.1 times greater odds (95% CI 1.6 – 5.7) of demonstrating histological changes consistent with significant photo-ageing (e.g. dermal elastosis) than lower grade casts. This association persisted even after adjustment for other influential variables including age, sex, occupation, skin colour, tanning ability and smoking. Inter-rater agreement of cast scores was high - 88% of casts were given the same grade by both raters (weighted kappa statistic 0.86; very good agreement). Intra-rater agreement was also high (weighted kappa statistic > 0.80 for both raters). Lucas et al (440) compared the skin cast grades of 534 adults (aged 18 – 61 years) with examination findings (e.g. skin complexion, solar keratosis, spectrophotometry findings), interview data regarding past sun exposure (including in childhood), and demographic information. Even after adjustment for age, sex and natural skin tone, the authors found that higher skin cast grades were significantly associated with higher lifetime cumulative UVR dose (adjusted OR 1.39; 95% CI 1.11-1.75 per 1000 kJ/m^2), higher number (>10) of life-time sunburns compared with no sunburns (adjusted OR 2.86; 95% CI 1.50-5.43) and history of solar keratosis (adjusted OR 1.55; 95%CI 1.03 – 2.32).

Counter to the findings of these studies, Seddon et al (366) found the correlation between higher histological changes (elastotic degeneration) on skin biopsy (dorsum left hand) and higher skin cast grade did not persist after adjustment for age and other independent predictors (e.g. male gender, smoking, lighter skin complexion, lighter iris colour, lower education status, sunburn tendency) ($r=0.18$; $p=0.13$). The small numbers in this study ($n=96$) suggest the sample was insufficiently statistically powered to show a significant association

between the variables of interest, particularly with the number of covariates included in the regression model.

5.2 Measuring potential confounders

5.2.1 Physical activity

Physical activity has been shown to modulate the immune response to immunisation (See Section 3.3.6). There are a multitude of questionnaires that are designed to measure physical activity, varying by target population, specific study aims, types of activity included and/or reference time periods (446). There is no gold standard method for assessing physical activity for clinical studies, although various instruments are available to verify the construct validity of questionnaires, including the doubly labelled water method (DLW), accelerometers, pedometers and actometers (447). Measures of physical fitness such as maximum oxygen uptake (VO_2 max) can also be useful, as fitness is related to physical activity (448). Each of these objective measures of physical activity has limitations restricting their use in epidemiological studies, including the need for equipment and training, time commitment and expense.

Amongst the questionnaire instruments for measuring physical activity, the International Physical Activity Questionnaire (IPAQ) has become the most widely used questionnaire for clinical studies where physical activity is measured (449), and was also used in the AusUVI Study.

International Physical Activity Questionnaire

The IPAQ was designed by an International collaboration to provide a questionnaire-based tool to obtain "comparable population estimates of health-enhancing physical activity" (450) (pp. 753). There are two IPAQ versions - a long and short form. For the AusUVI Study, the short form (IPAQ-SF) was used. It asks participants to recall the frequency and duration of the following forms of activity over the previous seven days: vigorous intensity (e.g. aerobics, heavy lifting, digging), moderate intensity (e.g. carrying light loads, cycling at regular pace, playing doubles tennis), walking and sitting. The IPAQ-SF generates scores based on the 'metabolic equivalent of task' (MET) that is attributed for different levels of activity intensity, and is expressed as MET-minutes per day or week. Scoring algorithms also allow for categorisation into low, moderate and high physical activity populations based on the frequency of physical activity and/or MET-minutes per week (www.ipaq.ki.se).

Validation studies have shown that the IPAQ-SF has good reliability (test-retest agreement) and initial assessment showed promising construct validity (451). However, more recent studies have cast doubt on the construct validity of the IPAQ-SF (446).

5.2.2 Psychological state

Many studies have shown an association between psychological state and an individual's immune status (see Section 3.3.4). For the AusUVI Study, validated tools that reliably measure aspects of psychological state most likely to influence immune function were required (e.g. distress, stressful events, chronic stress). The questionnaire-based tools that were chosen were based on previous psychoneuroimmunological studies, and are described in more detail below.

Measurement tools

Mental Health Inventory

The Mental Health Inventory (MHI) (www.rand.org) is a 38-item questionnaire developed to measure psychological well-being and distress over the previous 30 days in a non-psychiatric population. Participants choose from between five and six descriptive responses to questions that cover five domains: anxiety, depression, loss of behavioural and emotional control, positive affect and emotional ties (453). The MHI has been demonstrated to possess excellent internal consistency, test-retest reliability and construct validity after being validated in several large populations (452–454). The MHI has a complicated scoring system, though the final measurement outcomes are: One global higher-order score, the MHI score; two secondary higher-order global scores, the Distress Index and the Wellbeing Index; and five low-order scores covering the domains listed above.

The MHI has been used in previous studies looking at the effect of psychological distress on the primary immune response to immunisation (with KLH). Snyder et al (51) showed that participants with increased MHI-measured psychological distress had a decreased KLH antibody response, and those with increased MHI-measured wellbeing scores had increased KLH antibody response. In a subsequent study, the authors showed that increased MHI-measured distress was associated with a decreased cellular response to primary KLH immunisation (386).

Profile of Mood States

The Profile of Mood States (POMS) was developed to assess transient mood states. The original version contained 65 items and asked participants to describe their current mood (e.g. lively, forgetful, unhappy) on a five-point scale ranging from 'not at all' to 'extremely' (455). Six sub-scales are measured: tension-anxiety, depression-dejection, anger-hostility, fatigue-inertia, vigour-activity and confusion-bewilderment (see Table 5.4). A shortened form of the POMS, 'POMS-SF', was later developed using only 37 items which reduced the time taken to complete the questionnaire significantly but maintained the psychometric properties of the longer version (456,457). As with the long form, POMS-SF gives individual sub-scale scores as well as an overall global index score, the so-called 'Total Mood Disturbance score' (sum of the individual sub-scales minus the score for the vigour sub-scale).

The POMS-SF is considered an excellent tool where a brief measure of distress is required (457). The POMS questionnaire has been previously used in studies assessing the influence of distress (as measured by the POMS global index score) on the immune response to vaccination (335,342).

Table 5.4 Profile of Mood Score – Short Form subscale items

Sub-scale	Items included	Sub-scale total
Depression	Unhappy, sad, blue, hopeless, discouraged, miserable, helpless, worthless	8
Vigour	Lively, active, energetic, cheerful, full of pep, vigorous	6
Confusion	Confused, unable to concentrate, bewildered, forgetful, uncertain about things	5
Tension	Tense, on edge, uneasy, restless, nervous, anxious	6
Anger	Angry, peeved, grovely, annoyed, resentful, bitter, furious	7
Fatigue	Worn-out, fatigued, exhausted, weary, bushed	5

5.2.3 Nutritional state

Both macro- and micro-nutrient deficiency as well as obesity can influence human immune function (see Section 3.3.5). As nutrition encompasses a wide range of components (e.g. total energy intake, macro- or micro-nutrient intake), there is no universal measure of nutritional state. For the AusUVI Study - where under-nutrition in particular was not thought to play a large role given the inclusion and exclusion criteria - two relatively coarse measures of nutrition were used: body mass index and serum albumin.

Body mass index

The body mass index (BMI) is derived from the calculation: Weight (kg) / (Height (metres))^2. It is a simple, convenient and commonly utilised measure which correlates with body fat percentage (458). Various authorities have given guidance as to how to interpret BMI to delineate between underweight, normal weight and obese persons (www.cdc.gov/obesity) (See Table 5.5).

Table 5.5 Body mass index classification

Weight category	Body mass index (kg/m^2)
Underweight	<18.5
Normal	18.5 – 24.9
Overweight	25.0 - 29.9
Obese	>30

Source: www.cdc.gov/obesity

At the population-level, the distribution of BMI is a useful surrogate for population body fat levels (460,461). At the individual-level, BMI has been shown to correlate with objective measures of total body fat (e.g. dual-energy x-ray absorptiometry). However, as BMI cannot differentiate lean muscle mass from fat mass, there are numerous caveats that limit its applicability at an individual level (459). For example, for the same BMI, women generally have more body fat than men; older persons more body fat than younger persons; and muscular athletes less body fat than non-muscular individuals (460,461). There are differences in BMI between ethnic groups also that is related to genetic determinants of body composition (460).

Serum albumin

Protein malnutrition is very unlikely in the participants of this study due to the strict inclusion and exclusion criteria (see Table 5.6). Serum albumin reflects underlying protein stores which may be depleted during prolonged periods of poor oral intake (462,463). A low serum albumin

is not a specific test for chronic malnutrition as numerous conditions can lead to hypoalbuminaemia, such as inflammatory states, hepatic synthetic dysfunction (i.e. cirrhosis) or protein losing states (e.g. protein losing enteropathy, nephrotic syndrome) (464).

5.2.4 Assessing vitamin D status

As previously discussed, biologically active vitamin D (calcitriol) has immunomodulatory effects which may act in concert with, or independently of, direct UVR exposure. Assessment of vitamin D status is therefore important to include in explanatory models assessing the influence of UVR on the immune response to immunisation to delineate the relevant pathways of effect. As previously discussed, the usual method of assessing vitamin D stores in clinical studies is to quantify serum 25(OH)D levels.

Measuring 25(OH)D levels

There are numerous commercial assays which measure 25(OH)D serum levels, including competitive protein-binding assays, radioimmunoassays, enzyme immunoassays, chemiluminescence immunoassays, high performance liquid chromatography (HPLC), and liquid chromatography tandem mass spectrometry (LC-MS/MS) (Reviewed in 66).

In recent years, inter- and intra-assay differences in 25(OH)D level determination have been highlighted for many of the assays listed above (465,466). LC-MS/MS is now regarded by many as the "gold standard" assay (467) with very good key performance characteristics as well as the ability to differentiate and measure 25(OH)D_2 and 25(OH)D_3 (468,469).

5.2.5 Skin pigmentation

Skin pigmentation is an important factor modifying the effect of UVR exposure on various biological sequelae, (e.g. skin erythema, epidermal cell damage, vitamin D synthesis) and potentially immune function (See Sections 1.1.6; 1.2.4). There are ethnic differences in constitutive skin pigmentation as well as wide variability within any specific ethnic group. The ability to measure and adjust for skin pigmentation is therefore an important consideration when assessing the potential associations between UVR exposure and immune function. Additionally, skin pigmentation changes following UVR exposure (i.e. tanning effect) and hence the difference in pigmentation between sun exposed and non-exposed skin can reflect personal UVR exposure.

As briefly discussed in Section 1.2.S, the Fitzpatrick Skin Phototype scale provides a primarily qualitative tool for researchers to assess the association between UVR-related disease (e.g. skin cancer) and skin complexion / sun reactivity. However, this scale relies on participants' answers to subjective questioning (e.g. "Do you tan at the first average sun exposure?" (83)). Ethnicity also can influence (and bias) the categorisation of individuals into a particular skin

phototype category (where, for example, hair and eye colour are also included in the phototyping criteria) (470).

Objective measures of skin pigmentation include fluorescence video, reflective colorimetry and melanin visualisation with a Wood's lamp (470). Reflective spectrophotometry (RS) has emerged as a leading non-invasive method for assessing skin pigmentation.

Reflectance spectrophotometry

The method of RS is based on the differential attenuation of reflected light from skin of varying pigmentation. Most light in the visible spectrum (400 – 700nm) reaching the skin passes through the epidermis and follows a tortuous path either leading back out of the skin or to absorption by light chromophores – melanin, oxy-haemoglobin (oxy-Hb) or deoxy-haemoglobin (deoxy-Hb) (note difference with UVR chromophores related to immune system mediation; see Section 2.3.3) (471,472). Maximum visible light absorption for oxy-Hb occurs at 412nm (with lesser peaks at 542nm and 577m); and for deoxy-Hb, maximum absorption occurs at 430nm (and a lesser peak at 555nm). Melanin exhibits no maximum absorption 'peak', but instead declines exponentially at longer wavelengths (473). Changes in blood circulation therefore (e.g. following application of a blood pressure cuff or changes to vasculature following sun exposure), can influence the absorption of light, dependent on the relative concentrations of melanin, oxy-Hb and deoxy-Hb (474).

Spectrophotometers are hand-held instruments that emit light via a small aperture placed directly on the skin and analyse the wavelengths of light that are reflected back (473). There are various algorithms for estimating melanin density from skin reflectance measurements, although the most robust algorithms take into account the relative absorption of light by the various chromophores (reviewed in 75).

Dwyer et al (475) developed an algorithm to account for the visible light absorptive properties of cutaneous haemoglobin. They found that the difference in light reflectance at 400nm and 420nm correlated well (r=0.68) with histopathological grading (via skin punch biopsies) of cutaneous melanin of the upper inner arm in a predominantly Caucasian study population. Furthermore, estimated cutaneous melanin density derived via this method was predictive of skin cancer occurrence, particularly amongst men in a Tasmanian study population (476).

The anatomical sites for measurement using RS have varied by study, but the upper inner arm remains a common site for measurement of constitutive skin type in epidemiological research. Of note, a modest, but significant, seasonal variation in upper inner arm reflectance measurements has been demonstrated (likely due to some summer-time UVR exposure at this "protected site"). The buttock is another potential protected site for assessing constitutive

skin pigmentation, however can be practically difficult to access and can be influenced by body hair (477).

SECTION II: The AusUVI Study design, protocol and methods

5.3 Study design and statistical considerations

5.3.1 Research aim and hypotheses

The AusUVI Study aimed to quantify the effect of personal UVR exposure on the primary immune response to immunisation in young healthy adults at UVR exposure levels relevant to daily activities; and to examine the moderating effect of other potentially relevant factors (e.g. sex, age, skin pigmentation, 25(OH)D level) on this association.

Research Hypotheses

The following hypotheses were generated based on the current photoimmunology literature:

- Higher personal acute UVR exposure is associated with impaired cell-mediated and / or humoral immune response to primary immunisation.
- Higher cumulative personal UVR exposure does not lead to photoadaptation to the immunomodulatory effects of UVR.
- Vitamin D status modulates the antigen-specific response to immunisation.

5.3.2 Study design and setting

In order to test these hypotheses, this study was required to:

- Recruit healthy, young adult participants with a wide range of personal UVR exposures;
- Assess personal UVR dose (acquired via usual daily activities) using validated instruments, including both short-term and cumulative UVR exposures;
- Examine immune function by measuring the response to primary immunisation with a safe, reliable and novel T-cell dependent antigen;
- Measure potential moderating effects of other factors using validated instruments;
- Measure aspects of humoral and cell-mediated immunity using validated instruments and assays;
- Undertake data collection across a full year to include coverage of all seasons;
- Undertake all laboratory measurements of immune function in a standardized manner, in a single laboratory, to minimize inter-laboratory variability.

5.3.3 Participant selection and recruitment strategy

Participants in the AusUVI Study were volunteers, recruited through advertisement at universities, local hospitals, social and sporting clubs, local media and by word-of-mouth. A number of information sessions for the purposes of recruitment were held at the settings listed above. Volunteers were considered likely to have above-average motivation to complete study tasks and a volunteer sample allowed selective recruitment to ensure the broadest breadth of UVR doses. For example, if it became apparent during the study that indoor workers predominated, outdoor sporting groups or workers could be targeted for recruitment, to diversify the likely personal UVR exposure range.

While participants randomly selected from the population might improve generalisability (external validity) of study results, the potentially low adherence and/or completion rate for a study requiring considerable commitment over several weeks would have negated this advantage, in addition to greatly increasing the recruitment workload. With regard to potential selection bias, there was no evidence (nor reasoned likelihood) to suggest that the relationship between immune response to immunisation and UVR exposure would differ by volunteer status. Importantly, potential confounders and effect modifiers would be measured and accounted for.

Study promotions were professionally developed and included posters and brochures (see Appendix B). Study investigator contact details were provided on study promotional material for those wishing to express interest in participating.

An information sheet was sent (e-mailed, mailed or given directly – depending on individual preference) to interested parties following initial contact. A mutually convenient time was arranged for the first study visit, where, after appropriate counselling, written informed consent was obtained (see Appendix C). By way of compensation of a participant's time, effort and travel costs in attending study visits and completing study tasks, a $A50 gift voucher (redeemable at a chain of popular department stores) was provided to each volunteer at the last study visit, as well as feedback regarding examination findings and blood test results.

Table 5.6 lists the inclusion and exclusion criteria for the AusUVI Study.

Table 5.6 Inclusion and exclusion criteria for study participants

Inclusion criteria • Healthy adults (self-reported) • Aged 18 – 40 years • Willing and competent to sign informed consent
Exclusion criteria • Allergy to shellfish • Any infection within 30 days of study entry • Known pre-existing immunosuppressed state (including diabetes, HIV, chronic renal or liver disease, recurrent infections, IgA deficiency, solid organ transplant recipient) • Use of immunosuppressive agents within 30 days (for systemic agents, including inhaled) or 7 days (for topical agents) of study entry • Immunisation within 30 days of study entry • Currently pregnant or breastfeeding • Unable to attend all study visits

These criteria were chosen to maximise the internal validity of study findings. That is, we aimed to recruit a relatively homogeneous sample from which participants with non-quantifiable potential confounding characteristics could be excluded.

There is extensive literature demonstrating that older age (312,358), recent infection or immunisation (478), co-morbid medical conditions or immunosuppressive medications (388,479) can influence the subsequent immunisation response or immune testing, and therefore participants reporting any of these conditions were excluded (or had their entry into the study delayed until they no longer met any exclusion criteria). A list of immunosuppressive medications is provided in Appendix D).

Although there have been no reports of anaphylaxis or other Type-1 hypersensitivity reaction with KLH administration, participants were excluded if they reported a shellfish allergy, in line with other comparable studies (358,387). As there had been no studies assessing the effect of KLH immunisation in pregnant or breastfeeding females, these potential participants were also excluded.

5.3.4 Study setting

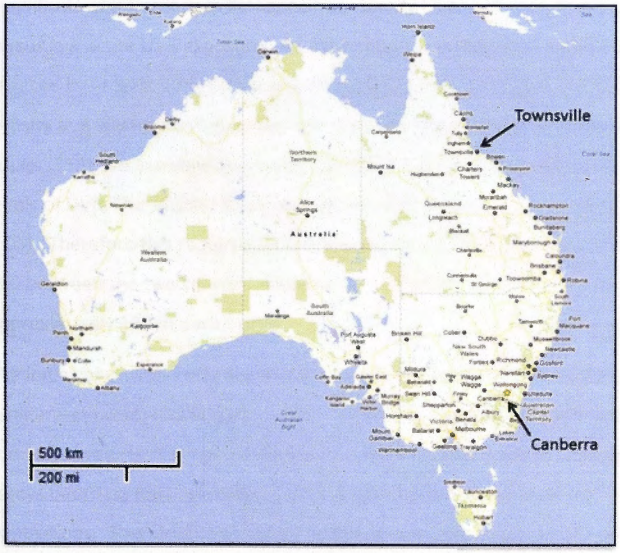
The AusUVI Study was conducted in two study cities – Canberra, Australian Capital Territory (35°15'S, 149°08'E) and Townsville, Queensland (19°15'S, 146°45'E) (see Figure 5.1). These cities were chosen because they experience large seasonal differences in ambient UVR (see Table 5.7), are large regional centres with suitable populations from which to recruit, and have appropriate biomedical facilities that can support this level of research.

Table 5.7 Average noon clear-sky UV Index by season for Canberra and Townsville

	Summer UVI (Dec – Feb)	Autumn UVI (March – May)	Winter UVI (June – Aug)	Spring UVI (Sept – Nov)
Canberra	11 - 12	5 - 6	2 - 3	7 - 8
Townsville	13	10	7	12

Source: Bureau of meteorology (www.bom.gov.au) Accessed: 7 April 2012

Figure 5.1 Location of AusUVI Study cities



Source: Google Maps (www.google.com)

5.3.5 Statistical and data considerations

Descriptive statistics were used to summarise participant demographics, examination findings and blood test results. Comparison of proportions between groups was undertaken using χ^2 statistic or Fisher's Exact Test, as appropriate. T-tests were used for comparison of groups with normally distributed data. Non-normally distributed continuous outcomes were

transformed as necessary. Where normal transformation could not be performed, non-parametric analyses were undertaken as indicated (e.g. Wilcoxon rank-sum test). Sinusoidal functions in time-of-year were used to assess and adjust for seasonal variation for immune outcomes variables. Multiple linear regression and / or ordinal logistic regression models examined associations between outcome and exposure variables, adjusting for other variables found to be confounders. Regression diagnostics were performed to confirm that regression assumptions had not been violated. Differences between groups were deemed significant at p values < 0.05. Where p values are reported, they are derived from two-tailed t-tests. Descriptive estimates (i.e. mean, standard deviation) and regression coefficients were reported to one decimal place. Where reporting to one decimal place did not reveal at least two significant figures, more decimal places were reported (i.e. the following examples would achieve the reporting standard: 12.0, 1.2, 0.12, 0.012). Data were analysed using the statistical software package Stata, v12 (College Station, TX: StataCorp. LP).

Apart from the pilot study, there were no previous studies to guide an estimate of the likely magnitude of difference in KLH-specific antibody titres between those experiencing high versus low acute UVR exposure. An assumption was therefore made that titres would be reduced by at least 20%. Previous work suggests that mean KLH anti-IgG1 corrected optical density at 3 weeks post-immunisation would be approximately 0.55±0.1 in healthy young adults (358); we assumed this would fall to 0.45±0.1 in those exposed to high (above median) levels of UVR. The AusUVI Study was designed to have a minimum of 80% power (at α level of 0.05). Therefore this required 95 subjects above and 95 below median exposure, i.e. a total of 190 between the two centres. Allowing for 10% attrition, this meant recruiting 212 subjects, or approximately 106 in each centre.

The initial laboratory tests planned for *ex vivo* cell-mediated immunity were lymphocyte proliferation assays (LPA) with results reported as mean stimulation indices (MSI). However, soon after commencement of the study, a decision was made to change to more modern assays based on flow cytometry / FACS to give better information on T-helper subset populations. Therefore the original power calculations based on MSI are not reported.

Seasonality of immune response was investigated using cosinor analysis. Assuming 190 evaluable subjects accrued uniformly across 12 months, simulations show that this model has 90% power to detect cycles of amplitude 0.4 standard deviations or more. Thus for humoral immunity, variations in titre of ±0.04 would be detectable corresponding to peak immunity 27% above the annual minimum.

Questionnaires were designed to be electronically entered ('teleformed') into a Microsoft Access (Redmond, WA USA) database. The database was checked for nonsensical, erroneous

or missing data by automated and manual methods and verified against paper copies of questionnaires. Outlier or influential points were identified using routine regression diagnostic techniques (e.g. Cook's D test).

5.3.6 Ethics approval and regulatory considerations

This study was conducted after approval by the Human Research Ethics Committees (HREC) of the Australian National University, Australian Capital Territory Health Department and James Cook University. Written informed consent was obtained from all participants on enrolment into the study. This study was also registered with the Therapeutic Goods Administration of Australia under the Clinical Trial Notification scheme (Trial No: 2010 / 0291) and the Australian and New Zealand Clinical Trials Registry (ACTRN12610000234011).

High molecular weight KLH for immunisation was purchased from Biosyn Corporation (Carlsbad, USA) in a clinical grade form suitable for use in human clinical studies. Certification of Good Manufacturing Process standard was provided along with evidence of testing for endotoxin, metal ions and potential viral and bacterial infective agents (See Appendix E). KLH was imported to Australia for the purposes of this study under an Australian Quarantine and Inspection Service permit (IP0008186).

Researchers independent of the AusUVI Study were appointed to a Safety Monitoring Board (SMB) to assess potential adverse events arising from use of the KLH vaccine (or any other aspect of the AusUVI Study). The SMB was authorized to make recommendations to the relevant HRECs regarding change of protocol or cessation of study, where necessary.

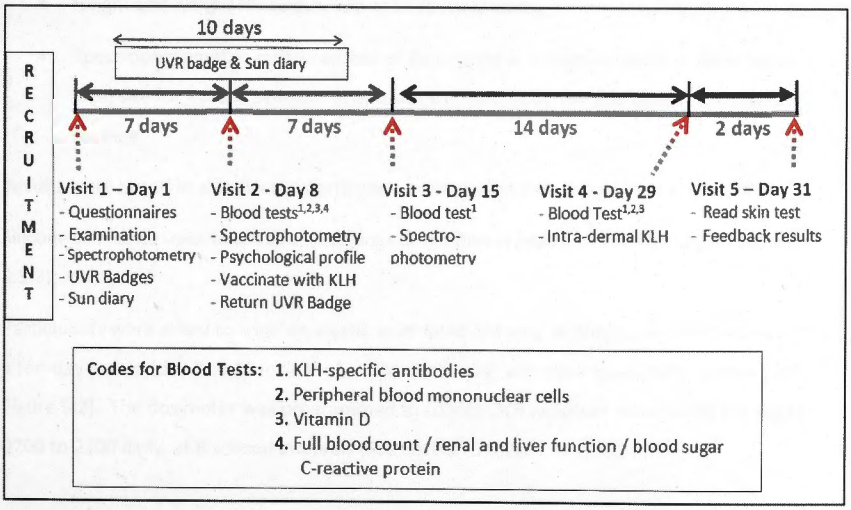
Funding for the AusUVI Study was primarily derived from a project grant from the National Health and Medical Research Council (NHMRC) (Grant No: 585489) which was obtained following a highly competitive, peer-reviewed process.

5.4 Study protocol

There were two enrolment sites in Canberra (Australian National University city campus and the Canberra Hospital, located approximately 10kms south of the city centre) and one Townsville enrolment site, based at the Townsville Hospital. Participants were requested to attend five study visits over a 31 day period. There was an assigned study officer in each city whose responsibilities included recruiting participants, administrating questionnaires, completing examinations and keeping a detailed record of all activities. At the Canberra sites, the study officer (the author of this thesis and registered medical practitioner) also took participants' blood and administered immunisations. At the Townsville site, a registered nurse was separately employed to take blood and administer immunisations. At both sites, qualified laboratory scientists were employed to process and store blood samples for subsequent immune function testing.

Figure 5.2 summarises the tasks undertaken at and between visits. At the outset, it was envisaged that two participants on average would be enrolled per site per week for 52 weeks (to attain the target of 105 participants per site). The remainder of this section summarises the study visits. A Manual of Procedures and Policies (MOPP) was developed for study officers at each site to ensure standardisation of methods of recruitment, measurement and laboratory issues.

Figure 5.2 AusUVI Study protocol



5.4.1 Study visits

VISIT 1 Initial Assessment (Day 1)

Questionnaires (See Appendix F) were administered relating to:

- Demographic Information: age, sex, ethnicity of parents, place of birth, places of current residence (and duration), education, employment status, current occupation;
- General Health: including chronic illness, menstrual patterns, smoking and alcohol intake, family history of immune related diseases;
- Medications: including vitamin D supplements;
- Previous immunisations: adverse events;
- Allergies (including shellfish);
- Physical activity: using the International Physical Activity Questionnaire.

Sun exposure history: skin response to sun exposure at various times of year, frequency of sunburn, sun protection behaviours (amount of skin exposed, type of clothing worn, sunscreen usage), recent sun exposure (over previous month).

A brief physical examination was undertaken, which included:

- Eye and natural hair colour (by comparison to photographs to ensure standardization across centres);
- Height and weight for calculation of body mass index;
- Spectrophotometric measurement of skin colour at sun-protected (i.e. inner upper arm) site for natural skin colour and sun-exposed body site (i.e. cheek) to estimate tanning.

Results were noted in a dedicated participant examination data record (see Appendix G).

Silicone skin casts were taken of a small area of the dorsal aspect of each hand (see Section 5.5.3).

Participants were asked to wear an elastic wrist band carrying an electronic UVR dosimeter for a ten-day period (day 3 to 12 inclusive), while continuing with their usual daily routine (see Figure 5.2). The dosimeter was programmed to collect UVR exposure data during the hours 0700 to 2100 daily, at 8 second intervals (See Section 5.5.2).

Finally, participants were instructed on the use of the self-administered sun exposure diary, to be completed daily for the ten days concurrently with electronic dosimeter wearing. The diary asked for detailed recording of time in the sun, use of sun protection (type of clothing, sunscreen) and exercise patterns (See Appendix H).

VISIT 2 Blood test and immunisation (Day 8)

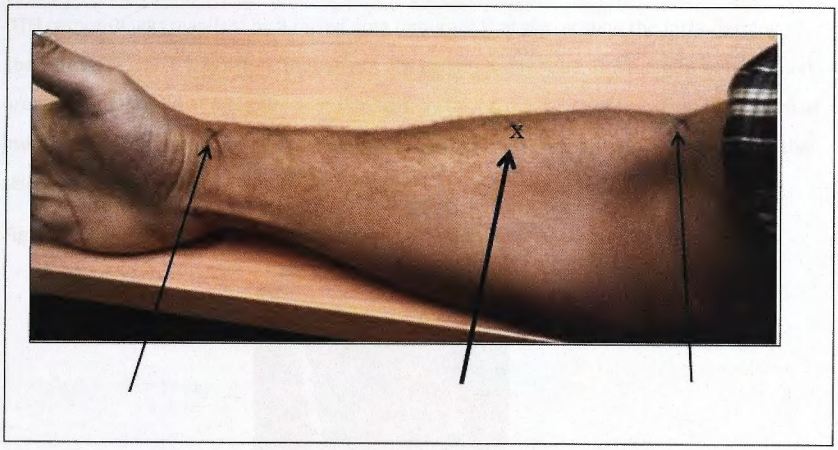
A questionnaire on psychological well-being was administered which comprised the Profile of Mood States (Short form) and Mental Health Inventory (See Appendix F). Participants were specifically asked about whether they had experienced sunburn in the previous week – details regarding frequency, site and severity were noted (See Appendix G).

Repeat spectrophotometric measurement of skin at the inner upper left arm and left cheek was taken.

A venous blood sample was taken for full blood examination, random blood glucose level, liver and renal function tests. Serum for 25(OH)D level and KLH-specific antibody testing and whole blood for peripheral blood mononuclear cells (for cellular immune assays) were processed (See Appendix K) and stored for analysis at study completion.

Participants were immunised subcutaneously with 125µg HMW KLH (0.125mL of 1g/1mL solution) (cGMP grade; Biosyn Corporation, USA) using a 29 gauge diabetic needle (BD catalogue no. 591823). The site of injection was strictly defined as one third of the straight-line distance from the insertion of the brachioradialis tendon at the elbow to the base of the radial styloid at the wrist, with the elbow resting on a flat surface and the wrist held in a neutral position (i.e. neither supinated nor pronated) (See Figure 5.4). This site was marked with a pen. This site was chosen for immunisation as it represented a sun exposed area (i.e. anterior aspect of forearm), had ample subcutaneous tissue for injection, and was accessible and easy to locate using the anatomical markers described. The wrist where the participant wore the electronic dosimeter was also the arm immunised.

Figure 5.4: Anatomical markers for KLH immunisation site



The electronic dosimeter was collected and data downloaded to a personal computer via a Universal Serial Bus (USB) cable. The dosimeter memory was cleared and re-set to commence data recording as before. Participants were instructed to wear the dosimeter until day 13 had been completed (to complete the ten days of dosimeter wearing).

The sun diary was also reviewed to ensure that it was being completed correctly.

VISIT 3 Blood test and spectrophotometry reading (Day 15)

Spectrophotometric skin measurements were again taken from the upper inner left arm and left cheek.

A venous blood sample was taken: serum was subsequently aliquoted and stored for KLH-specific antibody determination at study completion.

The electronic dosimeter was collected and data were downloaded to a personal computer. The sun diary was also checked and collected. Specific enquiries regarding sunburn in the previous week and any adverse events associated with the immunisation were made.

VISIT 4 Skin test and blood test (Day 29)

Participants underwent intra-dermal injection with 10μg of KLH (0.01 mL of 1g/1mL KLH formulation) at the same point on the forearm as was immunised at Visit 2. This was also administered with a 29 gauge diabetic needle.

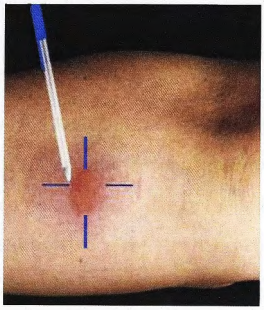
A venous blood sample was taken: serum for KLH-specific antibody determination was aliquoted and stored and whole blood for peripheral blood mononuclear cells was processed.

Details of sunburn episodes since the last visit were recorded.

VISIT 5 Reading of skin test and feedback (Day 31)

DTH response was manifest by a raised area (indurated) of skin around the intra-dermal challenge site which was often (though not always) erythematous. The margins of the raised area were measured at 48 hours post skin test, by taking the mean of two diameters (mm) of induration perpendicular to each other. Induration was detected by running a pen along the skin until it met resistance (see Figure 5.5).

Figure 5.5 "Pen test": measuring induration secondary to DTH challenge



Participants were given a feedback form which recorded their blood pressure, BMI and screening blood test results (full blood count, liver and renal function, random blood sugar level). The department store gift voucher was given to participants at this time also.

5.5 Methods of specific tasks undertaken in the AusUVI Study

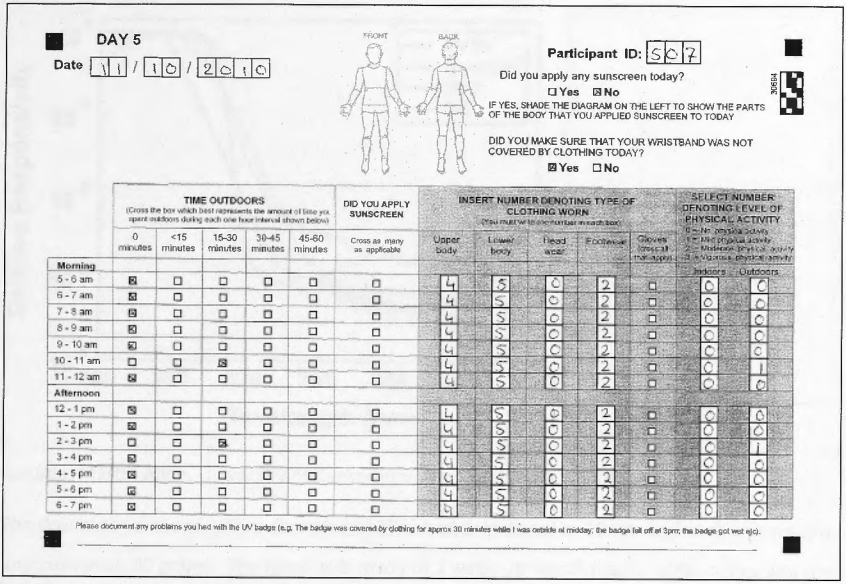
The following section will provide technical detail regarding the various measurement tools used in the AusUVI Study protocol. The rationale for their use has been discussed in the first section of this chapter. Along with the MOPP, four face-to-face meetings and monthly teleconference meetings of study personnel were held for training purposes, standardisation of methods, testing and calibration of equipment and to discuss concerns. The face-to-face meetings occurred prior to recruitment and at three month intervals thereafter, either in Canberra (one meeting) or Townsville (three meetings).

5.5.1 Sun diary and questionnaires

Participants were given a Sun and Activity Diary ("sun diary") to complete for the ten days during which they also wore a UVR dosimeter on their wrist (See Figure 5.6 and 5.8; Appendix H). The sun diary was to be completed each day to minimise recall error. The diary was divided into several parts: i) time spent outside in the sun for each hour of the day from 5am to 7pm (to the closest quarter hour); ii) clothing worn during each hour divided into headwear, upper body, lower body, footwear and gloves; iii) sunscreen use (timing, frequency and distribution) and iv) physical activity (indoors and outdoors) per hour.

Finally, frequency of sunburn was asked for each season over the previous 12 months. For Visits 2 - 5, participants were asked by the research officer whether they had experienced an episode of sunburn since the last visit, and, if so, its site and severity (See Appendix G).

Figure 5.6: Sun and Activity Diary example of daily sun exposure, clothing worn and physical activity



DAY 5

Date 11 / 10 / 2010

Participant ID: 502

Did you apply any sunscreen today?
☐ Yes ☒ No

IF YES, SHADE THE DIAGRAM ON THE LEFT TO SHOW THE PARTS OF THE BODY THAT YOU APPLIED SUNSCREEN TO TODAY

DID YOU MAKE SURE THAT YOUR WRISTBAND WAS NOT COVERED BY CLOTHING TODAY?
☒ Yes ☐ No

	TIME OUTDOORS (Cross the box which best represents the amount of time you spent outdoors during each one hour interval shown below)					DID YOU APPLY SUNSCREEN	INSERT NUMBER DENOTING TYPE OF CLOTHING WORN (You must write a number in each box)					SELECT NUMBER DENOTING LEVEL OF PHYSICAL ACTIVITY (0 – No physical activity; 1 – Mild physical activity; 2 – Moderate physical activity; 3 – Vigorous physical activity)	
	0 minutes	<15 minutes	15-30 minutes	30-45 minutes	45-60 minutes	Cross as many as applicable	Upper body	Lower body	Head wear	Footwear	Gloves (cross all that apply)	Indoors	Outdoors
Morning													
5 - 6 am	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
6 - 7 am	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
7 - 8 am	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
8 - 9 am	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
9 - 10 am	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
10 - 11 am	☐	☐	☒	☐	☐	☐	4	5	0	2	☐	0	1
11 - 12 am	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
Afternoon													
12 - 1 pm	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
1 - 2 pm	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
2 - 3 pm	☐	☐	☒	☐	☐	☐	4	5	0	2	☐	0	1
3 - 4 pm	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
4 - 5 pm	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
5 - 6 pm	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0
6 - 7 pm	☒	☐	☐	☐	☐	☐	4	5	0	2	☐	0	0

Please document any problems you had with the UV badge (e.g. The badge was covered by clothing for approx 30 minutes while I was outside at midday; the badge fell off at 3pm; the badge got wet etc).

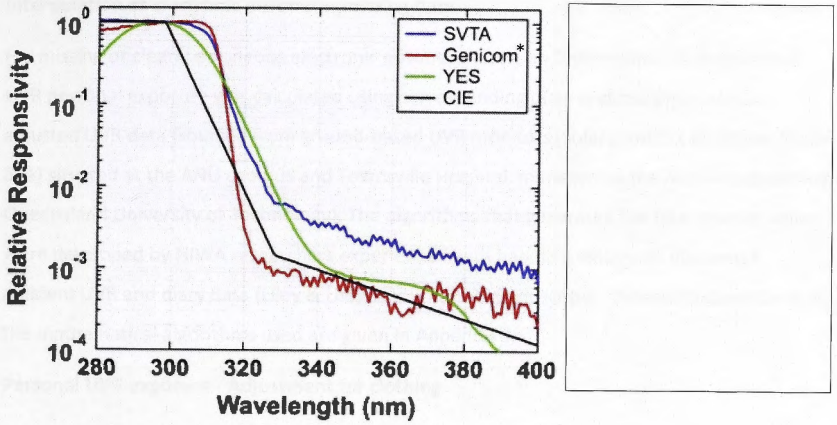
This is an example from the Sun and Activity Diary that participants were requested to complete at the end of each day. For each hour of the day (5am to 7pm), participants indicated time spent outdoors, if they applied sunscreen, what type of clothing was worn on different regions of the body and the type and duration of physical activity. Also, the distribution of sunscreen application was also required.

5.5.2 Use of electronic dosimeters

Electronic dosimetry was the preferred method for measuring an individual's ambient UVR exposure for the AusUVI Study. Electronic dosimeters, manufactured in New Zealand, had been tested and validated in various settings, including with skiers and workers at a ski resort, school children, outdoor workers and an older adult population (25, 416).

The dosimeter utilises a ternary semiconductor system with a photodiode (Genicom) that exhibits a spectral response that mimics the CIE erythemal action spectrum (particularly at wavelengths 325 – 400nm) (See Figure 5.7). On exposure to UVR, the photodiode sensor produces a minute current (1 – 10nA) which is amplified to a measurable voltage (0 – 2.4V), then sampled at pre-determined time intervals (i.e. 8 seconds in the AusUVI Study) by a microcontroller. The data are stored in non-volatile memory (416).

Figure 5.7: Spectral response of electronic dosimeters compared with the CIE erythemal action spectrum



(Source: Martin Allen, University of Canterbury, New Zealand (Unpublished))

The dosimeter is round with a diameter of 35mm and thickness of 10mm and each unit weighs approximately 20 grams. The housing is made of a water-resistant plastic white casing and the badge is secured to the participant's wrist by an elastic wristband. The dosimeters are programmed with customised software via a USB cable attached to a personal computer. In the AusUVI Study, dosimeters were programmed to commence collecting data at Visits 1 and 2 for each participant, with downloading of UVR exposure data at the following visits (i.e. Visits 2 and 3, respectively). Dosimeters were programmed to commence data collection at 0700 hours and cease at 2100 hours, and to sample UVR exposure at eight second intervals. Participants were asked to wear the badge on the outside of clothing (e.g. jacket) or to roll their sleeves up to expose the dosimeter's sensor to sunlight. Participants were requested to wear the dosimeter on the same wrist each day. If engaged in outdoor activity where the dosimeter could not be worn (e.g. swimming, competitive sport), the dosimeter was to be placed on a surface in the open (e.g. beach towel) to approximate the participant's exposure (and this variation noted in sun diary). Batteries (3V lithium cells) were changed monthly to ensure data were not lost or compromised due to low voltages.

UVR exposure was measured in standard erythemal doses (SED). Calibration of electronic dosimeters was undertaken against a Yankee Environmental Systems spectroradiometer at the National Institute for Water and Atmospheric (NIWA) Research (Lauder, Otago, New Zealand) before and after the badges were deployed to Australia. Raw UVR exposure data from the digital dosimeters were downloaded to a personal computer and stored as digital files (Microsoft Notepad, Redmond, USA). On completion of recruitment, all data files underwent

extensive checking for errors, repeat segments, wavering baseline and "noise", by staff at NIWA (see Appendix I).

Interpolation of electronic dosimeter missing data

For missing or clearly erroneous electronic dosimeter data, an "interpolation" of estimated UVR personal exposure was calculated using corresponding diary and maximum cloud-adjusted UVR data (sourced from ground-based UVR monitors (Solar Light Co, Biometer Model 501) situated at the ANU campus and Townsville Hospital, managed by the AusSun Laboratory, Queensland University of Technology). The algorithms that generated the interpolated values were developed by NIWA researchers experienced with handling electronic dosimeter, ambient UVR and diary data (Liley & Liley, unpublished manuscript). Detailed explanations of the mathematical algorithms used are given in Appendix J.

Personal UVR exposure - Adjustment for clothing

The sun diary required participants to detail the types of clothing worn for each hour (between 0500 and 1900) of the ten days that the electronic dosimeter was worn. Participants chose the relevant item from a set of pictures illustrating the most common types of clothing for each body region (see Figure 5.8).

Based on the work of Lee et al (482) where detailed analysis of the body surface coverage of many common clothing types (including of feet, hand and head coverings) was undertaken, a body surface area coverage (BSAC) adjustment coefficient was assigned for each clothing item shown in Figure 5.8. These are presented in Table 5.8.

Figure 5.8: Clothing options for Sun and Activity Diary

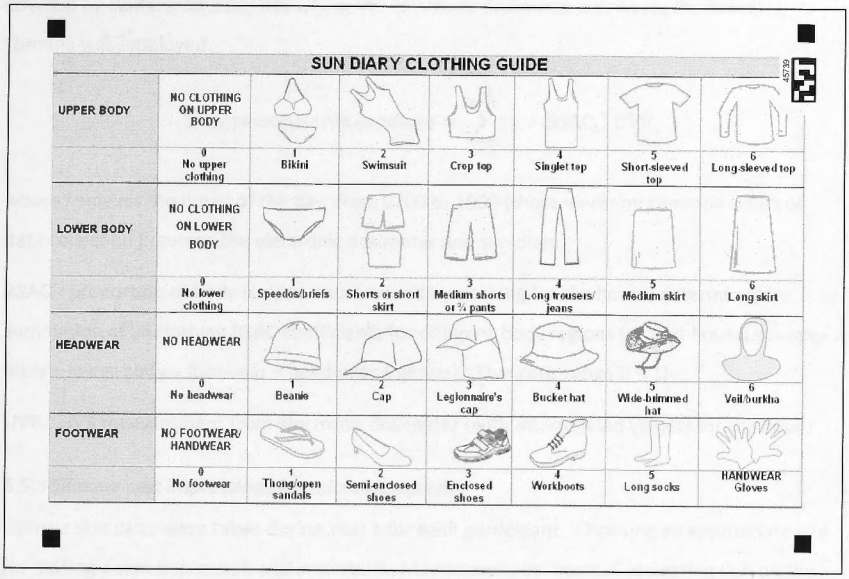


Table 5.8: Assigned body surface area covered coefficients for individual clothing types*

	0	1	2	3	4	5	6
Upper body	No clothing 0	Bikini top 0.041	Swimsuit top 0.354	Crop top 0.128	Singlet top 0.207	Short-sleeved top 0.254	Long sleeved top 0.358
Lower body	No clothing 0	Speedos / briefs 0.070	Shorts or short skirt 0.200	Medium shorts or 3/4 pants 0.294	Long trousers / jeans 0.423	Medium skirt 0.304	Long skirt 0.431
Headwear	No headwear 0	Beanie 0.049	Cap 0.049	Legionnaire's cap 0.061	Bucket hat 0.054	Wide-brimmed hat 0.071	Veil / burkha 0.065
Footwear	No footwear 0	Thongs / open sandals 0.032	Semi-enclosed shoes 0.056	Enclosed shoes 0.077	Workboots 0.111	Long socks 0.154	
Gloves	No gloves 0	Gloves 0.049					

* Adapted from (482)

To determine a participant's daily personal UVR exposure adjusted for the body surface area covered by clothing for each day where the electronic dosimeter was worn, the following formula was employed:

$$\text{Daily personal UVR exposure} = \sum_{i=1}^{12} (1 - \text{BSAC}_i)\,\text{UVR}_i$$

where ***i*** indexes the hours of the day, from 0700 to 1900 (these were the common hours of data collection between the electronic dosimeter and sun diary)

BSAC$_i$: proportion of body surface area covered by clothing for the hour *i*; determined by summation of all clothing BSAC coefficients for different body regions for that hour (i.e. upper body + lower body + footwear + headwear + gloves) (Theoretic range 0 – 1)

UVR$_i$: UVR measurement from electronic dosimeter (with interpolated values) for the hour *i*

5.5.3 Silicone cast impressions for microtopography

Silicone skin casts were taken during Visit 1 for each participant. Choosing an appropriate site for making a skin impression was important. Macroscopically 'normal' appearing skin on the dorsum of the left and right hand was required, free of scars, veins or other blemishes. Participants were seated comfortably with their hands resting on a flat surface (i.e. desk or table). Participants loosely held a plastic cylinder (diameter ~ 4cm) in each of their hands with palms facing down, ensuring that the skin surface on the back of the hand was flat and the hand was held still. A cartridge containing Affinis Fast Body silicone cast material (Coltene, Altstätten, Switzerland) was loaded into the supplied applicator gun. The applicator nozzle could optionally be attached to the applicator gun. Silicone material (comprising two reagents) was squeezed onto a mixing tray and thoroughly mixed with a metal spatula, with care to avoid bubbles. Using the spatula, mixed silicone material was applied to the dorsum of both hands to cover the area of a 50 cent coin (~2.5 x 2.5cm). If the area contained many hairs, then an increased thickness of silicone was applied. The timer was set for eight minutes and the participant instructed not to move their hands from the table. Following setting of the impressions, they were peeled from the skin and examined. If there was evidence of bubbles or smudging, the procedure was repeated. The casts were placed in a clear plastic pocket and labeled on the opposite side of the impression with the date, study number and whether the cast was from the left or right hand.

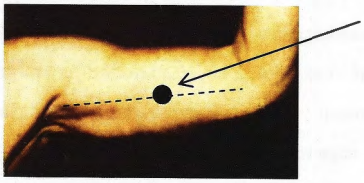
5.5.4 Reflectance spectrophotometry

An estimate of cutaneous melanin density was obtained by reflectance spectrophotometry. Two skin sites were chosen - a non-sun exposed area (left upper inner arm) and a sun exposed area (left cheek) – as they were easy to access, were relatively flat surfaces and had been used in previously published studies (440,483,484).

Skin reflectance was measured at study Visit 1, 2 and 3 using a hand-held spectrophotometer (Konica Minolta; Model: M-2500d, Japan). Skin reflectance measurements were undertaken before blood pressure assessment or venepuncture, as these procedures could affect local blood flow and hence complexion. Facial cosmetic make-up was removed where possible, and those having applied recent tanning products were excluded from reflectance measurements. The spectrophotometer was calibrated prior to each session. Identification of the appropriate site for reflectance measurements was critical and therefore much emphasis was placed on correct positioning of the participant and identification of anatomical landmarks.

For the inner arm measurement, the participant was seated with their left arm resting on a white pillow with the elbow at approximately the height of the heart. A pen mark was made just above the halfway point of a straight line between the mid-point of the axilla and the medial epicondyle of the humerus (See Figure 5.9).

Figure 5.9: Skin reflectance measurement: Left upper inner arm representing non-sun exposed skin



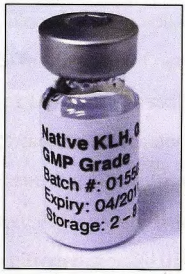
The reading from the left cheek was taken at approximately 2.5 cm lateral to the inferior aspect of the left nostril.

Three reflectance readings were taken at each site. After each measurement the reading was checked and the spectrophotometer re-positioned. Reflectance results were deemed acceptable if all three readings were within a 1% range. If outside this range, further set/s of three readings would be taken until the required consistency was attained. Skin reflectance measurements at 400nm and 420nm wavelengths at each anatomical site were recorded on the data collection sheet.

5.5.5 Vaccine-related issues

The high molecular weight (HMW) KLH vaccine (Biosyn Corporation, Carlsbad, USA) was custom manufactured for the AusUVI Study. Vaccine was packaged in borosilicate glass vials with butyl-rubber stopper closures and flip-top aluminium seals (see Figure 5.10). Each vial contained 600µg HMW-KLH in phosphate buffer solution at concentration 1mg/mL. The initial expiration / retest date was set at 12 months with stability and sterility testing performed at 3 monthly intervals following this to ensure ongoing suitability for use. The vials were stored within the recommended range of 2 – 8°C in refrigerators at the Canberra Hospital and Townsville Hospital pathology laboratories that had generator back-up systems. At regular intervals, the temperature logs for both fridges were checked to ensure that there had been no fluctuation of temperature outside of the recommended storage range.

Figure 5.10: Customised HMW-KLH in glass vial with rubber stopper



As each vial contained 600µg KLH, an individual vial could be used more than once to obtain KLH for immunisation (125µg per dose) or DTH testing (10µg per dose), although a maximum limit of three "accesses" per vial within a maximum period of four hours of first use, was set. A new, sterile diabetic needle was used for each vial access and the rubber stopper decontaminated with an alcohol wipe prior to each dosing. The vials were kept refrigerated between uses. When participants were seen at the ANU study site, vaccine vials were collected from the Canberra Hospital just prior to use and transported in an insulated transport container (e.g. Esky™) with ice (following National Vaccine Storage Guidelines) to maintain cold-chain integrity. Importantly also, the KLH solution was gently mixed by swirling prior to each dosing to ensure an even distribution of antigen.

5.5.6 Blood samples for biochemistry, haematology and 25(OH)D analysis

For the AusUVI Study, blood was collected by venesection from participants at Visit 2 (study day 8), Visit 3 (day 15) and Visit 4 (study day 29). Blood collected in serum separator tubes

(SSTII; BD Catalogue no. 367782) was centrifugally spun at 1000g for 10 minutes within four hours of collection. Biochemistry (electrolytes, urea and creatinine; liver function tests; random blood glucose; C-reactive protein) was determined by automated chromatography assay at the clinical pathology laboratories at Canberra Hospital and Townsville Hospital. In addition, a full blood count was determined by an automated cell counter from whole blood collected in an EDTA tube (BD catalogue no. 366437).

Serum was also transferred in 1 to 1.5 mL aliquots to three labelled vials (Nunc CryoTubes® Cat. No 347627) and stored in designated freezer boxes at -20°C. On completion of recruitment at both study sites, one vial of stored serum for each participant at Visits 2 and 4 was transported on dry ice in one batch to Royal Melbourne Institute of Technology (RMIT) Drug Discovery Technologies for 25(OH)D analysis by LC-MS/MS. Thirty duplicate serum samples (with re-coded labels) were also included for quality control testing. The remaining frozen serum was stored for later anti-KLH antibody assays.

At Visit 2 and Visit 4, blood was also collected in acid citrate dextrose (ACD) tubes (BD Catalogue no 455055) for isolation of peripheral blood mononuclear cells (PBMCs). A maximum time limit of 16 hours was set from collection of blood to PBMC isolation, although in the vast majority of cases, this was performed within 4 - 6 hours of collection. Isolation of PBMCs was by Ficoll centrifugation over a step gradient as per standard protocols (See Appendix K). Separated PBMCs were then carefully frozen in cryovials in a -80°C freezer, then transferred for liquid nitrogen storage until further analysis.

5.5.7 Immunoassays used for the AusUVI Study

Assessment of KLH-specific humoral response was by indirect ELISA against IgG1. The decision to proceed with this IgG subset alone was based on the results of the Pilot Study where this assay demonstrated the lowest background noise and gave the clearest signal. However, serum has been stored for later analysis of other IgG classes as required. The ELISA protocol is otherwise as described in Section 4.4.3 and Appendix A. Anti- KLH IgG1 values are presented as a "corrected" optical density (OD) by dividing the raw absorbance by the result for the standard positive control sample that was also run on each plate (387). The assay's "background noise" was subtracted from the day 7 and day 21 post-immunisation readings. Background noise was defined as the difference in optical density reading between the negative control and a participant's baseline serum.

Assessment of cell-mediated immunity *ex vivo* was undertaken using flow cytometry / FACS and intracellular staining of cytokines to quantify the proportion of Th1, Th2, Th17 and regulatory T cells (FoxP3+ CD127lo) in PBMC samples isolated at baseline and day 21-post-

immunisation (also See Table 5.8). Due to logistic and time constraints, this assay was limited to a subset of AusUVI participants with extremes of personal UVR exposure. Of note, the assays were conducted using cells proliferated with polyclonal mitogens only (Concanavalin A / phytohaemagglutinin). Incubation with KLH alone did not generate enough stimulated lymphocytes to enable further analysis (although work in this area is ongoing). Appendix K contains the protocol details for the cellular assays conducted.

Table 5.8: Cellular targets for FACS analysis of PBMCs from AusUVI Study participants

T-helper subset	Cell surface markers	Intracellular cytokine	Transcription factor
Th1	CD3+ CD4+	IFN γ	
Th2	CD3+ CD4+	IL-4	
Th17	CD3+ CD4+	IL-17	
Regulatory T cell	CD3+ CD4+ $CD25^{hi}$ $CD127^{lo}$	IL-10	Foxp3

5.6 Summary

The AusUVI Study was designed as a prospective longitudinal study aiming to assess the relationship between personal UVR exposure, at doses relevant to day-to-day living, on the immune response to primary immunisation with a T-cell dependent antigen.

Reliable and accurate measurements of short-term and cumulative personal UVR exposure were critical for the AusUVI Study. Personal UVR dosimetry allows for highly detailed measurements within the periods adjacent to immune challenge with immunisation, whilst sun diaries additionally collate corollary information such as clothing and sunscreen. Assessment of cumulative sun exposure was achieved by questionnaires and measurement of UVR-induced skin damage by cutaneous microtopography.

Measurement of factors potentially confounding the association between UVR exposure and the immunisation response were achieved via a combination of examination techniques (e.g. BMI, reflectance spectrophotometry), questionnaires (e.g. assessing physical activity or psychological state) or blood testing (e.g. vitamin D status). As with measurement of UVR exposure, choice of assessment tool was a balance between accuracy, reliability, cost-effectiveness and participant acceptability.

The AusUVI Study was conducted in two cities – Canberra and Townsville - that had appropriate biomedical facilities and a wide ambient UVR differential. Based on sample size calculations, 105 participants from each site were to be recruited over 52 weeks. Inclusion criteria were designed to optimise the study's internal validity by identifying a healthy, young volunteer cohort of adults without characteristics that could affect immune status (i.e. medications or co-morbid conditions). KLH was obtained in a clinical grade form appropriate for immunisation. Participants were seen at five visits over 31 days. Baseline information was collected detailing participant characteristics, 25(OH)D level, skin pigmentation and cumulative UVR exposure. Detailed personal UVR measurements (via electronic UVR dosimetry and sun diary) were planned for five days either side of subcutaneous KLH immunisation to the forearm. An interpolation function for missing or erroneous dosimeter data was developed.

Subsequent visits assessed changes in skin reflectance (tanning), serum 25(OH)D level and changes in immune parameters compared with baseline (KLH-specific antibodies, DTH response and cellular assays).

Subsequent chapters will detail the results from the AusUVI Study.

Chapter 6: AusUVI Study Results: Participant characteristics and UVR exposure data

Synopsis

This chapter describes results of the AusUVI Study in three sections: i) participant enrolment and demographic characteristics, ii) potential immunomodulatory factors, and iii) personal UVR exposure data. Chapter 7 will describe the results of the immune assays and associations with UVR exposure and other variables.

6.1 Demographic data and participant characteristics

6.1.1 Participant recruitment

Two-hundred and twenty two participants completed the AusUVI Study, 110 recruited from Canberra and 112 from Townsville. Recruitment commenced in mid-July 2010 and finished in mid-July 2011 in Canberra and early-August 2011 in Townsville. Graph 6.1 illustrates the cumulative total and monthly rate of enrolment at both sites over the study period. Townsville recruitment was temporarily suspended in September 2010 due to technical issues with the electronic UVR dosimeters and associated software. Due to this unavoidable delay, Canberra recruited more participants in spring 2010 compared with Townsville, with the latter 'catching up' with recruitment by the following winter (2011) (see Table 6.1). Recruitment was suspended in December 2010 for the Christmas-New Year holiday period, and re-commenced in January 2011.

Graph 6.1: AusUVI Study recruitment – Monthly and cumulative totals by study site

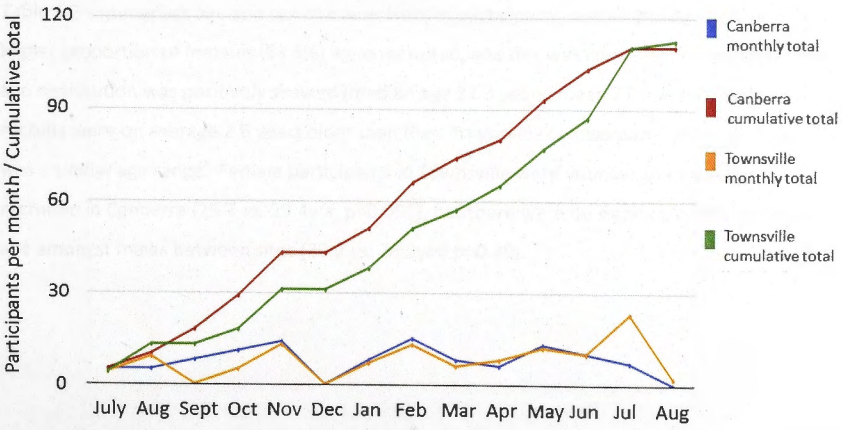


Table 6.1: Participant recruitment: By season

Period	Canberra n (%)	Townsville n (%)	Overall n (%)
Winter (July – Aug 2010 & June – Aug 2011)	27 (24.6)	49 (43.8)	76 (34.2)
Spring (Sept – Nov 2010)	34 (30.9)	18 (16.1)	52 (23.4)
Summer (Dec 2010 – Feb 2011)	20 (18.2)	20 (17.9)	40 (18.0)
Autumn (March – May 2011)	29 (26.4)	25 (22.3)	54 (24.3)
Total	110 (100)	112 (100)	222 (100)

Participants were seen at either of two sites in Canberra depending on participant preference. Fifty-one participants (46%) were seen at the Australian National University campus (in central Canberra) and the remainder at the Clinical Trials Unit at Canberra Hospital (south Canberra). All Townsville participants were seen at the James Cook University's Skin Cancer Research Unit located at the Townsville Hospital, Queensland.

In Canberra, two participants withdrew after commencing the study, one because of interstate employment relocation and the other after learning of pregnancy (before KLH immunisation was given). In Townsville, seven participants commenced the study and subsequently withdrew - three due to employment relocation from Townsville, three due to busy schedules and one for unknown reasons. Participants who withdrew from the study were not counted in the 222 completed study participants and were excluded from analysis.

6.1.2 Participant characteristics

Table 6.2 summarises age and sex characteristics of participants overall and by study site. A higher proportion of females (63.5%) were recruited, and this was consistent across sites. The age distribution was positively skewed (median age 27.3 years; mean 27.9 years). Canberra recruits were on average 2.6 years older than their Townsville counterparts, although there was a similar age range. Female participants in Townsville were younger than females recruited in Canberra (25.7 *vs.* 29.4yrs; $p<0.001$), but there were no significant differences in age amongst males between sites (29.2 *vs.* 28.2yrs; $p=0.49$).

Table 6.2: Participant characteristics – Age and Sex

Participant characteristic	Canberra	Townsville	Overall
Number, n (%)	110 (49.5%)	112 (50.5%)	222
Female, n (%)	71 (64.5%)	70 (62.5%)	141 (63.5%)
Age (years), mean (SD)	**29.2 (5.8)**	**26.6 (6.5)****	27.9 (6.3)
(Range)	(18.3 – 40.9)	(18.2 – 40.6)	(18.2 – 40.9)
**Significant at p<0.01 using t-test after log transformation of positively skewed age distributions			

6.1.3 Ethnicity

There was a wide ethnic diversity amongst AusUVI participants, with 34 different countries of birth. Table 6.3 displays data showing region of birth. The majority of participants were born in Australia (71.6%), followed by south-east Asia (6.3%) and northern Europe (5.9%). Similar proportions were seen at both sites.

Table 6.3: Participant's country of birth grouped by geographic regions

Region of Birth	Canberra	Townsville	Overall
Australia	77 (70.0%)	82 (73.2%)	159 (71.6%)
New Zealand	3 (2.7%)	3 (2.7%)	6 (2.7%)
Northern Europe (including UK)	5 (4.5%)	8 (7.1%)	13 (5.9%)
Central or Southern Europe	1 (0.91%)	2 (1.8%)	3 (1.3%)
South Asia	4 (3.6%)	3 (2.7%)	7 (3.1%)
South East Asia	11 (10.0%)	3 (2.7%)	14 (6.3%)
East Asia	3 (2.7%)	1 (0.91%)	4 (1.8%)
Middle East	1 (0.91%)	2 (1.8%)	3 (1.3%)
Africa	1 (0.91%)	1 (0.91%)	2 (0.91%)
North America	3 (2.7%)	5 (4.5%)	8 (3.6%)
Central / South America	0 (0%)	2 (1.8%)	2 (0.91%)
Pacific	1 (0.91%)	0 (0%)	1 (0.45%)

The ethnicity of participants based on nomination of their parent's ethnic background was also diverse. Table 6.4 shows the maternal and paternal ethnic groups based on the question - "What is your parent's ethnic origin (that is, the place where most of their ancestors came

from)?" Townsville participants had a statistically higher proportion of mothers with northern European ethnicity.

Table 6.4: Ethnic Group of Participant's Mother and Father

	Mother			Father		
Ethnic group	**Overall n (%)**	**Canberra n (%)**	**Townsville n (%)**	**Overall n (%)**	**Canberra n (%)**	**Townsville n (%)**
Northern European	168 (75.7)	**77 (70.0)**	**91 (81.3)***	164 (73.9)	78 (70.9)	86 (76.8)
Central/Southern European	10 (4.5)	5 (4.6)	5 (4.5)	18 (18.1)	6 (5.5)	12 (10.7)
Pacific Islander/ATSI#	5 (2.3)	0 (0)	5 (4.5)	3 (1.4)	0 (0)	3 (2.7)
South Asian	13 (5.9)	9 (8.2)	4 (3.6)	12 (5.4)	8 (7.3)	4 (3.6)
South East Asian	12 (5.4)	9 (8.2)	3 (2.7)	10 (4.5)	8 (7.3)	2 (1.8)
East Asian	12 (5.4)	9 (8.2)	3 (2.7)	12 (5.4)	9 (8.2)	3 (2.7)
Middle Eastern	2 (0.91)	1 (0.91)	1 (0.91)	2 (0.9)	1 (0.91)	1 (0.91)
African	0 (0)	0 (0)	0 (0)	1 (0.45)	0 (0)	1 (0.91)

#ATSI – Aboriginal or Torres Strait Islander *Significant at $p < 0.05$ using χ^2 test

For data analysis where the ethnicity of participants was examined, it was useful to categorise participants into ethnic groups where both parents shared the same ethnicity. Overall, 198 participants (89.2% of all participants) had common parental ethnicity (Table 6.5). Only the proportion of participants with south-east Asian parents was significantly different between sites. The majority of participants had both parents with northern Europe ancestry (156/222 = 70.3%).

Table 6.5: Participants with common parental ethnicity

Ethnic group	Canberra n (%)	Townsville n (%)	Overall n (%)
Northern European	73 (73.7)	83 (83.4)	156 (78.9)
Central /Southern European	3 (3.0)	4 (4.0)	7 (3.5)
Pacific Islander or ATSI#	0 (0)	2 (2.0)	2 (1.0)
South Asian	8 (8.1)	4 (4.0)	12 (6.1)
South-east Asian	**8 (8.1)**	**2 (2.0)***	10 (5.1)
East Asian	7 (7.1)	3 (3.0)	10 (5.1)
Middle Eastern	0 (0.0)	1 (1.0)	1 (0.5)
Total	99 (100)	99 (100)	198 (100)

#ATSI – Aboriginal or Torres Strait Islander
*Significant at p <0.05 using Fisher's exact test

6.1.4 Education and Employment

Canberra participants were significantly more likely to have a Bachelor degree or post-graduate qualification compared with Townsville participants (82% *vs.* 39%; p<0.001) (Table 6.6). Almost half of Townsville's participants had the Higher School Certificate as their highest educational qualification.

Table 6.6: Highest educational qualification attained

Highest Educational Qualification attained	Canberra n (%)	Townsville n (%)	Overall n (%)
High school (not including Year 12)	1 (0.91)	2 (1.8)	3 (1.4)
Higher school certificate (Year 12)	**12 (10.9)**	**54 (48.7)*** **	66 (29.9)
Trade / Apprenticeship	1 (0.91)	3 (2.7)	4 (1.8)
Certificate or Diploma	6 (5.5)	8 (7.2)	14 (6.3)
Bachelor's Degree	**54 (49.1)**	**27 (24.3)*** **	81 (36.7)
Postgraduate degree	**36 (32.7)**	**17 (15.3)****	53 (24.0)

** Significant at p < 0.01 *** Significant at p < 0.001 using χ^2

Table 6.7 displays the occupations of participants, with students and professionals constituting the majority. There were significantly more professionals enrolled at the Canberra sites and students at the Townsville site.

Table 6.7: Occupation held for longest period

Occupation	Canberra n (%)	Townsville n (%)	Overall n (%)
Student	**49 (44.6)**	**70 (62.5)****	119 (53.6)
Manager or administrator	9 (8.2)	4 (3.6)	13 (5.9)
Professional (e.g. engineer, doctor, teacher)	**46 (41.8)**	**22 (19.6)*****	68 (30.6)
Tradesperson (e.g. Carpenter, electrician)	1 (0.9)	5 (4.5)	6 (2.7)
Clerk (e.g. typist, receptionist, data processor)	3 (2.7)	5 (4.5)	8 (3.6)
Salesperson or personal service worker	0 (0)	3 (2.7)	3 (1.4)
Member of defence force	2 (1.8)	1 (0.9)	3 (1.4)
Other	0 (0)	2 (1.8)	2 (0.9)

** Significant at $p < 0.01$ *** Significant at $p < 0.001$ using χ^2

Table 6.8 shows the employment status of participants on enrolment into the study. The majority of participants were either working full-time or enrolled in studies, with differences between sites consistent with previous data.

Table 6.8: Current employment status

Current employment status	Canberra n (%)	Townsville n (%)	Overall n (%)
Unemployed	0 (0)	4 (3.6)	4 (1.8)
Home duties	0 (0)	1 (0.9)	1 (0.5)
Part-time work	13 (11.8)	23 (20.5)	36 (16.2)
Full-time work	**68 (61.8)**	**35 (31.3)*****	103 (46.4)
Student	**29 (26.4)**	**47 (42.0)***	76 (34.2)
Other	0 (0)	2 (1.8)	2 (0.9)

* Significant at $p < 0.05$ *** Significant at $p < 0.001$ using χ^2

Table 6.9 shows the proportion of participants' occupations described as being mainly indoors, outdoors or half-indoors/half outdoors vocations. The vast majority of participants described their occupation as being 'mainly indoors'; however, there were some significant differences between study sites. Townsville participants had a greater proportion of occupations described as "half indoors/half outdoors", and lower proportion of occupations that were "mainly indoors" compared with Canberra participants.

Table 6.9: Indoor - outdoor occupation status

Indoor - outdoor status of current occupation	Canberra n (%)	Townsville n (%)	Overall n (%)
Mainly indoors (e.g. office worker)	**104 (94.6)**	**93 (83.0)****	197 (88.7)
Half indoors and half outdoors (e.g. Physical ed. teacher)	**5 (4.6)**	**16 (14.3)***	21 (9.5)
Mainly outdoors	1 (0.9)	3 (2.7)	4 (1.8)
* Significant at p<0.05 ** Significant at p< 0.01 using χ^2 test			

6.1.5 Income level

Table 6.10 shows the average personal income (before tax) from all sources. Consistent with the above data, Canberra participants reported significantly higher incomes than Townsville participants, reflecting the differences in education levels and occupations.

Table 6.10: Personal income before tax#

Personal annual income before tax	Canberra n (%)	Townsville n (%)	Overall n (%)
Low (<$30K)	**30 (28.3%)**	**68 (61.2%)*****	98 (45.2%)
Middle ($30K - $70k)	34 (32.1%)	26 (23.4%)	60 (27.6%)
High ($>70K)	**42 (39.6%)**	**14 (12.6%)*****	56 (25.8)
# Based on 214 responses; ***Significant at p< 0.001 using χ^2 test			

6.1.6 Suitability for enrolment

Enrolment for the AusUVI Study was conditional on meeting the inclusion and exclusion criteria. Inclusion criteria were based on age (18 – 40 years inclusive) and self-report of good health. Section 6.1.1 shows data confirming all participants met the age criteria. Table 6.11 shows data of participants' self-rating of "general health over the past month". All participants reported fair to excellent recent health, and this was consistent across sites.

Table 6.11: Participant self-report of general health over previous month

General health over past month	Canberra n (%)	Townsville n (%)	Overall n (%)
Excellent	32 (29.4)	29 (26.1)	61 (27.7)
Very good	52 (47.7)	50 (45.1)	102 (46.4)
Good	21 (19.3)	28 (25.2)	49 (22.3)
Fair	3 (3.7)	4 (3.6)	8 (3.6)
Poor or Very poor	0 (0)	0 (0)	0 (0)

There were a number of exclusion criteria for this study (See Table 5.6). At enrolment, no participants met exclusion criteria (although a small number were required to delay their entry into the study because of recent infection or medication).

Although participants reported having had no infection over the past 30 days prior to enrolment, a number did report infections in the previous three months (Table 6.12).

Table 6.12: Reported infections over the past three months prior to entry into the study

Infection site (number of responses)	Canberra n (%)	Townsville n (%)	Overall n (%)
Upper respiratory tract (n=217)	41 (38.3)	47 (42.7)	88 (40.6)
Gastrointestinal (n=214)	10 (9.5)	10 (9.2)	20 (9.4)
Skin or nail (n=214)	10 (9.5)	12 (11.0)	22 (11.2)
Urinary or genitourinary (n=213)	1 (1)	9 (8.2)	10 (4.7)
Other	2 (2.4)	6 (5.9)	8 (4.3)

No participants reported suffering from any chronic heart, kidney or liver condition. Seven participants reported 'chronic respiratory illness' whose aetiology was not specified, though in most this represented asthma that did not require inhaled or oral steroids at the time of, or in the lead up to, the study. Three participants reported a diagnosis of coeliac disease.

To support the self-report of "good health" at time of enrolment, a number of screening blood tests were performed at Visit 2 (the day of immunisation), that aimed to detect inflammatory states (indicative of active infection or inflammation), diabetes and chronic kidney or liver disease.

In this study, the markers used to detect clinically significant inflammation were C-reactive protein (CRP) and white cell count (and differential). Local laboratories were used at the two study sites - ACT Pathology at Canberra Hospital and Queensland Pathology Service at

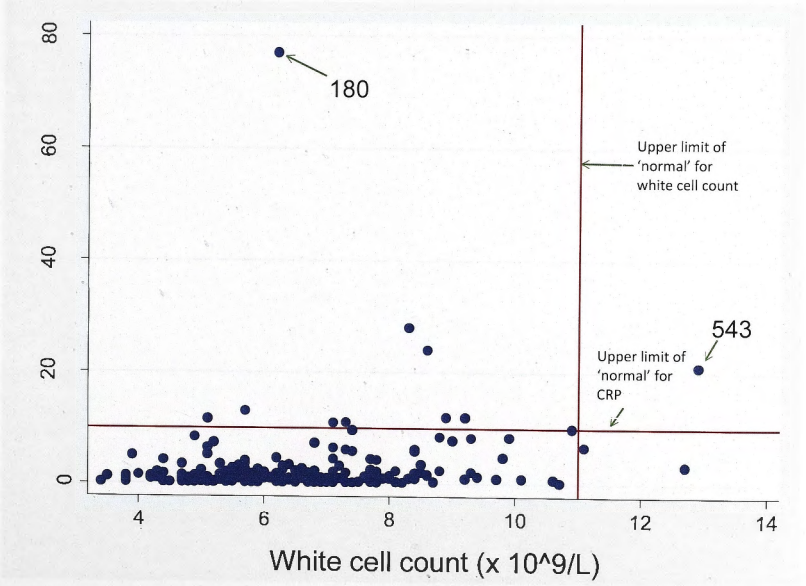
Townsville Hospital. This led to the potential for variation in test results due to differences in methodology, reagents and equipment. However, both laboratories were accredited under the National Association of Testing Authorities (www.nata.com.au) and were mandated to undertake quality control testing. As the reference ranges reported by these labs were similar for these tests, the results between sites were compared directly for the purposes of screening participants for eligibility. The results are summarised in Table 6.13.

Table 6.13: C-reactive protein, white cell count, lymphocytes and neutrophils

Laboratory Test (Reference range)	N	Mean	Median	SD	Range	Number of participants above reference range
C-reactive protein (<10 mg/L)	220	2.7	1	6.2	0.2 – 77.0	10
White cell count ($4.0 – 11.0 \times 10^9$/L)	222	6.4	6.1	1.6	3.4 – 12.9	3

For participants with CRP levels above the reference range (n=10), the mean and median were 22.1mg/L and 12.5mg/L respectively. Graph 6.2 plots CRP against WBC to identify participants where both inflammatory marker tests were raised.

Graph 6.2: Scatterplot of C-reactive protein *vs.* white cell count



One participant (Study ID 543) had both C-reactive protein and white cell count above the reference range. This participant, a female non-smoker on the oral contraceptive pill, rated her health over the past one month as "very good" and reported no infections in the previous three months and no history of chronic illness. Symptoms of inflammation or infection were not reported at subsequent visits. Participant 180 had a markedly elevated C-reactive protein (77mg/L). This was attributed to an accident several days prior to the blood test where an injured ankle was sustained. Again there was no evidence of recent infection on history, or evident at subsequent visits.

Previous studies have shown an association between increased BMI and raised inflammatory markers, supporting evidence that obesity confers a state of low grade inflammation (485). There is a moderate positive association between C–reactive protein and BMI in the AusUVI Study population also ($r=0.33$; $p<0.001$). Notably, participants 543 and 180 both recorded BMI readings in the obese range.

Analysis of random blood glucose level (BGL) readings for all participants did not show any individual with overt hyperglycaemia that would warrant additional testing for diabetes mellitus (i.e. with random BGL > 7.8mmol/L). One participant had an elevated serum creatinine level (114 µmol/L). However, this was explained by this participant's high muscle mass (he was a rugby player, weighing 95kg). Additionally, he had a non-elevated serum urea level (7.8mmol/L), another marker of renal function.

6.1.7 Summary

Two hundred and twenty two participants were recruited into the AusUVI Study over a 13 month period. Approximately two thirds of volunteers were women. The Townsville participants differed from their Canberra counterparts in the following ways – they were generally younger, had attained lower educational qualifications, were on lower incomes, and comprised a higher proportion of students and workers involved with non-indoors employment. Over three-quarters of participants were of northern European heritage, whilst the others were of very varied ethnic backgrounds. All participants self-reported as being healthy, however a small proportion had blood tests at baseline suggesting underlying inflammatory activity, the aetiology of which was uncertain in most cases but may be related to obesity.

6.2 Potential confounding factors and biologically relevant variables

6.2.1 Measures of nutrition

There were no participants with evidence of moderate or severe hypoalbuminaemia which may indicate protein-energy malnutrition or an inflammatory state (mean 43.4 g/L; range 35 – 52 g/L)) (486). Participants' body mass index (BMI) values were similar across sites (see Table 6.14). Canberra had a higher proportion of participants in the overweight category than Townsville, based on BMI measurements.

Table 6.14: Measures of nutrition

Measure of nutrition		Canberra	Townsville	Overall
Body Mass Index ($kg\ m^{-2}$)	Mean	24.0	24.3	24.2
	Median	23.6	22.8	23.3
	Range	17.8 – 33.6	17.0 – 53.4	17.0 – 53.4
	Standard deviation	3.3	5.3	4.4
BMI by category	Underweight (<18.5)	3 (2.7%)	1 (0.9%)	4 (1.8%)
	Normal weight (18.5 – 24.9)	66 (60.0%)	75 (67.0%)	141 (63.5%)
	Overweight (25 – 29.9)	**35 (31.8%)**	**22 (19.6%)***	57 (25.7%)
	Obese (>30)	6 (5.5%)	14 (12.5%)	20 (9.0%)
* Significant at $p < 0.05$ using χ^2 test *** Significant at $p < 0.001$ using t-test				

6.2.2 Smoking

Data regarding smoking habits of participants are shown in Table 6.15. A higher proportion of males than females were "current smokers" although this was not statistically significant (10% *vs.* 3.5%, p=0.07).

Table 6.15: Proportion of participants who were active or past smokers

Category of smoker	Canberra, n (%)	Townsville, n (%)	Overall n (%)
Have ever been a regular smoker (221 responses)	15 (13.6%)	20 (18.0%)	35 (15.8%)
Current regular smoker (222 responses)	5 (4.5%)	8 (7.1%)	13 (5.8%)

6.2.3 Exercise and physical activity

Physical activity status of participants was assessed by the International Physical Activity Questionnaire - Short Form (IPAQ-SF) (see Section 5.2.1.2). The IPAQ-SF assessed activity in three domains: walking, moderate-intensity and vigorous-intensity activities. Based on recall of activities during the previous week, a continuous score was derived for each domain based on that activity's "metabolic equivalent" (measure of intensity), number of active sessions per week and number of minutes spent per session. An overall activity categorical score – low, moderate or high – was also obtained, based on the continuous score and/or number and type of activities engaged in. Table 6.16 shows the categorical data for the AusUVI Study participants with comparisons made between relevant groups. "High activity" constituted the greatest proportion of participants in each comparator group. There were significantly more participants in the low activity group from Canberra compared with Townsville. There was no statistical association between age and activity score (data not shown).

Table 6.16: IPAQ-SF Categorical scores: comparison by site, sex and body mass index

		Site		Sex		BMI	
Activity category	Overall	Canberra	Townsville	Male	Female	<25	≥25
Low	14 (6.3)	**12 (10.9)**	**2 (1.8)****	5 (6.3)	9 (6.4)	6 (4.2)	8 (10.3)
Moderate	72 (32.6)	38 (34.6)	34 (30.6)	22 (27.5)	50 (35.7)	48 (33.3)	24 (31.2)
High	135 (61.0)	60 (54.6)	75 (67.6)	53 (66.3)	81 (57.9)	90 (62.5)	45 (58.4)

** Significant at p <0.01 using Fisher's exact test

6.2.4 Psychological state

Aspects of psychological states were assessed by validated questionnaires – Profile of Mood States and Mental Health Inventory.

Profile of Mood States

Table 6.17 shows data for the different domains measured by the Profile of Mood States (POMS) questionnaire, as well as the overall global index score 'Total Mood Disturbance' (TMD). There was no statistical difference evident between study sites, or according to gender or age groups (comparing younger with older participants) with regard to the TMD score (using t-tests).

Table 6.17: Profile of Mood States: Sub-scores and global indices

		Site Mean score (SD)		Gender Mean score (SD)		Age Mean score (SD)	
POMS scale	**Overall (n=219)**	**Canberra (n=108)**	**Townsville (n=111)**	**Male (n=79)**	**Female (n=139)**	**<Median age (27.3yrs) (n=110)**	**≥Median age (27.3yrs) (n=109)**
Subscale scores							
Tension-anxiety	2.8 (3.3)	3.0 (3.6)	2.4 (3.0)	2.70 (3.2)	2.9 (3.4)	3.2 (3.9)	2.4 (2.5)
Depression-dejection	1.5 (2.5)	1.4 (2.5)	1.5 (2.5)	1.24 (2.1)	1.6 (2.7)	1.3 (2.13)	1.6 (2.8)
Anger-hostility	0.9 (1. 9)	1.01 (1.9)	0.86 (1.9)	0.91 (1.8)	0.9 (2.0)	0.88 (2.0)	0.98 (1.8)
Fatigue-inertia	4.3 (3.9)	4.4 (4.3)	4.2 (3.6)	4.41 (3.9)	4.2 (4.0)	4.2 (3.9)	4.3 (4.0)
Vigour-activity	10.5 (5.0)	10.5 (5.2)	10.5 (5.2)	11.3 (4.6)	10.1 (5.1)	11.2 (5.0)	9.9 (4.8)
Confusion-bewilderment	2.5 (2.5)	2.4 (2.4)	2.7 (2.5)	2.49 (2.5)	2.6 (2.5)	2.8 (2.7)	2.3 (2.2)
Total Mood Disturbance*	1.5 (13.4)	1.3 (13.0)	1.7 (13.8)	0.49 (11.7)	2.1 (14.3)	1.2 (14.1)	1.8 (12.7)

* NB: Range of scores: -20 to 56; Median -1

Mental Health Inventory

Table 6.18 displays data from the Mental Health Inventory (MHI) questionnaire. There are six sub-scale scores (higher scores indicating more of the construct described by the sub-scale name), two global scales indicating the degree of psychological distress (higher scores indicating greater distress) or psychological wellbeing (higher scores indicating greater wellbeing), and a global mental health index score with higher scores indicating greater psychological wellbeing and relatively less psychological distress.

Statistically different global scores were noted according to gender, with females scoring higher overall distress, lower overall wellbeing and lower overall index score compared with males. There was no statistical difference in global indices between younger and older participants, or by study site.

Table 6.18: Mental Health Inventory: Sub-scores and global indices

		Site Mean score (SD)		Sex Mean score (SD)		Age Mean score (SD)	
MHI sub-scales and global indices	Overall (n=219)	Canberra (n=108)	Townsville (n=111)	Male (n=79)	Female (n=139)	<Median age (27.3yrs) (n=110)	≥Median age (27.3yrs) (n=109)
Sub-scale scores							
Anxiety	20.0 (5.7)	20.1 (5.2)	19.9 (6.2)	18.4 (5.0)	21.0 (5.9)	20.2 (5.8)	19.8 (5.7)
Depression	8.1 (2.5)	8.2 (2.6)	8.0 (2.5)	7.5 (2.1)	8.4 (2.7)	7.9 (2.4)	8.3 (2.6)
Loss of behavioural and Emotional control	15.7 (4.7)	15.9 (5.0)	15.5 (4.5)	14.1 (3.5)	16.6 (5.1)	15.7 (4.3)	15.8 (5.2)
General Positive affect	40.9 (7.1)	40.7 (6.8)	41.1 (7.5)	42.2 (6.4)	40.1 (7.4)	41.8 (6.8)	40.0 (7.4)
Emotional ties	9.0 (2.4)	9.2 (2.4)	8.8 (2.4)	9.1 (2.5)	9.0 (2.3)	9.0 (2.3)	9.1 (2.5)
Life satisfaction	4.3 (1.0)	4.2 (1.0)	4.4 (1.0)	4.4 (0.92)	4.2 (1.0)	4.4 (1.0)	4.2 (1.0)
Global indices							
Overall Distress	49.2	49.5	49.0	**45.0**	**51.6*** **	49.2	49.3
	(12.8)	(12.5)	(13.1)	**(10.5)**	**(13.5)**	(12.2)	(13.5)
Overall-Wellbeing	58.3	58.2	58.4	**60.0**	**57.3***	59.4	57.2
	(10.0)	(9.6)	(10.4)	**(9.0)**	**(10.4)**	(9.4)	(11.0)
Global mental health score	167.8	166.8	168.7	**172.0**	**165.3*** **	169.4	166.1
	(23.1)	(25.0)	(21.2)	**(25.8)**	**(21.2)**	(19.5)	(26.2)

Statistical testing for global scores only and assessed by t-test: * p<0.05 *** p<0.001

Consistency of questionnaires in measuring similar psychological constructs

To check agreement between the POMS and MHI questionnaires for the various sub-scale and global measures of similar psychological constructs, a correlation matrix was constructed. It showed a strong correlation between the MHI global mental health score and POMS TMD (r=-0.60; p<0.0001); moderate correlation between MHI overall wellbeing score and POMS vigour-activity subscale (r=0.44; p<0.0001); and moderate to strong correlation between MHI overall distress score and all POMS sub-scales (excluding vigour-activity) (all r>0.40; p<0.0001).

6.2.5 Skin phototype and spectrophotometry measurements

Skin Phototype

Table 6.19 details the Fitzpatrick skin phototype for AusUVI Study participants based on questionnaire responses and observation of skin, eye and hair characteristics. Using an ordinal logistic regression model, there was a statistical difference in the proportion of skin phototypes amongst participants between study sites (p=0.033).

Table 6.19: Fitzpatrick phototype classification

Fitzpatrick Skin Phototype	Description	Canberra	Townsville	Overall
I	White skin, burns easily, never tans, red/blonde hair	3 (2.7%)	4 (3.6%)	7 (3.2%)
II	Fair, burns easily, tans minimally with difficulty	35 (31.8%)	45 (40.2%)	80 (36.0%)
III	Burns moderately, tans moderately and uniformly	42 (38.2%)	48 (42.9%)	90 (40.5%)
IV	"Mediterranean Complexion" Burns minimally, tans moderately and easily	20 (18.2%)	9 (8.0%)	29 (13.1%)
V	Rarely burns, tans profusely, South Asian complexion	10 (9.1%)	6 (5.4%)	16 (7.2%)
VI	Black skin, never burns, tans profusely	0	0	0

Spectrophotometry readings

Skin reflectance readings were taken of the left upper inner arm (to represent non-sun exposed skin) and left upper cheek (to represent sun exposed skin). Previous work (see Section 5.5.4) has shown that the difference in measured reflectance at 400 and 420nm wavelengths correlates with cutaneous melanin density (a technique validated in participants of northern European descent) (476).

Estimate of cutaneous melanin density was derived using the following equation:

$$\textit{Melanin density (MD)}_{400} = 100 \times (0.035307 + 0.009974\ (R_{420} - R_{400})$$

Where: MD_{400} = estimate of the percentage of epidermis containing melanin

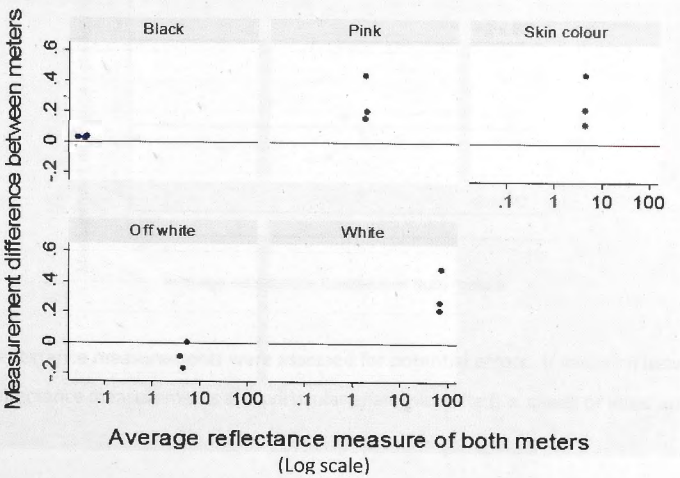
R = reflectance at the specified wavelength

Calibration and error checking of spectrophotometry readings

Skin reflectance readings were taken by a single researcher at each study site using a spectrophotometer of the same model type. Three readings were taken at each anatomic site and averaged (or repeated if the readings differed by more than 1% in skin reflectance). Inter-rater reliability testing was undertaken in two ways. At three of the four study investigator meetings (November 2010, March and July 2011), both spectrophotometers were tested on a set of coloured 'swatch' fabrics (black, pink, "fair" skin colour, off-white, white) to assess consistency of results. Calibration readings were also taken on 'test' individuals using both spectrophotometers.

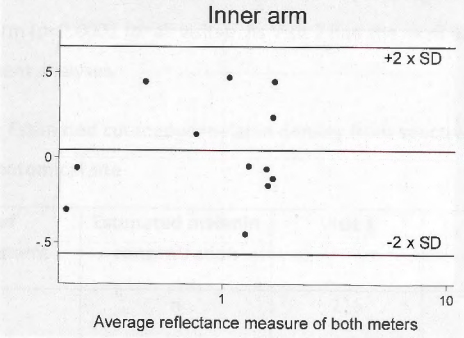
Graph 6.3a shows the difference in reflectance measurements (420nm – 400nm) between spectrophotometers (Canberra meter – Townsville meter) against the average of reflectance measurements for each of the swatch colours. The difference between meters was relatively small in proportion to the average meter reading. The bias towards higher Canberra meter readings for pink, skin colour and white swatches were considered not clinically significant, and hence no correction was made for later participant readings.

Graph 6.3a: Level of agreement between spectrophotometers – coloured fabric

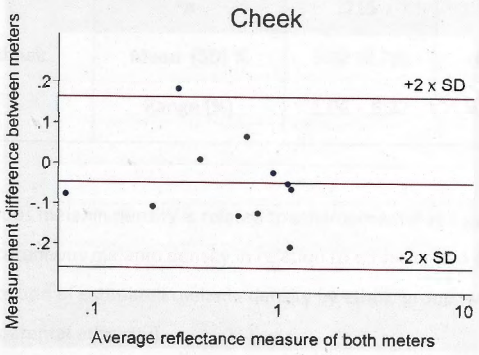


Graph 6.3b and 6.3c show the difference in reflectance measurements (420nm – 400nm) between spectrophotometers (Canberra meter – Townsville meter) against the average of the reflectance measurements for 12 volunteers, for the inner arm and left cheek, respectively. The central red line represents the average difference between spectrophotometers and the two outer lines represent 95% confidence limits. The graphs show that mean difference between meters was close to zero at both anatomical sites, and there was no bias in the distribution of data points above or below zero (i.e. point of no difference). This calibration study (albeit with limited numbers) supported the use of the spectrophotometers at each site, with no correction of raw measurements required.

Graph 6.3b: Level of agreement between spectrophotometers – volunteers' inner arms



Graph 6.3c: Level of agreement between spectrophotometers – volunteers' cheeks



Reflectance measurements were assessed for potential errors. If variation between reflectance measurements at a particular anatomical site (i.e. cheek or inner arm) for an

individual over the three study visits was high (defined as a standard deviation of >1% reflectance), data for that participant were reviewed – and removed if a particular reading was clearly erroneous (based on the other arm and cheek readings). Using this method, 8 (1.5%) of readings were deleted from the cheek data (Visit 1: participant 166, 506; Visit 2: 111, 135, 509, 571, 574; Visit 3: 208). For the inner arm, only two data points (<0.4%) were removed – Visit 1: participant 146; and Visit 2: 151.

Skin reflectance results for AusUVI Study participants

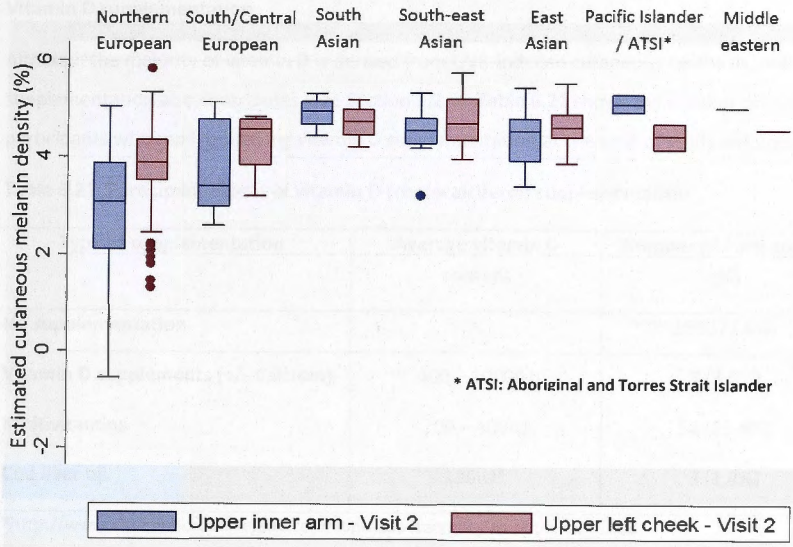
Table 6.20 displays the calculated melanin density data from spectrophotometer readings for all participants for each study visit by anatomical site. The data were normally distributed and there was no significant difference in readings between visits taken at a given site. As expected, the estimated cutaneous melanin density was higher at the cheek compared with the inner arm ($p<0.0001$ for all visits). As Visit 2 had the most data points, Visit 2 was used for all subsequent analyses.

Table 6.20: Estimated cutaneous melanin density from spectrophotometer readings by study visit and anatomical site

Site of measurement	Estimated melanin concentration	Visit 1	Visit 2	Visit 3
Left upper inner arm	n	216	220	215
	Mean (SD) %	3.30 (1.27)	3.30 (1.29)	3.29 (1.26)
	Range (%)	-0.44 – 5.6	-0.54 – 5.4	-0.02 – 5.6
Left cheek	n	215	216	212
	Mean (SD) %	3.88 (0.78)	4.01 (0.78)	3.92 (0.78)
	Range (%)	1.06 – 6.32	1.07 – 5.83	1.06 – 6.56

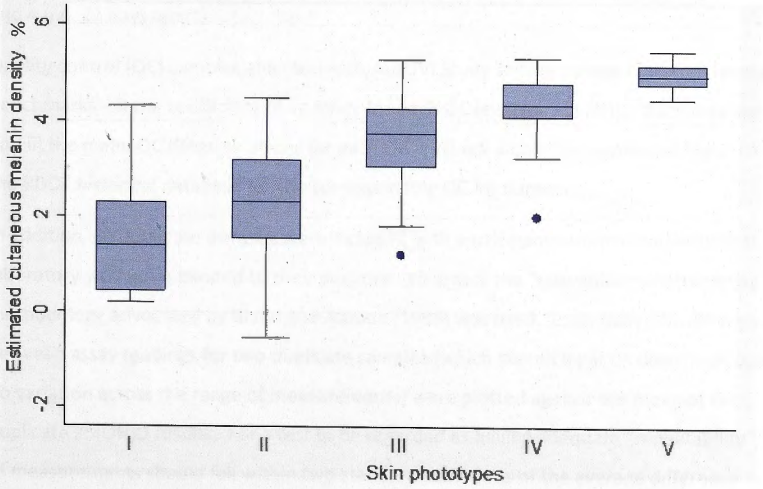
As cutaneous melanin density is related to environmental and genetic factors, the next section assesses cutaneous melanin density in relation to ethnicity and environment. Graph 6.3 shows the distribution of estimated melanin density by ethnic group (where participants have common parental ethnicity).

Graph 6.3: Estimated melanin density distribution by common parental ethnicity



Graph 6.4 shows the strong relationship between constitutive skin pigmentation as measured by estimated melanin density (from skin reflectance) at the inner arm and Fitzpatrick skin phototypes.

Graph 6.4: Estimated cutaneous melanin density by Fitzpatrick skin phototype – measurements taken from left upper inner arm



6.2.6 Vitamin D status

Vitamin D supplementation

Although the majority of vitamin D is derived from UVR-induced cutaneous synthesis, oral supplementation also contributes (see Section 1.2.5). Table 6.21 shows the number of AusUVI participants who reported taking vitamin D supplementation at the time of study enrolment.

Table 6.21: Participant report of vitamin D (cholecalciferol) supplementation

Type of supplementation	Average vitamin D content	Number of Participants (%)
No supplementation	-	159 (71.6%)
Vitamin D supplements (+/- Calcium)	400 – 1000IU	8 (3.6%)
Multivitamins	200 – 400IU	52 (23.4%)
Cod liver oil	130IU*	3 (1.4%)

*http://www.vitaminherbuniversity.com/topic.asp?categoryid=3&topicid=1108

There was no sex difference in the proportion taking vitamin D supplementation (males 29.6% *vs.* females 27.7%; p=0.83). There was no seasonal variation in vitamin D supplementation (winter 31.6%; spring 25.0%; summer 30.0%; autumn 25.9%; p=0.83).

Serum 25-hydroxyvitamin D levels

Quality control

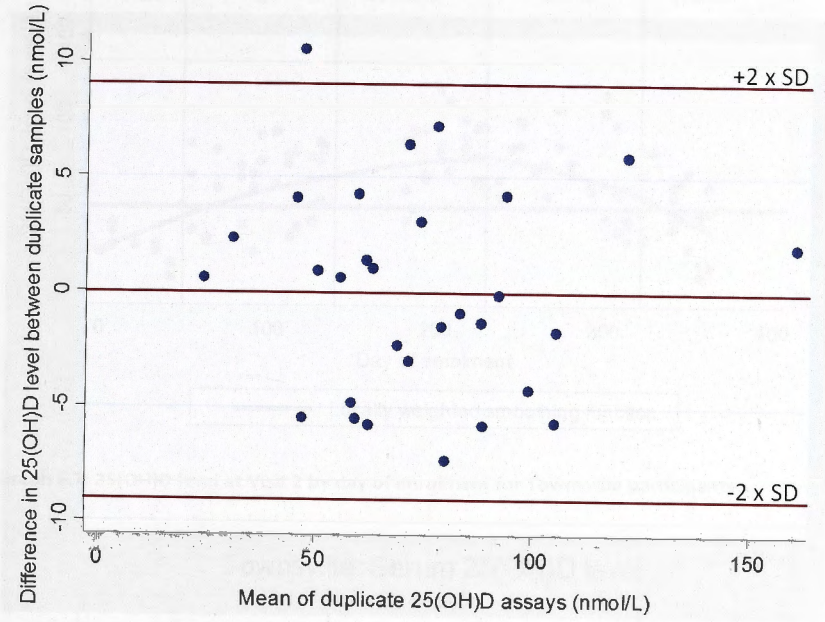
Serum 25-hydroxyvitamin D (25(OH)D) levels were obtained from blood taken at study Visits 2 and 4 (i.e. 21 days apart).

Quality control (QC) samples analysed with AusUVI Study samples passed internal laboratory benchmarks – i) the coefficient of variance for each QC level for 25(OH)D_3 did not exceed 6.9%; and ii) the mean QC determinations for each QC level fell within the upper and lower limits of the RDDT historical database for the corresponding QC lot numbers.

In addition, 30 duplicate samples were included with participant samples, coded such that laboratory staff were blinded to their purpose. To assess the "repeatability of the assay", the methodology advocated by Bland and Altman (1986) was used. Essentially, the differences between assay readings for two duplicate samples (which should be at or close to zero, with no variation across the range of measurements) were plotted against the mean of the duplicate 25(OH)D results. For a test to be regarded as having adequate "repeatability", 95% of measurements should fall within two standard deviations of the average difference in

duplicate samples. Graph 6.5 shows these data plotted. As only one duplicate (out of 30) fell outside the 95% confidence interval, the assay was deemed to have acceptable repeatability.

Graph 6.5: Difference in 25(OH)D level between duplicate tests plotted against mean of the duplicate tests.

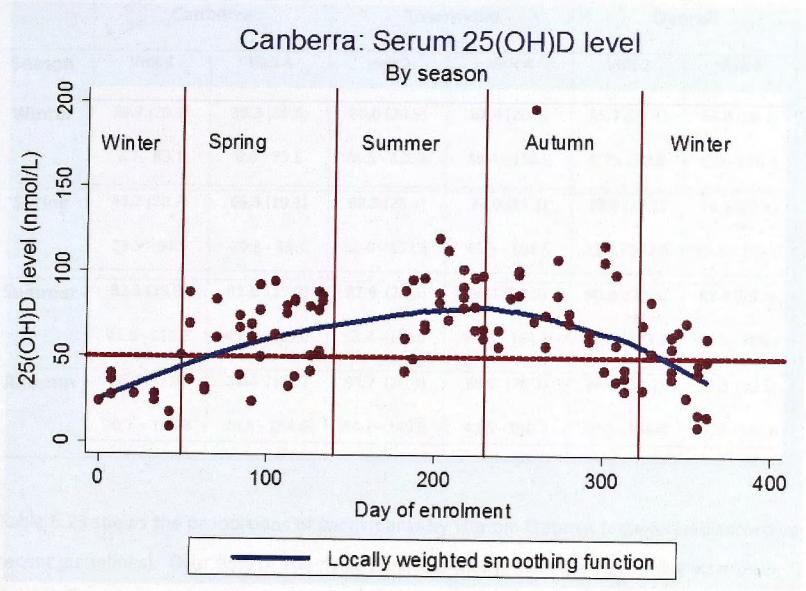


AusUVI Study participants' results

Graphs 6.6 and 6.7 show the serum 25(OH)D result (at Visit 2) for each participant by day of their enrolment, where July 14, 2010 was day 1, and day 385 was the last day of enrolment which occurred in August 2011. The level of serum 25(OH)D 'sufficiency' is indicated on the graphs at 50nmol/L. Table 6.22 shows the serum 25(OH)D data by season and site for Visit 2 (Day 8) and Visit 4 (Day 29).

There was a sinusoidal seasonal variation in 25(OH)D levels across both sites of enrolment (summer > autumn > spring > winter). The amplitude of the seasonal change in 25(OH)D levels from winter to summer was most pronounced for Canberra participants. The difference in 25(OH)D levels over the 21 days between Visit 4 and Visit 2 was most pronounced in spring (~+3 nmol/L) and autumn (~-4 nmol/L) seasons reflecting the direction of ambient UVR changes occurring during these periods.

Graph 6.6: 25(OH)D level at Visit 2 by day of enrolment for Canberra participants



Graph 6.7: 25(OH)D level at Visit 2 by day of enrolment for Townsville participants

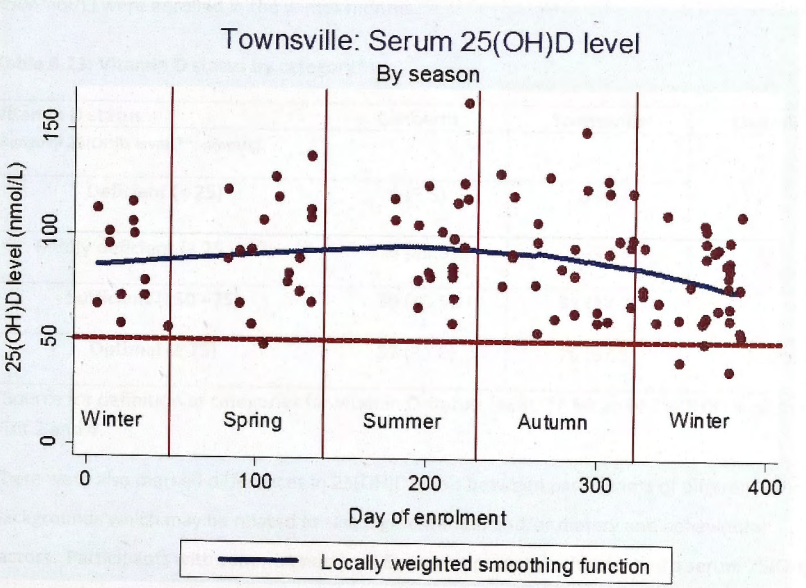


Table 6.22: Serum 25(OH)D level (nmol/L) by season and study visit (mean, SD, range)

	Canberra		Townsville		Overall	
Season	Visit 2	Visit 4	Visit 2	Visit 4	Visit 2	Visit 4
Winter	**39.7** (20.1)	**39.3** (18.6)	**80.0** (20.9)	**81.4** (20.5)	**65.7** (28.3)	**66.6** (28.2)
	8.7 - 83.1	6.0 - 75.8	36.5 - 120.6	38.4 - 116.9	8.7 - 120.6	6.0 - 116.9
Spring	**63.7** (20.7)	**66.3** (19.1)	**92.8** (25.2)	**95.0** (23.1)	**73.8** (26.1)	**76.5** (24.6)
	23.5 - 94.5	29.8 - 93.3	48.0 - 137.6	45.9 - 134.5	23.5 - 137.6	29.8 - 134.5
Summer	**82.1** (19.8)	**81.6** (19.7)	**97.9** (24.9)	**98.1** (24.0)	**90.0** (23.6)	**89.4** (23.1)
	41.8 - 119.8	45.5 - 119.0	58.4 - 163.2	65.3 - 164.6	41.8 - 163.2	45.5 - 164.6
Autumn	**76.2** (32.6)	**71.4** (31.7)	**93.7** (26.9)	**89.7** (26.3)	**84.1** (31.1)	**79.9** (30.5)
	30.7 – 195.8	26.9 - 184.6	54.2 - 149.9	43.5 - 150.7	30.7 - 195.8	26.9 - 184.6

Table 6.23 shows the proportions of participants by vitamin D status (categorised according to recent guidelines). Over 95% of Townville participants were categorised in the vitamin D 'sufficient' or 'optimal' range, compared with 69% from Canberra ($p<0.0001$). Of note, 56% (19/34) of Canberra participants with vitamin D deficient/mildly deficient status (i.e. 25(OH)D <50nmol/L) were enrolled in the winter months.

Table 6.23: Vitamin D status by category*

Vitamin D status (Range of 25(OH)D levels**; nmol/L)	Canberra	Townsville	Overall
Deficient (< 25)	6 (5.5)	0 (0)	12
Mildly deficient (≥ 25 – 50)	28 (25.5)	5 (4.5)	33
Sufficient (≥50 - 75)	39 (35.5)	31 (27.7)	70
Optimal (≥ 75)	37 (33.6)	76 (67.9)	113

*Source for definition of categories for vitamin D status: (488); ** Mean of 25(OH)D level from Visit 2 and 4.

There were also marked differences in 25(OH)D levels between participants of different ethnic backgrounds which may be related to skin pigmentation, and/or dietary and behavioural factors. Participants with common northern European parental ethnicity had a serum 25(OH)D level 17.2nmol/L higher than those with non-northern European parents ($p<0.001$) (adjusted for season and site).

6.2.7 Summary

This section examined participant characteristics that may also affect the immune response to immunisation. Approximately two-thirds of participants were in the "normal" weight range as defined by BMI, with one-third categorised as overweight or obese. A small proportion of participants were current cigarette smokers. The majority of participants were classed as being in the moderate to high physical activity categories. The Mental Health Inventory demonstrated gender differences in global indices of distress, well-being and overall mental health, with females scoring higher in the distress constructs of the various scales. Assessment of Fitzpatrick skin phototypes showed the majority of participants were of "Caucasian" skin type (Phototype I-III). There were a higher proportion of Canberra participants with skin phototypes IV and V compared with Townsville participants. Higher cutaneous melanin density (as estimated by skin reflectance measurements) was demonstrated: i) at the cheek compared with the inner arm for those of European ethnicity, ii) in participants of non-European parentage compared with European parentage; and iii) in participants with higher skin phototypes. Finally, serum 25(OH) levels showed marked seasonal variation, particularly amongst Canberra participants. Differences in serum 25(OH)D levels were also demonstrated according to ethnic group.

6.3 UVR exposure

Participants' level of UVR exposure during the study was measured in several ways. Short-term UVR exposure was measured using i) electronic dosimeter badges worn on the wrist; ii) sun diary data; and iii) ambient UVR levels (measured by an outdoor monitor). Cumulative UVR exposure over the life-course was measured by taking silicone impression casts of the dorsum of the hand to assess degree of actinic damage.

6.3.1 Electronic dosimeter UVR measurements

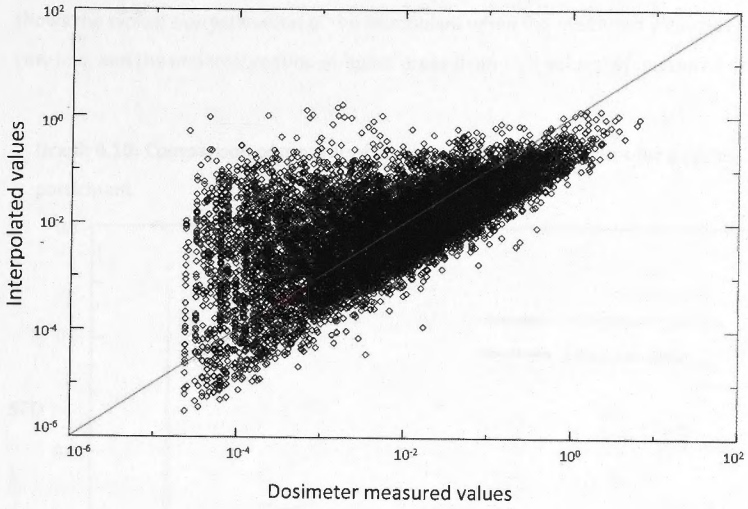
Short-term personal UVR exposure was measured by electronic dosimeters worn on the wrist for ten days (with KLH immunisation occurring on the sixth day) with UVR readings taken every eight seconds.

Assessing the performance characteristics of interpolant UVR data

As described in Section 5.5.2, an interpolation function was used to generate UVR data to replace raw data that were missing (e.g. dosimeter not worn or low battery error) or removed because of repeats, oscillations or clearly erroneous data. The average hourly dosimeter measured UVR value was 0.026 SED. The average hourly interpolant value, where measured values were available, was 0.022 SED. The average daily difference between measured totals and interpolated totals on corresponding days was 0.050 SED, a small difference when compared with the average daily measured total of 0.38 SED.

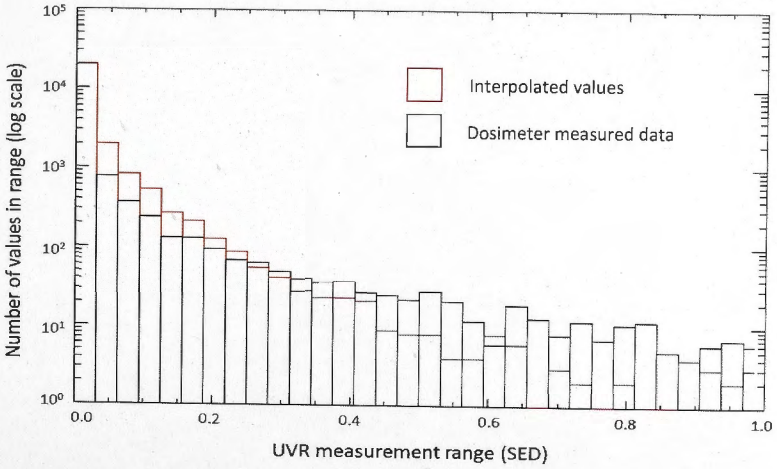
Graph 6.8 directly compares dosimeter measured data against the interpolation function derived value. The graph shows a wide range of interpolated values at the lower end of measured values (tending to overestimate the measured value); whilst at the higher end, interpolated values tended to underestimate measured values. However, there was reasonable correlation across the range of intermediate values.

Graph 6.8: Direct comparison of measured data and corresponding interpolated values



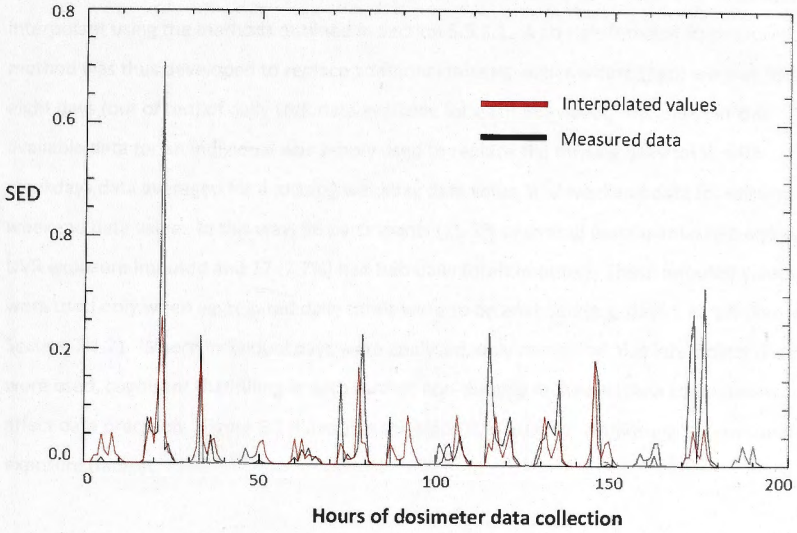
Graph 6.9 shows the frequency distribution of dosimeter measured and interpolated values (note the logarithmic y-axis scale). Measured and interpolated values were clustered in the lower UVR range. There were more interpolated than measured values in the lower UVR measurement range, whilst there were comparatively more measured values than interpolated values in the higher UVR measurement range.

Graph 6.9: Distribution of interpolated and measured values



Graph 6.10 is an example of a participant's dosimeter measured UVR over the ten days of measurement (in hours) compared with the corresponding interpolated values. Again, this shows the typical overestimation of the interpolant when the measured values were zero or very low, and the underestimation in 'spike' areas (high UVR values) of measured data.

Graph 6.10: Comparison of measured and interpolated UVR values for a typical participant

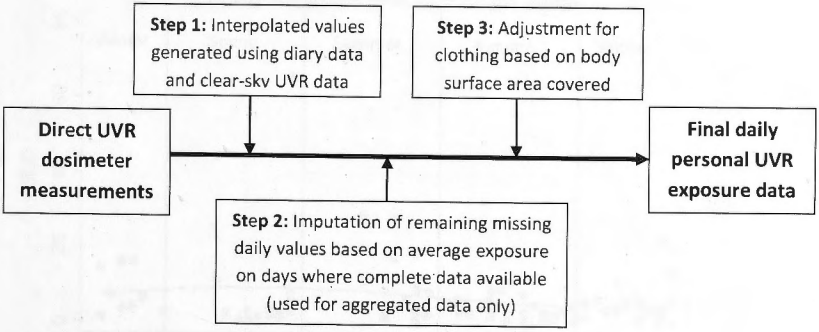


Use of interpolated values for AusUVI Study participants

Interpolated values were used to replace missing or removed measured UVR data only when there was less than 20 minutes of measured data for any given hour. Overall, a minority of missing measured values were replaced by an interpolated value in the final UVR data set (Canberra 9.1%; Townsville 5.8%).

The interpolation algorithm was not able to replace all missing values where there was incomplete UVR dosimeter badge data, diary data or insufficient data to be able to generate an interpolant using the methods outlined in Section 5.5.2.1. A straightforward 'imputation' method was thus developed to replace additional missing values where there were at least eight days (out of ten) of daily UVR data available for each individual. The mean of the available data for an individual was simply used to replace the missing daily total, with weekdays data averaged for a missing weekday data value, and weekend data for missing weekend data value. In this way, 56 participants (25.2% of overall participants) had one daily UVR exposure imputed and 17 (7.7%) had two daily totals imputed. These imputed values were used only when aggregated daily totals were to be analysed (e.g. days 1 to 10) (See also Section 7.1.2). Where individual days were analysed, only measured plus interpolant data were used, cognisant that filling in with further non-directly measured data could adversely affect data precision. Figure 6.1 illustrates the algorithm used for generating the personal UVR exposure dataset.

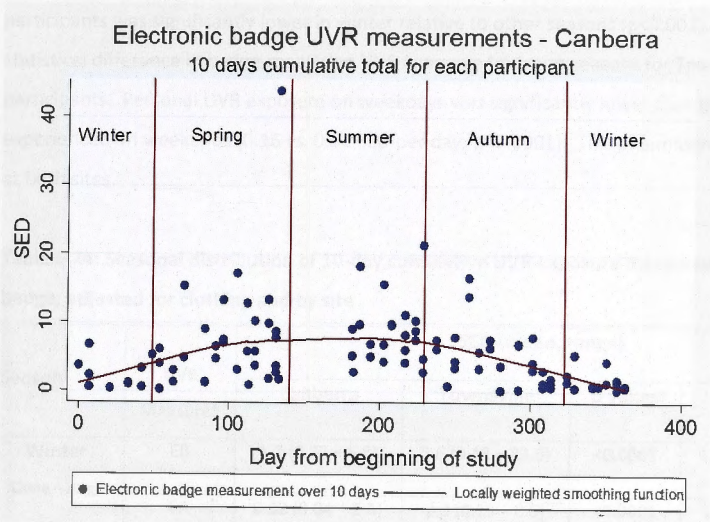
Figure 6.1 Algorithm for determining participant's personal UVR measurements



AusUVI Study electronic badge measurements

Graphs 6.11 and 6.12 demonstrate the electronic badge 10-day cumulative UVR exposure (measured plus interpolated plus imputed) for Canberra and Townsville participants over the ~13 month study course.

Graph 6.11: Electronic UVR badge output with interpolated values: Canberra



Graph 6.12: Electronic UVR badge output with interpolated values: Townsville

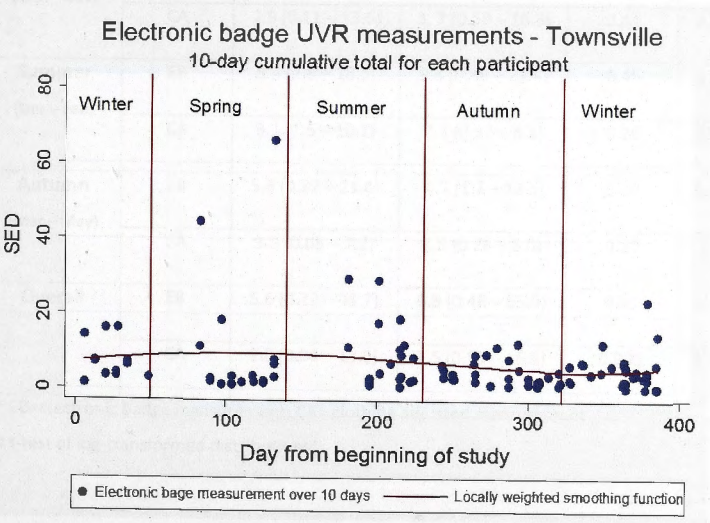


Table 6.24 shows the range of cumulative UVR readings by season and site. UVR exposure, as directly measured by the electronic badge, varied by season in Canberra and Townsville participants. Townsville participants had significantly higher UVR measurements compared with Canberra participants in the winter months (5.6 *vs.* 1.7 SED; p<0.0001), but were not significantly different at other times of year. The amplitude of seasonal variation was more marked for Canberra than Townsville participants. Badge measured UVR exposure in Canberra participants was significantly lower in winter relative to other seasons (p<0.001). There was no statistical difference in badge-measured UVR exposure between seasons for Townsville participants. Personal UVR exposure on weekdays was significantly lower than that experienced on weekends (0.16 *vs.* 0.32 SED per day; p<0.0001). This phenomenon was seen at both sites.

Table 6.24: Seasonal distribution of 10-day cumulative UVR exposure measured by electronic badge, adjusted for clothing and by site

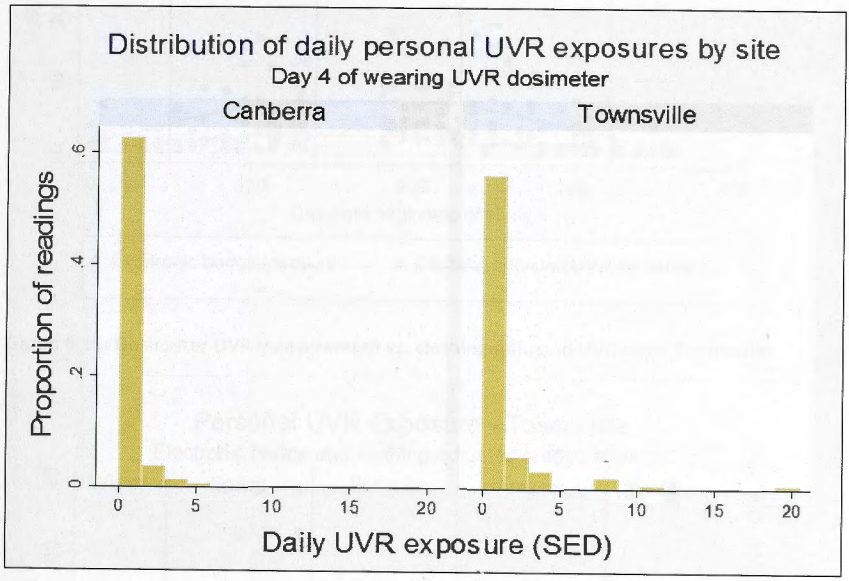
Season	UVR Measure*	SED (mean, range)			
		Canberra	Townsville	p value#	Overall
Winter (June – Aug)	EB	1.7 (0.27 – 6.8)	5.6 (0.48 – 23.6)	**<0.0001**	4.2 (0.27 – 23.6)
	CA	0.32 (0.04 – 1.4)	2.3 (0.13 – 8.5)	**<0.0001**	1.6 (0.04 – 8.5)
Spring (Sept –Nov)	EB	7.4 (0.35 – 43.7)	10.6 (1.3 – 65.9)	0.87	8.5 (0.35 – 65.9)
	CA	2.5 (0.11 – 13.6)	3. 7 (0.58 – 16.8)	0.44	2.9 (0.11 – 16.8)
Summer (Dec – Feb)	EB	8.4 (2.8 – 18.3)	9.3 (0.88 – 29.4)	0.45	8.9 (0.88 – 29.4)
	CA	3.1 (1.5 – 10.1)	2.7 (0.37 – 8.2)	0.26	2.9 (0.37 – 10.1)
Autumn (Mar – May)	EB	5.3 (0.22 – 21.4)	4.7 (1.2 – 12.5)	0.98	5.1 (0.22 – 21.4)
	CA	1.5 (0.05 – 7.2)	1.8 (0.26 – 5.0)	0.20	1.7 (0.05 – 7.2)
Overall	EB	5.6 (0.22 – 43.7)	6.9 (0.48 – 65.9)	0.15	6.2 (0.22 – 65.9)
	CA	1.8 (0.04 – 13.6)	2.5 (0.13 – 16.8)	**0.003**	2.1 (0.04 – 16.8)

* EB=electronic badge measurement; CA= clothing adjusted measurement

t-test of log-transformed distributions

Graph 6.13 shows the distribution of daily personal UVR exposure for a typical day (in this case, the fourth day of wearing the dosimeter) for Canberra and Townsville participants. The majority of readings were concentrated around zero (i.e. no UVR exposure); however there was a spread of measures, with Townsville participants having a greater range than their Canberra counterparts.

Graph 6.13: Distribution of daily electronic badge measured UVR exposures for a typical day

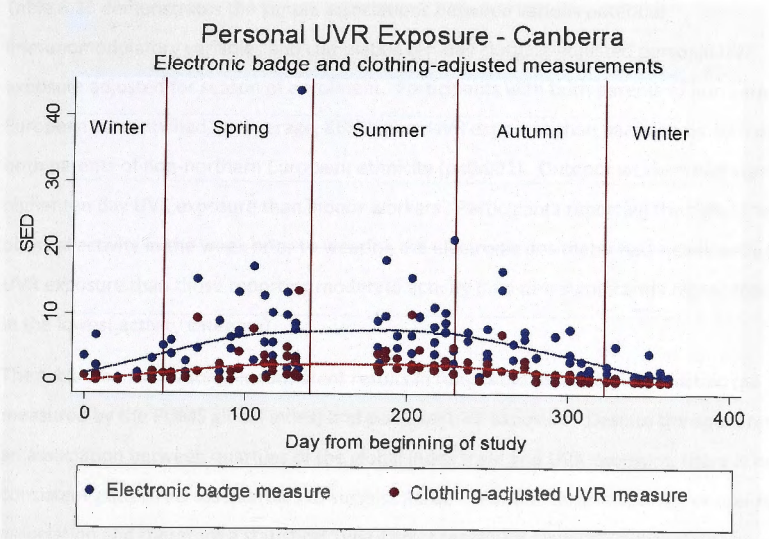


Adjustment for clothing: determining personal UVR exposure

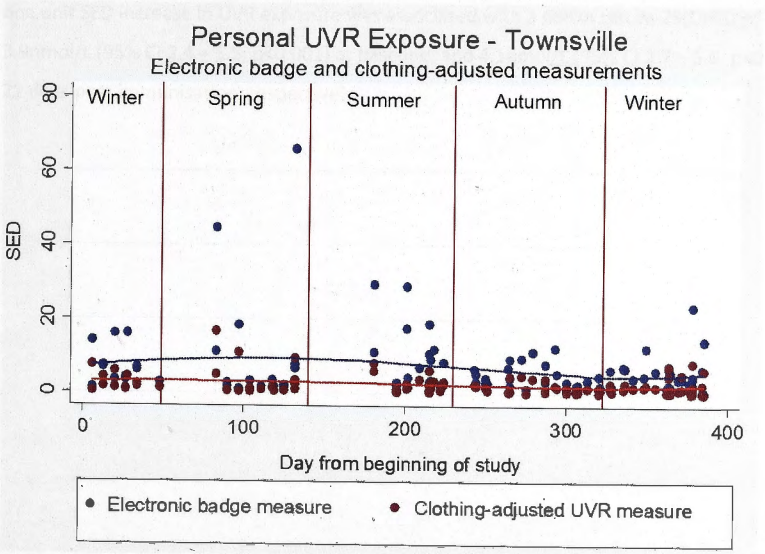
As described in Section 5.5.1, daily personal UVR exposure was derived by multiplying the electronic badge UVR data for a given hour by the proportion of the body surface area not covered by clothing for that same hour, and summing the total for that day.

Graphs 6.14 and 6.15 show the seasonal change in clothing-adjusted UVR exposure (over ten days) compared with electronic badge measured data for Canberra and Townsville. Table 6.24 also shows these data by season and by site. On average, clothing-adjusted UVR exposure was approximately 1/3 the total electronic-badge measurement, although this ratio changed by season and site. Overall, participants from Townsville had a higher clothing-adjusted UVR exposure over 10 days compared with Canberra participants (2.5 *vs.* 1.8 SED; p=0.003). The largest difference between sites occurred in the winter season (Townsville 2.3 *vs.* Canberra 0.32 SED; p<0.0001).

Graph 6.14: Dosimeter UVR measurement vs. clothing-adjusted UVR dose: Canberra



Graph 6.15: Dosimeter UVR measurement vs. clothing-adjusted UVR dose: Townsville



6.3.2 Association between UVR exposure and other immunomodulatory variables

Table 6.25 demonstrates the simple associations between various potential immunomodulatory variables and cumulative ten-day clothing-adjusted personal UVR exposure adjusted for season of enrolment. Participants with both parents of northern European ethnicity had, on average, 86% higher UVR exposure than participants with one or both parents of non-northern European ethnicity ($p<0.001$). Outdoor workers had significantly higher ten day UVR exposure than indoor workers. Participants reporting the highest level of physical activity in the week prior to wearing the electronic dosimeter had significantly higher UVR exposure than those reporting moderate activity (and non-significantly higher than those in the lowest activity category).

The table also shows some inconsistent results in relation to psychological function (as measured by the POMS global index) and personal UVR exposure. Despite the appearance of an association between quartiles of the global index scale and UVR exposure, there is no consistent pattern across quartiles to suggest a true linear (i.e. dose-response) or non-linear association and therefore a statistical Type-I error remains a plausible explanation.

Vitamin D status was strongly linearly associated with clothing-adjusted UVR exposure. Each one unit SED increase in UVR exposure was associated with a rise in serum 25(OH)D of 3.9nmol/L (95% CI 2.4 – 5.5; $p<0.001$) at baseline, and 4.1nmol/L (95% CI 2.7 – 5.6; $p<0.001$) at 21 days-post immunisation, respectively.

Table 6.25: Association between clothing-adjusted UVR exposure and other potential immune-modulator variables

Potential immune modulators	Sub-group (n)	Mean SED (SD)	SED Range	p value*
Overall	**(216)**	2.1 (2.4)	0.043 – 16.8	
Sex	**Male (79)**	2.2 (2.4)	0.051 – 2.4	0.85
	Female (137)	2.1 (2.4)	0.043 – 16.8	
Site	**Canberra (108)**	1.8 (2.2)	0.043 – 13.6	**<0.001**
	Townsville (108)	2.5 (2.6)	0.13 – 2.6	
Age (years)	**18 – 24 (83)**	2.1 (2.1)	0.051 – 11.1	Reference
	25 – 29 (67)	1.9 (1.9)	0.043 – 9.5	0.47
	30 – 34 (31)	2.5 (3.4)	0.074 – 16.8	0.83
	35 – 40 (41)	2.3 (2.9)	0.094 – 13.6	0.75
Parental ethnic background	**Northern European (152)**	2.5 (2.6)	0.043 – 16.8	**<0.001**
	Non-Northern European (64)	1.3 (1.6)	0.051 – 10.1	
Indoors / Outdoors Worker status	**Indoors worker (193)**	2.1 (2.4)	0.043 – 16.8	Reference
	Half indoors/ half outdoors (19)	2.3 (1.3)	0.11 – 5.10	0.14
	Outdoors worker (4)	5.3 (3. 8)	1.6 – 9.6	**0.038**
Smoking	**Non-smoker (204)**	2.1 (1.5)	0.043 – 16.8	0.29
	Current smoker (13)	3.0 (2. 7)	0.31 – 6.6	
Body mass index	**Underweight (3)**	1.1 (1.3)	0.13 – 2.6	0.37
	Normal weight (138)	2.2 (2.4)	0.051 – 16.8	Reference
	Overweight (56)	2.0 (2.3)	0.043 – 9.5	0.37
	Obese (19)	2.1 (2.9)	0.068 – 11.1	0.33
Psychological profile: POMS (TMD)	**Below first quartile score (54)**	2.8 (3.0)	0.11 – 13.6	Reference
	Between 1st & 2nd quartile (62)	1.5 (1.6)	0.043 – 9.5	**0.008**
	Between 2nd & 3rd quartile (50)	2.3 (2.3)	0.084 – 9.6	0.69
	Above fourth quartile (50)	2.7 (2.6)	0.051 – 16.8	0.084
Psychological profile: MHI (Global score)	**Below first quartile score (58)**	1.9 (2.7)	0.043 – 16.8	Reference
	Between 1st & 2nd quartile (55)	1.9 (2.3)	0.051 – 11.1	0.71
	Between 2nd & 3rd quartile (50)	2.5 (2.0)	0.084 – 9.5	0.052
	Above fourth quartile (50)	2.3 (2.7)	0.11 – 13.6	0.35
Physical activity IPAQ category	**Low activity (14)**	1.9 (2.8)	0.094 – 10.1	0.24
	Moderate activity (72)	1.5 (1.3)	0.043 – 1.3	**0.038**
	High activity (129)	2.5 (2.8)	0.051 – 16.8	Reference
Vitamin D status (Baseline blood test) (nmol /L)	**<25 (6)**	0.23 (0.30)	0.043 – 0.81	**<0.001**
	25 – 49.9 (33)	0.74 (0.64)	0.068 – 2.5	**<0.001**
	50 – 74.9 (69)	1.9 (1.8)	0.051 – 11.1	**0.018**
	> 75 (108)	2.8 (2.9)	0.11 – 16.8	Reference

* Linear regression models using log-transformed UVR exposure distributions and adjusted for season

6.3.3 Cumulative UVR exposure with silicone skin casts

Skin cast quality control

Silicone skin casts were taken from the dorsum of the left and right hand (i.e. sun exposed area) for all participants. The casts were graded according to the previously described Beagley and Gibson criteria by two independent scorers (AS & VM) who were blinded to the participant's enrolment and study details (see Section 5.5.3). Results that differed by more than two grades (44 casts (9.9% of total)) were re-graded by both scorers. The degree of cast grade agreement between scorers was determined by a linear-weighted kappa statistic (given the categorical nature of skin grading), where a conservative weighting matrix was applied as follows:

$$\begin{bmatrix} 1 & 0.66 & 0 & 0 & 0 & 0 \\ 0.66 & 1 & 0.66 & 0 & 0 & 0 \\ 0 & 0.66 & 1 & 0.66 & 0 & 0 \\ 0 & 0 & 0.66 & 1 & 0.66 & 0 \\ 0 & 0 & 0 & 0.66 & 1 & 0.66 \\ 0 & 0 & 0 & 0 & 0.66 & 1 \end{bmatrix}$$

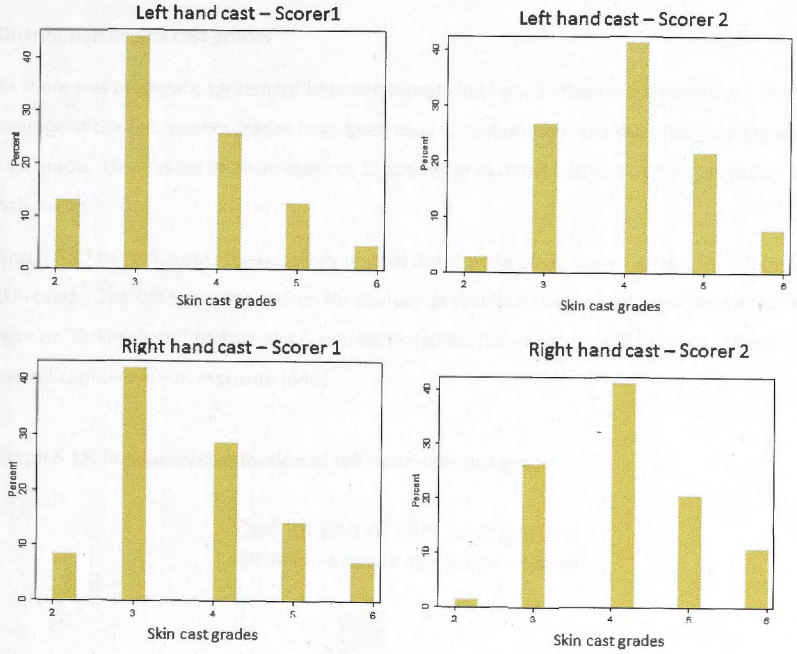
The kappa (κ) statistic as a measure of level of agreement can be interpreted as follows (489): 0 - 0.20, poor; 0.21-0.40, fair; 0.41 – 0.60, moderate; 0.61-0.80, good; 0.81-1.00, very good.

Table 6.25 and Graph 6.16 show the grading of casts by each scorer. Scorer 2 graded, on average, half a grade higher than Scorer 1 for both hands, although there was 'moderate' agreement between scorers according to the weighted kappa statistic. On average, both scorers had graded the right hand casts higher than the left.

Table 6.25: Grading of skin casts by two independent scorers.

	Scorer 1: Mean (SD)	Scorer 2: Mean (SD)	Weighted Kappa statistic
Left cast (n=214)	3.5 (1.0)	4.0 (0.94)	0.55 Moderate agreement
Right cast (n=213)	3.7 (1.0)	4.1 (0.97)	0.59 Moderate agreement

Graph 6.16 Histograms of left and right hand graded skin casts – comparing scorers



As a quality control measure to assess intra-rater grade reproducibility, both scorers re-graded 60 randomly selected skin casts. Scores for these duplicate casts were compared with the original grading using the same weighted kappa statistic as described above (Table 6.26). Both scorers demonstrated "good agreement" between original and re-graded scores.

Table 6.26: Regrading of duplicate casts to assess intra-rater agreement

		Original grading	Re-grading	Level of agreement *
Scorer 1	**Mean (SD)** **25th / 75th percentile**	3.62 (1.19) 3 / 4.5	3.63 (1.02) 3 / 4	0.66 Good agreement
Scorer 2	**Mean (SD)** **25th / 75th percentile**	4.08 (1.00) 3 / 5	3.82 (1.07) 3 / 4	0.65 Good agreement

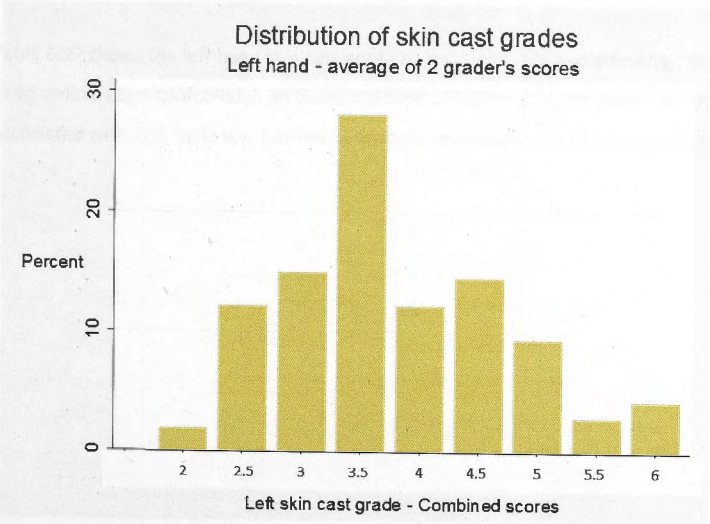
* Weighted kappa statistic

Distribution of skin cast grades

As there was moderate agreement between scorers (and good intra-rater agreement), the average of the two scorers grades have been used for subsequent analyses involving the skin cast grade. This has led to an increase to 12 scoring gradations because of the generation of half marks.

Graph 6.17 demonstrates the relatively normal distribution of combined grades for left hand skin casts. The left hand was chosen for analysis as previous studies have also shown that the right or "driving hand" in Australia is subject to higher UVR exposure and may not reflect overall cumulative sun exposure (440).

Graph 6.17: Frequency distribution of left hand skin cast grades



Cumulative sun exposure was also related to age. The box plots (Graph 6.18) shows a positive relationship between age and left hand skin cast grade. An ordinal regression model revealed that for each 1 year increase in age, odds of an increased skin cast grade (1/2 grade increment) increased by 0.11 (95% CI: 0.07-0.15); p <0.0001).

Graph 6.18: Left hand skin cast grade *vs.* age category

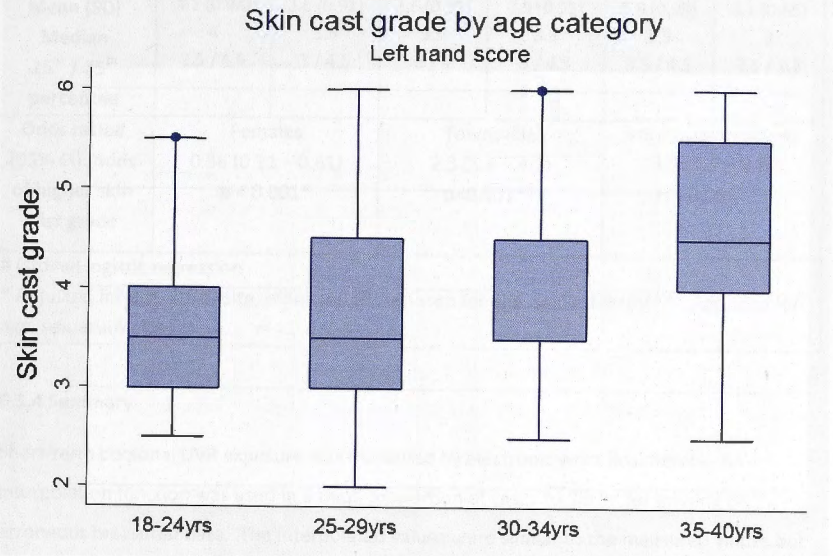


Table 6.27 shows the left hand skin cast grade by study site, sex and ethnicity. When included in an ordinal regression model, an increased odds of higher skin cast grade was strongly associated with age, male sex, northern European parentage and Townsville residence.

Table 6.27: Association between left hand skin cast and skin, ethnicity and study site

	Sex		Study site		Ethnicity	
	Male	**Female**	**Canberra**	**Townsville**	**Northern European parents**	**Non-Nth. European parents**
Number	78	136	108	106	160	31
Mean (SD)	4.1 (0.94)	3.6 (0.91)	3.6 (0.91)	3.9 (0.95)	3.9 (0.93)	3.1 (0.65)
Median	4	3.5	3.5	3.5	3.5	3
25th / 75th percentile	3.5 / 4.5	3 / 4.5	3 / 4	3 / 4.5	3.5 / 4.5	2.5 / 3.5
Odds ratio# (95% CI): odds of higher skin cast grade	Females 0.36 (0.21 – 0.61) p < 0.001*		Townsville 2.3 (1.4 – 3.7) p=0.001**		Nth European parents 3.3 (1.9 – 5.8) p < 0.001***	

Ordinal logistic regression

* Adjusted for age, study site, ethnicity ** Adjusted for age, sex, ethnicity *** Adjusted for age, sex, study site

6.3.4 Summary

Short-term personal UVR exposure was measured by electronic wrist dosimeters. An interpolation function was used in a small proportion of cases to 'fill in' for missing or erroneous measured data. The interpolated values were similar to the measured values but tended to overestimate values near zero and underestimate high measured (or 'spike') UVR values. For remaining missing daily UVR values, a simple imputed value was generated by averaging the remaining days for that individual (differentiated by weekday or weekday). This imputed value was only used where aggregated daily data (i.e. more than one day of daily data) were required.

Canberra participants had lower cumulative electronic-badge measured UVR exposure over ten days compared with Townsville participants in the winter season, but this was not statistically different for other seasons. When adjusted for clothing, Townsville participants had significantly higher UVR exposure over ten days compared with Canberra participants overall, and in the winter season. In Canberra, participants enrolled in winter had the lowest UVR exposures over ten days compared with other seasons. Clothing adjusted UVR exposure was approximately one-third of the corresponding electronic badge measurement, although this also varied with season and site of enrolment.

Cumulative UVR exposure over the life course was assessed by quantifying the degree of actinic damage observed in silicone skin casts taken of the dorsum of hands. In this study,

agreement between independent scorers of skin casts was moderate and intra-observer agreement was good. Factors associated with increased skin cast grade (and hence cumulative UVR exposure) were: right hand, increasing age, male sex, northern European heritage and Townsville residence.

The next chapter describes the results from the immune assays and regression models which aim to determine associations with UVR exposure and other potential immunomodulatory factors.

Acknowledgments

Christel van den Boogaard and Kylee Parsons were the Townsville research officers who organised and collected all data from that study site. Martin Allen and Richard McKenzie (NIWA) assisted with the electronic dosimeter badges particularly troubleshooting software and hardware problems as they arose. Ben Liley (NIWA) assisted with calibrating the electronic badges at the end of the study. James Liley (NIWA) was instrumental in checking for erroneous dosimeter data and developing the interpolation function. Rachel Slatyer (based at ANU) digitally photographed the silicone casts. Vincent Mantio (based at JCU) assisted with skin cast graders. Ryan Murray (QUT) extracted data from the questionnaire via teleform-specific software. Libby Hattersley (NCEPH) assisted with checking and correcting diary data.

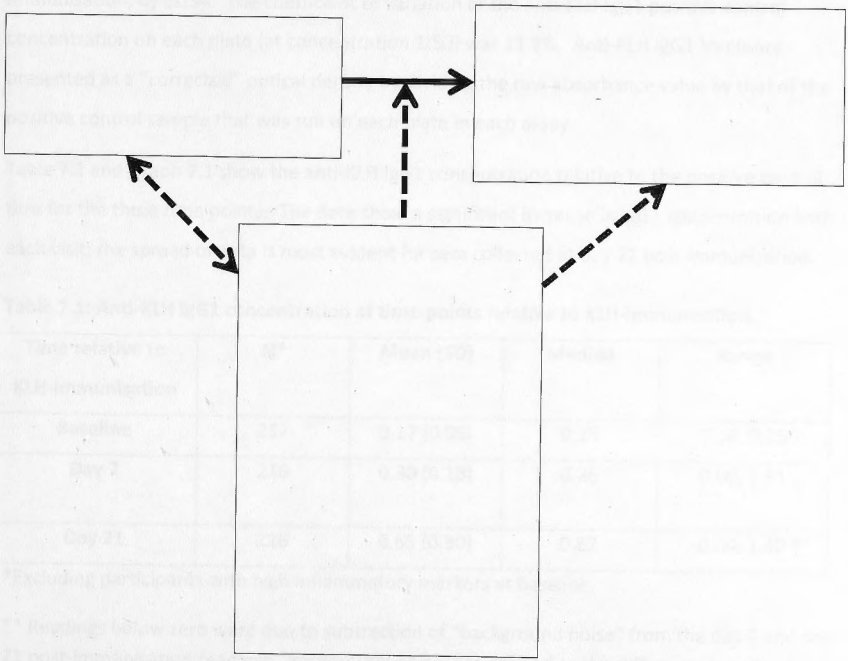
Chapter 7: Associations between UVR exposure and immune response

Synopsis

This chapter discusses the relationship between personal UVR exposure and primary immune responses to KLH immunisation. Figure 7.1 illustrates the postulated relationships between UVR exposure variables, immune outcome measures and potential confounding and/or effect modifying factors. Linear regression models were developed to fully examine these associations.

This chapter is divided into two sections: i) immune responses (sub-divided into humoral and cell-mediated immune response); and ii) vaccine-related safety issues. The first section details the distribution of immune responses, their (univariate) association with potential immunomodulatory factors and UVR exposure(s), respectively, and finally, the results of multiple linear regression modelling.

Figure 7.1: Postulated relationship between UVR exposure variables, vaccine-associated immune outcomes and potential immunomodulatory factors



7.1 Immune responses

7.1.1 Exclusion of participants with high baseline inflammatory markers

Participants with screening test results (C-reactive protein (CRP) and white cell count (WCC)) that suggested an inflammatory state at the time of KLH immunisation were excluded from further analysis. The threshold level for exclusion was set at three times the standard deviation above the study population mean for each screening test. The threshold for CRP was 20.7mg/L which led to exclusion of four participants, and for WCC was 11.3 x 10^9/L, excluding two participants. As one participant had both a CRP and WCC above these thresholds, a total of five participants were excluded from analysis leaving 217 evaluable participants. Of note, the average BMI of excluded participants was significantly higher than the remaining population (37.7 *vs.* 23.8; p=0.03), but they were similar in other characteristics.

7.1.2 Humoral immune response

Distribution of anti-KLH IgG1 response

KLH-specific IgG1 antibody responses were assessed at baseline, 7 and 21-days post-KLH immunisation, by ELISA. The coefficient of variation of the anti-KLH IgG1 positive control concentration on each plate (at concentration 1:50) was 11.8%. Anti-KLH IgG1 levels are presented as a "corrected" optical density by dividing the raw absorbance value by that of the positive control sample that was run on each plate in each assay.

Table 7.1 and Graph 7.1 show the anti-KLH IgG1 concentration relative to the positive control titre for the three time points. The data show a significant increase in IgG1 concentration with each visit; the spread of data is most evident for sera collected at day 21 post-immunisation.

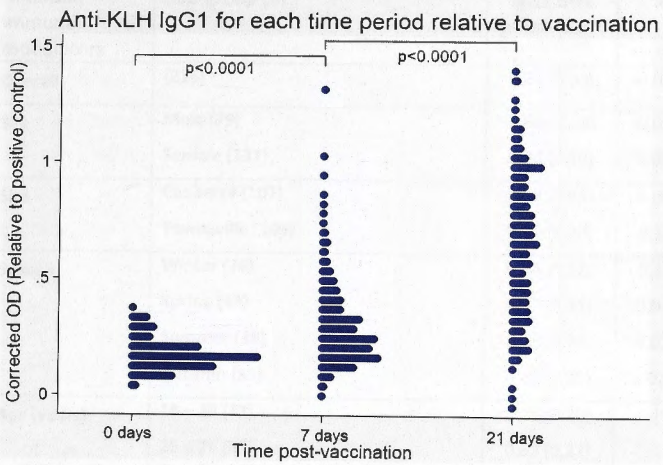
Table 7.1: Anti-KLH IgG1 concentration at time-points relative to KLH-immunisation

Time relative to KLH-immunisation	N*	Mean (SD)	Median	Range
Baseline	217	0.17 (0.06)	0.16	0.04, 0.35
Day 7	216	0.30 (0.18)	0.26	0.00, 1.31
Day 21	216	0.65 (0.30)	0.67	-0.04, 1.40 **

*Excluding participants with high inflammatory markers at baseline

** Readings below zero were due to subtraction of "background noise" from the day 7 and day 21 post-immunisation readings. Background noise was defined as the difference in optical density reading between the negative control and a participant's baseline serum.

Graph 7.1: Frequency distributions of anti-KLH IgG1 at baseline, 7 and 21 days post-immunisation

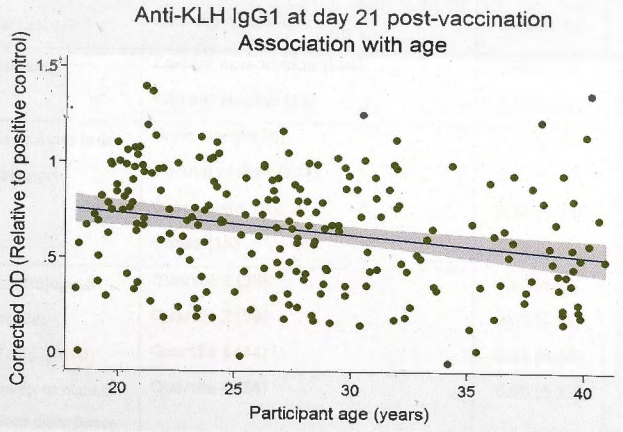


NB: p values derived from paired t-tests

Association between anti-KLH IgG1 and potential immunomodulatory variables

Table 7.2 shows the relationship between anti-KLH IgG1 response at day 21 post-immunisation and potential immunomodulatory variables. Females demonstrated a 20% higher anti-KLH IgG1 response than males (p=0.006). Additionally, a negative linear association between age and antibody response was demonstrated (See Graph 7.2). The youngest age group (18 – 24 years) had a 39% higher antibody response compared with the oldest age group (35 – 40 years) (p<0.001).

Graph 7.2: Scatterplot of anti-KLH IgG1 vs. age of AusUVI participant



Regression coefficient: -0.012 (95% CI -0.018, -0.0061); p <0.001

Table 7.2: Anti-KLH IgG1 at day 21 post-immunisation vs. potential immune modulators

Potential immune modulators	Sub-group (n)	IgG1 titre mean (SD)*	Range	p value
Overall	**(216)**	0.65 (0.30)	-0.040, 1.40	
Sex	**Male (79)**	0.58 (0.29)	-0.040, 1.26	**0.006**
	Female (137)	0.70 (0.30)	-0.010, 1.40	
Site	**Canberra (107)**	0.62 (0.29)	-0.040, 1.40	0.065
	Townsville (109)	0.69 (0.30)	0.15, 1.37	
Season	**Winter (76)**	0.64 (0.32)	0.17, 1.37	Reference
	Spring (49)	0.67 (0.31)	-0.040, 1.40	0.58
	Summer (38)	0.66 (0.26)	-0.010, 1.17	0.68
	Autumn (53)	0.65 (0.29)	0.024, 1.26	0.79
Age (years)	**18 – 24 (81)**	0.75 (0.28)	0.19, 1.40	Reference
	25 – 29 (67)	0.62 (0.27)	-0.010, 1.17	**0.005**
	30 – 34 (30)	0.61 (0.32)	-0.040, 1.26	**0.022**
	35 – 40 (38)	0.54 (0.32)	0.024, 1.36	**<0.001**
Parental ethnic background	**Northern European (both parents) (150)**	0.68 (0.29)	-0.040, 1.40	0.32
	Other (66)	0.64 (0.024)	-0.010, 1.37	
Melanin density Left inner arm (Lightest to darkest skin)	**Quartile 1 (54)**	0.60 (0.31)	-0.010, 1.40	Reference
	Quartile 2 (54)	0.67 (0.30)	-0.040, 1.36	0.18
	Quartile 3 (50)	0.68 (0.28)	0.18, 1.17	0.18
	Quartile 4 (56)	0.66 (0.31)	0.024, 1.37	0.24
Melanin Density Left upper cheek (Lightest to darkest skin)	**Quartile 1 (54)**	0.65 (0.33)	-0.040, 1.40	Reference
	Quartile 2 (51)	0.67 (0.28)	0.14, 1.13	0.62
	Quartile 3 (51)	0.64 (0.29)	0.15, 1.26	0.98
	Quartile 4 (54)	0.65 (0.31)	0.024, 1.36	0.89
Smoking	**Current non-smoker (204)**	0.66 (0.30)	-0.040, 1.40	0.76
	Current smoker (12)	0.62 (0.35)	0.16, 1.36	
Body mass index Category	**Underweight (4)**	0.73 (0.55)	0.024,1.37	0.73
	Normal weight (141)	0.67 (0.29)	-0.039, 1.40	Reference
	Overweight (56)	0.58 (0.30)	0.14, 1.22	**0.038**
	Obese (15)	0.72 (0.30)	0.29, 1.36	0.61
Psychological profile: POMS (TMD) Lowest to highest mood disturbance	**Quartile 1 (58)**	0.66 (0.32)	-0.040, 1.26	Reference
	Quartile 2 (58)	0.67 (0.28)	0.14, 1.40	0.91
	Quartile 3 (44)	0.63 (0.30)	0.16, 1.22	0.61
	Quartile 4 (54)	0.65 (0.30)	-0.010, 1.37	0.87

Psychological profile: MHI (Global) Lowest to highest well-being	**Quartile 1 (58)**	0.62 (0.31)	-0.010, 1.37	Reference
	Quartile 2 (55)	0.69 (0.27)	0.20, 1.40	0.23
	Quartile 3 (50)	0.67 (0.30)	0.14, 1.36	0.38
	Quartile 4 (51)	0.64 (0.66)	-0.040, 1.19	0.68
Physical activity IPAQ category	**Low activity (13)**	0.63 (0.34)	0.20, 1.22	0.68
	Moderate activity (71)	0.62 (0.31)	-0.040, 1.40	0.28
	High activity (131)	0.67 (0.29)	0.024, 1.37	Reference
25(OH)D level (nmol /L) (at baseline)	**<25 (6)**	0.56 (0.32)	0.19, 1.03	0.39
	25 – 49.9 (32)	0.57 (0.34)	-0.040, 1.40	0.08
	50 – 74.9 (68)	0.68 (0.29)	0.14, 1.37	0.91
	> 75 (110)	0.67 (0.29)	-0.010, 1.36	Reference

* Corrected OD relative to positive control titre

The data in Table 7.2 suggests a relationship between serum 25(OH)D and anti-KLH IgG1 when the former is considered as a dichotomous variable with a cut-off set at 50nmol/L. Indeed, a significantly higher mean anti-KLH IgG1 titre was seen in participants with serum 25(OH)D ≥ 50nmol/L compared with those with <50nmol/L (0.67 *vs.* 0.57; p=0.049).

Associations between personal UVR exposure and anti-KLH IgG1 response

Acute UVR exposure

Personal acute UVR exposure, as measured by electronic UVR dosimeters with interpolated data and adjusted for body surface area covered by clothing, was compared with anti-KLH IgG1 response at day 21 post-immunisation.

Due to the uncertainty regarding which days were most critical for UVR-induced immunomodulation, an exploratory approach was undertaken. Data were analysed for each individual day that the dosimeter was worn and by combinations of days (by summing the individual daily UVR exposures) as per the matrix shown in Table 7.3. Particular scrutiny was placed on the days adjacent to immunisation day, as this period was deemed *a priori* to have the maximum potential for UVR-associated immunomodulation. Only days with complete UVR data (i.e. no missing data) were used to generate the aggregated combinations of days. To assist with making this aggregated-day dataset as complete as possible, missing daily totals that remained after the interpolation process were replaced by a simplified imputation method (based on the average of the remaining days that had complete data, differentiated by weekday and weekend) (see Section 6.3). This most complete data set comprised ten days of daily UVR exposure totals for 216 participants (97.3% of the total sample).

Table 7.3 Combinations of UVR exposure daily totals used for regression models

		Days electronic dosimeter worn									
		1	**2**	**3**	**4**	**5**	**6***	**7**	**8**	**9**	**10**
Day combinations to be analysed #	A	1	2	3	4	5	6	7	8	9	10
	B		2	3	4	5	6	7	8	9	
	C			3	4	5	6	7	8		
	D				4	5	6	7			
	E					5	6				
	F					5	6	7			
	G					5	6	7	8		
	H					5	6	7	8	9	
	I				**4**	5					
	J			3	4	5					
	K		2	3	4	5					
	L	1	2	3	4	5					
	M						6	7			
	N						6	7	8		
	O						6	7	8	9	
	P						6	7	8	9	10

* Day of immunisation # Sum of daily UVR exposure totals (measured + interpolated + imputed)

Natural logarithm-transformed data were used for individual days and combinations of days to normalise the highly positively skewed UVR exposure data (see Figure 6.13). Table 7.4 shows the simple association between log-transformed personal UVR exposure and anti-KLH IgG1 response at day 21 post-immunisation. The data show no significant simple linear relationship with any measure of acute UVR exposure.

Table 7.4: Clothing-adjusted personal UVR exposure *vs.* anti-KLH IgG1 response at day 21 post immunisation: Simple linear analyses

Adjusted UVR exposure (log transformed; (SED)	Number of observations	Regression coefficient (x 1000)	95% Confidence interval (x 1000)	p value
Individual days				
Day 1	204	113	-164, 391	0.42
Day 2	205	165	-41, 372	0.12
Day 3	196	1.4	-119, 147	0.83
Day 4	190	-0.72	-152, 150	0.99
Day 5	197	3.9	-169, 248	0.71
Day 6*	211	-6.8	-308, 171	0.57
Day 7	203	7.8	-157, 314	0.51
Day 8	200	112	-5.5, 280	0.19
Day 9	192	1.0	-179, 200	0.92
Day 10	185	3.2	-111, 175	0.66
Combinations of days**				
A: Days 1 – 10	200	3.5	-3.5, 105	0.33
B: Days 2 – 9	200	3.3	-42, 108	0.38
C: Days 3 – 8	200	3.0	-51, 112	0.46
D: Days 4 – 7	200	6.5	-9.6, 109	0.90
E: Days 5 – 6	200	-12	-170, 146	0.88
F: Days 5 – 7	200	7.4	-122, 137	0.91
G Days 5 – 8	200	3.4	-7.3, 140	0.53
H: Days 5 – 9	200	2.4	-7.2, 119	0.63
I: Days 4 – 5	200	2.1	-101, 143	0.73
J: Days 3 – 5	200	3.1	-6.2, 125	0.51
K: Days 2 – 5	200	4.5	-4.4, 135	0.32
L: Days 1 – 5	200	4.7	-4.1, 134	0.30
M: Days 6 – 7	200	-1.8	-184, 147	0.83
N: Days 6 – 8	200	2.6	-9.4, 145	0.68
O: Days 6 – 9	200	1.4	-8.9, 117	0.79
P: Days 6 – 10	200	1.9	-7.0, 108	0.68

* Day of immunisation; ** Using imputed UVR data for missing days

Cumulative UVR exposure

Table 7.5 demonstrates anti-KLH IgG1 response at day 21 post-immunisation by cumulative UVR exposures as quantified by skin cast grade. There was no statistical difference between participants with the lowest skin cast grades (≤3.5) and those with the highest (≥4) (0.67 *vs.* 0.64; p=0.47). In a multiple regression model using skin cast grade as an ordinal explanatory variable (and adjusting for age, sex, site and ethnicity), a moderate but non-significant association between increasing skin cast grade and increasing anti-KLH IgG1 titre was seen (i.e. for every half-step increase in skin cast grade, there was an increase by 0.047 in IgG1 titre; 95% CI: -0.0035, 0.098; p=0.068).

Table 7.5: Skin cast grade versus anti-KLH IgG1 at day 21 post immunisation

Skin cast grade*	N	IgG1 titre mean (SD)**	95% CI Range
2	4	0.68 (0.14)	0.48, 0.81
2.5	26	0.66 (0.28)	0.17, 1.26
3	30	0.68 (0.27)	0.25, 1.11
3.5	59	0.66 (0.30)	-0.010, 1.40
4	25	0.59 (0.33)	0.19, 1.36
4.5	30	0.67 (0.32)	0.14, 1.37
5	20	0.69 (0.34)	-0.040, 1.19
5.5	6	0.44 (0.24)	0.20, 0.78
6	8	0.70 (0.32)	0.42, 1.22

* Average score of two independent assessors; ** Relative to positive control titre

Multiple linear regression analysis of UVR exposure and anti-KLH IgG1 response

The main goal in developing an explanatory model was to determine the association between the exposures of interest (i.e. acute and cumulative UVR exposure) and the outcome of interest (i.e. anti-KLH IgG1 response at day 21 post-immunisation). All potential immunomodulatory variables were included where there was *a priori* evidence &/or univariate evidence of an association with anti-KLH IgG1. As acute and cumulative personal UVR exposures were hypothesised to independently influence cell-mediated immune response, both were included in the regression model. As vitamin D status may lie on the causal UVR immunomodulatory pathway (particularly for cumulative UVR exposure), models with and without 25(OH)D levels at baseline were assessed.

Table 7.6 shows the results of the multiple regression models for different UVR exposures. The following variables were modelled as continuous measures: age (years), BMI, 25(OH)D level (at visit 2), skin reflectance at the left inner arm and cheek, and psychological profiles

(POMS-TMD and MHI-global index). Physical activity was modelled as a continuous variable using MET-minutes per week as recorded in the IPAQ (See Section 5.2.1.2). This variable was square-root transformed to normalise the initial positively-skewed distribution. Skin cast grade was modelled as an ordinal explanatory variable. The effect of season was accounted for by fitting a sine-cosine function across the study enrolment period. Separate seasonal terms were fitted for Canberra and Townsville given their distinct climates.

While no statistical association was found between anti-KLH IgG1 response and personal UVR exposure (either acute or cumulative), other covariates did demonstrate a significant association with immune response.

Table 7.6 Clothing-adjusted UVR exposures *vs.* anti-KLH IgG1 response at day 21 post-immunisation: Multiple linear regression analyses

Adjusted UVR exposure (log transformed; (SED)	Number of observations	Regression coefficient (x1000)	95% Confidence interval (x1000)	p value
Individual days				
Day 1	188	- 53	- 384, 278	0.75
Day 2	187	197	- 33, 426	0.093
Day 3	178	42	- 104, 188	0.57
Day 4	173	- 46	- 211, 119	0.58
Day 5	180	-11	- 249, 226	0.93
Day 6*	193	- 145	- 413, 122	0.29
Day 7	185	94	- 158, 347	0.46
Day 8	182	36	- 146, 219	0.70
Day 9	177	6.9	- 205, 219	0.95
Day 10	168	38	- 110, 186	0.61
Day combinations**				
A: Days 1 – 10	182	16	- 75, 107	0.72
B: Days 2 – 9	182	- 19	- 77, 115	0.70
C: Days 3 – 8	182	8.7	- 93, 111	0.87
D: Days 4 – 7	182	- 22	- 147, 102	0.72
E: Days 5 – 6	182	- 61	- 249, 127	0.52
F: Days 5 – 7	182	- 12	- 170, 146	0.88
G: Days 5 – 8	182	0.35	- 130, 130	1.0
H: Days 5 – 9	182	-1.4	- 119, 116	0.98
I: Days 4 – 5	182	0.088	- 139, 139	1.0
J: Days 3 – 5	182	27	- 82, 136	0.63
K: Days 2 – 5	182	48	- 59, 154	0.38
L: Days 1 – 5	182	38	- 70, 145	0.49
M: Days 6 – 7	182	- 39	- 232, 155	0.69
N: Days 6 – 8	182	- 19	- 160, 122	0.79
O: Days 6 – 9	182	- 17	- 140, 107	0.79
P: Days 6 – 10	182	- 6.2	- 111, 98	0.91

* Day of immunisation; ** Using imputed UVR data for missing days; Adjusted for age, sex, study site, season, BMI, physical activity (MET-minutes/week), psychological state (MHI-global index, POMS-TMD), skin reflectance at the inner arm and left cheek, smoking status, 25(OH)D level (on day of immunisation), cumulative sun exposure (left hand skin cast grade), parental ethnicity (both parents northern European *vs.* other)

Table 7.7 shows the results of the multiple regression model with acute UVR exposure combination K (Days 2 to 5) which had the best 'fit' for the data based on the model's R^2 value (and where all models contained the same number of participants; n=182). Female sex remained strongly associated with humoral immunisation response with a 14.6% higher anti-KLH IgG1 response than in males (p=0.004) at 21 days post-immunisation. Age was strongly linearly associated with anti-KLH IgG1 response, with antibody response declining by 1.6% (95% CI 0.63% - 2.5%; p=0.001) for every increased year of participant's age. Notably, serum 25(OH)D level (including when modelled as a dichotomous variable using a 50nmol/L cut-off) or cumulative UVR exposure was not significantly associated with anti-KLH IgG1 response.

Table 7.7: Association between covariates and anti-KLH IgG1 response at 21 days: A multiple linear regression analysis using the best fitting regression model#

Variable	Regression coefficient (x 1000)	95% confidence interval (x 1000)	p value
UVR Days 2 – 5 (Model K)	47	-58, 154	0.38
Skin cast grade (ordinal ½ step)	51	-9.5, 112	0.097
Age (years)	**-16**	**-25, -6.3**	**0.001**
Sex: Female	**146**	**47, 245**	**0.004**
Parental ethnicity: Northern European (both parents)	-88	-21, 35	0.16
BMI*	3.3	-9.2, 16	0.61
Melanin density* Inner arm	25	-22, 73	0.30
Melanin density* Left cheek	-24	-91, 42	0.47
Physical activity* IPAQ (MET minutes / week)	0.28	-1.7, 2.3	0.79
Psychological profile* POMS – TMD	1.0	-2.8, 4.9	0.22
Psychological profile* MHI – global score	1.7	-0.99, 4.4	0.21
Vitamin D (25(OH)D)*	-1.2	-3.1, 0.74	0.22
Current smoker	132	-86, 35	0.23

N= 182 * Modelled as a continuous variable

Checking regression assumptions

This section outlines the tests used to verify that the assumptions underpinning the use of linear regression modelling were met. As the best model in Table 7.6 with the best fit for the data, the aggregated personal UVR exposure over days 2 to 5 (combination 'K' from Table 7.5; n=182) is used to demonstrate robustness of regression assumptions.

Influential data points: Data points with high residual (deviation from the regression line) or 'leverage' can potentially influence regression coefficients. The most influential data points were checked for accuracy and transcription errors. When regression models were run with and without these data points, little change to the regression coefficients of the UVR exposure variable was found. Therefore all data points were retained.

Normality of residuals: The Shapiro-Wilk W test demonstrated that there was no evidence to reject the hypothesis that the residuals were normally distributed (p=0.96)

Homoscedasticity of residuals: The White's test and Breusch-Pagan test demonstrated no evidence to reject the hypothesis that there was no pattern of the residuals when plotted against fitted values (p=0.30 and p=0.57, respectively).

Multi-collinearity: Variance inflation factors applied to the explanatory variables did not show evidence of collinearity between variables in the model.

Summary

Following KLH immunisation, there was a rise in anti-KLH IgG1 titres at 7 and 21 days post-immunisation. The latter response demonstrated the greatest variation and was used as the end-point for further analyses.

Simple linear analysis demonstrated that higher anti-KLH IgG1 at 21 days post-immunisation was significantly associated with younger age, female sex and serum 25(OH)D levels greater than 50nmol/L. On multiple regression analyses, only the statistical associations with sex and age persisted. Measures of clothing-adjusted acute and cumulative personal UVR exposure were not found to be significant explanatory variables.

Diagnostic testing supported the underlying regression model assumptions.

7.1.3 *In vivo* cell-mediated immune response: DTH response

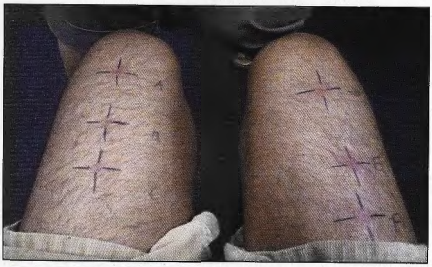
Delayed type hypersensitivity (DTH) responses to KLH were used as the *in vivo* measure of antigen-specific cell-mediated immunity. Twenty-one days following KLH immunisation to the forearm, intra-dermal injection of KLH was given at the same site and the subsequent DTH response was read 48 hours later.

Intra-rater and inter-rater agreement for DTH testing

At the Canberra study sites, a single observer (AS) measured all DTH responses. In Townsville, the majority of responses were measured by a single observer (KP). However, when KP was unavailable, a second study officer was required to measure responses (CvB).

For the purpose of assessing inter-rater agreement, sets of DTH responses were read by the three raters over three calibration visits. Each rater was blinded to the others' measures (and pen markings were removed between readings). DTH responses were derived from: i) multiple intra-dermal KLH injections (varying doses: 5 – 20 µg) administered to the thighs of a pre-sensitised volunteer[3] (see Figure 7.2); and, ii) a sample of AusUVI participants. The former strategy occurred on two occasions at calibration sessions when all assessors were present at the same site, and the latter strategy was opportunistic, using participants who were attending for their study visit at the time of the calibration visit.

Figure 7.2: Assessing inter-rater agreement in reading DTH responses



Use of the 'pen method' proved challenging for assessing the repeatability of DTH measurement. There was noticeably increased erythema and local swelling around the DTH response following pen marking of the skin. Nevertheless, 22 DTH responses were assessed by all three raters, and the two-way mixed-method intra-class correlation coefficient was 0.63 (95% CI: 0.38 – 0.80), indicating moderate to strong agreement between scorers.

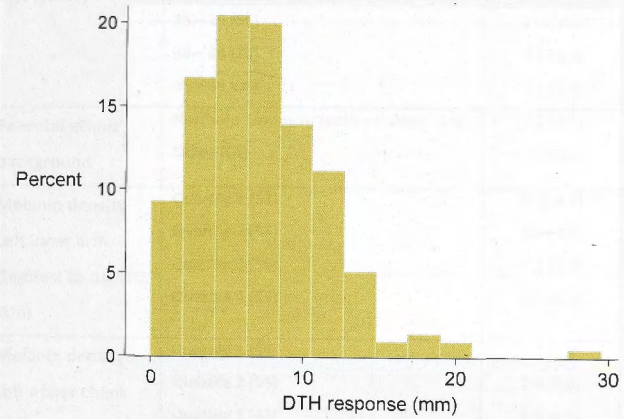
[3] The volunteer was a study investigator (AS)

DTH responses amongst AusUVI participants

For the AusUVI Study, 211 DTH responses were recorded (98% of evaluable participants) – six participants were unable to attend for skin test reading at the prescribed time.

Graph 7.3 demonstrates the distribution of DTH responses. The mean response was 7.4mm (SD 4.3mm) and median response 6.5mm with a range of 0 – 29.5mm. Twelve participants (5.7%) did not have a measureable DTH response (i.e. 0mm). Given the positively-skewed distribution, square-root transformation was required to allow for parametric testing and robust linear regression models.

Graph 7.3: Distribution of DTH responses



Participant 186 had the most extreme measured DTH response (29.5mm), above the 99th centile for the distribution and 55% higher than the next measured response (19mm). It was not clear from the corresponding photograph whether the measurement was of the erythema associated with the DTH response rather than the area of induration. In view of the lack of confidence in the accuracy of this measurement, the models presented in this section were generated with this value excluded given its propensity to be influential on regression coefficients (see also Section 7.1.3).

Table 7.8 shows the relationship between the DTH response and potential immunomodulatory variables. Females had a significantly higher DTH response than males (17%; p=0.03). Other putative immunomodulatory factors were not significantly associated with DTH response.

Table 7.8: Association between potential immunomodulatory factors and DTH response

Potential immune modulators	Sub-group (n)	Mean (SD) (mm)	Range (mm)	p value*
Overall	**(210)**	7.0 (4.0)	0 – 19	
Sex	**Male (79)**	6.5 (4.2)	0 – 19	**0.038**
	Female (131)	7.4 (3.8)	0 – 19	
Site	**Canberra (102)**	7.2 (4.2)	0 – 19	0.86
	Townsville (108)	6.9 (3.7)	0 – 19	
Season	**Winter (75)**	7.3 (4.4)	0 – 19	Reference
	Spring (47)	6.8 (3.7)	0 – 16	0.61
	Summer (37)	7.6 (4.0)	0 – 19	0.65
	Autumn (51)	6.4 (3.5)	0 – 17	0.33
Age (years)	**18 – 24 (79)**	6.7 (3.9)	0 – 19	Reference
	25 – 29 (66)	7.3 (3.8)	0 – 16	0.39
	30 – 34 (29)	7.1 (4.3)	0 – 19	0.69
	35 – 40 (36)	7.3 (4.3)	0 – 17	0.61
Parental ethnic background	**Northern European (both parents) (146)**	7.2 (3.9)	0 – 17	0.62
	Other (64)	7.0 (4.0)	0 – 19	
Melanin density Left inner arm (Lightest to darkest skin)	**Quartile 1 (52)**	7. 5 (4.1)	0 – 17.5	Reference
	Quartile 2 (51)	6.2 (3.6)	0 – 19	0.16
	Quartile 3 (50)	7.1 (4.0)	0 – 19	0.71
	Quartile 4 (55)	7.3 (4.2)	0 – 17	0.74
Melanin density Left upper cheek (Lightest to darkest skin)	**Quartile 1 (54)**	6.5 (3.3)	0 – 13.5	Reference
	Quartile 2 (56)	7.4 (3.9)	2 – 17.5	0.29
	Quartile 3 (44)	6.4 (4.5)	0 – 17	0.59
	Quartile 4 (53)	8.0 (4.2)	0 – 19	0.11
Smoking	**Non-smoker (199)**	7.0 (4.0)	0 – 19	0.74
	Current smoker (11)	7.1 (2.9)	2 – 13	
Body mass index Categories	**Underweight (4)**	6.5 (1.9)	4 – 8.5	0.87
	Normal weight (136)	7.2 (3.9)	0 – 19	Reference
	Overweight (55)	6.4 (4.4)	0 – 17.5	0.69
	Obese (15)	7.9 (3.0)	3.5 – 14	0.58
Psychological profile: POMS (TMD) Lowest to highest mood disturbance	**Quartile 1 (54)**	7.0 (4.1)	0 – 19	Reference
	Quartile 2 (56)	7.5 (4.1)	0 – 17.5	0.46
	Quartile 3 (44)	6.8 (4.5)	0 – 19	0.67
	Quartile 4 (53)	6.6 (3.3)	0 – 17	0.81

Psychological profile: MHI (Global score) Lowest to highest well-being	**Quartile 1 (57)**	6.5 (3.8)	0 – 19	Reference
	Quartile 2 (53)	7.9 (4.3)	0 – 17.5	0.083
	Quartile 3 (47)	6.9 (4.0)	0 – 16	0.65
	Quartile 4 (50)	6.6 (3.9)	0 – 19	0.86
Physical activity IPAQ category	**Low activity (12)**	6.1 (4.8)	0 – 14	0.26
	Moderate activity (70)	7.3 (4.2)	0 – 19	0.79
	High activity (128)#	7.0 (3.8)	0 – 19	Reference
25(OH)D level (nmol /L) (at baseline)	**<25 nmol /L (6)**	7.2 (7.8)	0 – 14	0.99
	25 – 49.9 nmol /L (31)	7.4 (4.6)	0 – 19	0.71
	50 – 74.9 nmol/L (65)	7.4 (4.4)	0 – 19	0.56
	> 75 nmol /L (108)	6.7 (3.5)	0 – 17	Reference
*Parametric testing or regression performed using square-root transformed distribution of DTH responses				

There was no association with DTH response when serum 25(OH)D level was analysed as a dichotomous variable with cut-off set at 75nmol/L (7.3mm vs. 6.8mm; p=0.58).

Associations between personal UVR exposure and DTH response

Acute UVR exposure

Table 7.9 shows the simple linear association between clothing-adjusted personal UVR exposure and DTH response.

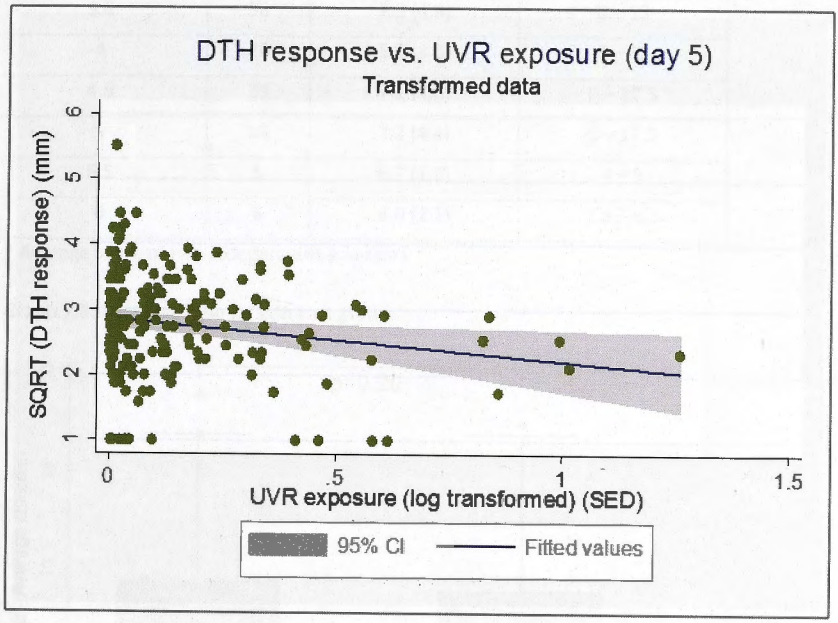
Table 7.9: Adjusted personal UVR exposure *vs.* DTH response: Simple linear analyses

Adjusted UVR exposure (log transformed; (SED) Individual days	Number of observations	Regression coefficient (x 100)	95% Confidence interval (x 100)	p value
Day 1	198	- 3.7	- 108, 32	0.29
Day 2	200	0.79	- 51, 53	0.98
Day 3	191	9.7	- 23, 43	0.56
Day 4	185	3.3	- 33, 40	0.86
Day 5	192	**- 60**	**-110, - 9.8**	**0.019**
Day 6*	206	25	- 34, 85	0.40
Day 7	198	- 40	- 97, 17	0.17
Day 8	195	- 26	- 68, 16	0.22
Day 9	187	- 31	- 78, 17	0.21
Day 10	181	7.4	- 31, 45	0.70
Day combinations **				
A: Days 1 – 10	195	- 8.3	- 26, 0.88	0.34
B: Days 2 – 9	195	- 9.3	- 27, 8.9	0.31
C: Days 3 – 8	195	- 7.5	- 27, 12	0.45
D: Days 4 – 7	195	- 13	- 38, 12	0.30
E: Days 5 – 6	195	- 21	- 59, 17	0.27
F: Days 5 – 7	195	- 23	- 55, 8.3	0.15
G: Days 5 – 8	**195**	**- 22**	**- 47, 4.1**	**0.100**
H: Days 5 – 9	**195**	**- 22**	**- 45, 1.0**	**0.060**
I: Days 4 – 5	195	- 16	- 46, 13	0.27
J: Days 3 – 5	195	- 4.9	- 28, 18	0.67
K: Days 2 – 5	195	- 6.2	- 28, 16	0.58
L: Days 1 – 5	195	- 9.6	- 31, 12	0.38
M: Days 6 – 7	195	- 11	- 51, 30	0.60
N: Days 6 – 8	195	- 17	- 46, 122	0.26
O: Days 6 – 9	195	- 18	- 43, 6.6	0.15
P: Days 6 – 10	195	- 9.9	- 32, 12	0.38

* Day of immunisation; ** Using imputed UVR data for missing days

Clothing-adjusted personal UVR exposure on Day 5 (i.e. the day prior to immunisation) was significantly associated with a decreased DTH response (p=0.019). Graph 7.4 illustrates this association. Additionally, UVR exposure over the Days 5 to 8 (i.e. the day before immunisation to day three following immunisation) and Days 5 to 9, showed low-moderate evidence of a simple linear association with decreased DTH response (p≤0.10).

Graph 7.4: DTH response vs. clothing-adjusted personal UVR exposure on Day 5



Cumulative UVR exposure

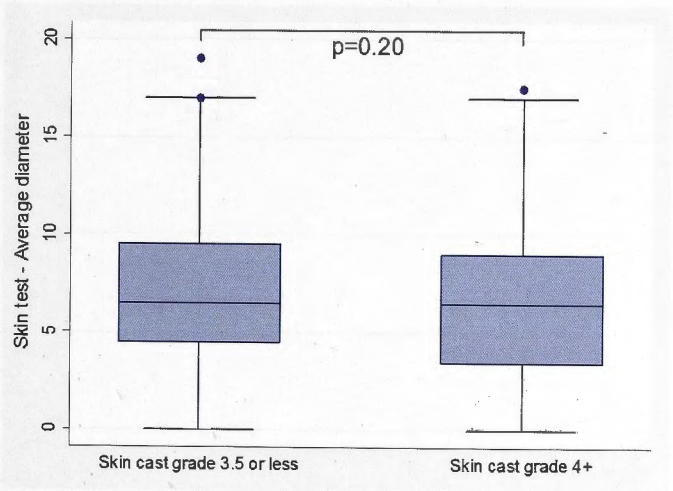
Table 7.10 compares the DTH response according to cumulative UVR exposure as quantified by skin cast grade. Participants with above-median grade skin casts (≥4) had a lower average DTH response compared with those with below-median(≤3.5) skin cast grades, however this did not reach statistical significance (6.6mm vs. 7.5mm; p=0.20) (See Graph 7.5). Adjustment for age, sex, site and ethnicity in a multiple regression model did not change the significance of this association.

Table 7.10: Skin cast grade *vs.* DTH response

Skin cast grade*	N	Measurement (mean, SD (mm))	Range (mm)
2	4	7.3 (2.6)	5 – 11
2.5	24	6.5 (2.7)	3 – 14.5
3	29	8.1 (4.1)	1.5 – 16
3.5	58	7.3 (4.4)	0 – 19
4	26	5.8 (3.6)	0 – 12
4.5	29	7.1 (4.8)	0 – 17.5
5	19	7.2 (4.4)	0 – 17.5
5.5	5	6.7 (1.8)	4 – 9
6	8	6.0 (2.1)	2.5 – 8.5

* Average score of two independent assessors

Graph 7.5: DTH response by skin cast grade



Multiple linear regression analysis of UVR exposure and DTH response

Table 7.11 displays the output of multiple regression models assessing the association between acute UVR exposure and DTH response, adjusted for various postulated immunomodulatory variables. Again, all variables in Table 7.7 were included in the multiple regression models as was the skin cast measure of cumulative UVR exposure.

Clothing-adjusted personal UVR exposure on Day 5 was significantly associated with a reduced DTH response (p=0.015). Additionally, aggregated UVR exposure over Days 5 to 8 and 5 to 9

were also significantly associated with a reduced DTH response 21 days later. Notably, the regression coefficients for all other aggregated acute UVR exposure variables, pre- and post-immunisation, demonstrated a negative (albeit non-significant) association with DTH response, providing some additional support of a suppressive UVR effect.

Table 7.11: UVR exposure *vs.* DTH response: Multiple linear regression analyses

Clothing-adjusted UVR exposure (SED) (log transformed)	Number of observations	Regression coefficient** (x 100)	95% Confidence interval (x 100)	p value
Individual days				
Day 1	183	-59	- 150, 32	0.20
Day 2	182	6.1	- 57, 69	0.85
Day 3	174	25	- 14, 65	0.21
Day 4	168	17	- 26, 59	0.45
Day 5	175	**-78**	**- 140, - 15**	**0.015**
Day 6*	188	50	- 23, 123	0.18
Day 7	180	- 47	- 115, 22	0.18
Day 8	177	- 45	- 95, 4.3	0.073
Day 9	172	- 34	- 93, 25	0.26
Day 10	164	21	- 24, 65	0.37
Day combinations				
A: Days 1 – 10	177	- 10	- 35, 14	0.41
B: Days 2 – 9	177	- 11	- 37, 14	0.38
C: Days 3 – 8	177	- 7.6	- 35, 19	0.58
D: Days 4 – 7	177	- 15	- 48, 18	0.38
E: Days 5 – 6	177	- 28	- 78, 21	0.26
F: Days 5 – 7	177	- 34	- 75, 7.7	0.11
G: Days 5 – 8	177	**- 36**	**- 70, - 1.7**	**0.039**
H: Days 5 – 9	177	**- 35**	**- 66, - 4.4**	**0.025**
I: Days 4 – 5	177	- 15	- 52, 21	0.41
J: Days 3 – 5	177	1.2	- 28, 30	0.93
K: Days 2 – 5	177	- 1.1	- 29, 27	0.94
L: Days 1 – 5	177	- 7.7	- 36, 21	0.60
M: Days 6 – 7	177	- 11	- 63, 40	0.66
N: Days 6 – 8	177	- 26	- 63, 11	0.16
O: Days 6 – 9	177	- 27	- 59, 5.4	0.10
P: Days 6 – 10	177	- 12	- 40, 17	0.42

* Day of immunisation

** Adjusted for age, sex, study site, season, BMI, physical activity (MET-minutes/week), psychological state (MHI-global index, POMS-TMD), skin reflectance at the inner arm and left cheek, smoking status, 25(OH)D level (on day of immunisation), cumulative sun exposure (left hand skin cast grade), parental ethnicity (both parents northern European *vs.* other)

Table 7.12 shows the results of a multiple linear regression model using acute UVR exposure on Day 5 which had the strongest association with DTH response. No other putative immunomodulatory factor (including sex, cumulative UVR exposure or serum 25(OH)D level) showed a significant association with DTH response.

Further exploratory analysis with interaction terms involving acute UVR exposure, cumulative UVR exposure, age and sex was conducted, but did not reveal any other significant associations.

Table 7.12: Association between covariates and anti-KLH DTH response at 21 days: A multiple linear regression analysis using acute clothing-adjusted UVR exposure on Day 5

Variable	Regression coefficient (x 100)	95% confidence interval (x 100)	p value
UVR exposure Day 5	**-78**	**-140, -15**	**0.015**
Skin cast grade (ordinal ½ step)	-12	-29, 4.3	0.15
Age (years)	1.0	-1.4, 3.6	0.38
Sex: Female	22	-4.0, 49	0.096
Parental ethnicity: Northern European (both parents)	-7.2	-39, 25	0.66
BMI*	-0.17	-3.5, 3.1	0.92
Melanin density* Inner arm	-4.8	-17, 7.1	0.43
Melanin density* Left cheek	2.9	-16, 21	0.76
Physical activity* IPAQ (MET minutes / week)	0.38	-0.19, 0.94	0.20
Psychological profile* POMS – TMD	-0.0010	-1.0, 1.0	1.0
Psychological profile* MHI – global score	0.32	-0.43, 1.0	0.39
Vitamin D (25(OH)D)* nmol/L	-0.11	-16, 21	0.66
Current smoker	16	-41, 73	0.58

* Modelled as continuous variable

Checking regression assumptions

As personal UVR exposure on Day 5 showed evidence of a significant inverse association with DTH response, this model was used as a typical example of the regression models in Table 7.11.

Influential data points: All 'influential' points were assessed in the data set and checked to ascertain validity of measurement (i.e. by reviewing primary data collection documents or photographs of DTH skin tests) and accuracy of data entry. As previously mentioned, the most influential data point related to the largest DTH response (29.5mm) that was recorded in participant 186. Inclusion of this data point in regression models confirmed that it had a highest residual, and measure of influence (Cook's D test). As there was concern regarding the validity of this particular skin test measurement, it was excluded from further analysis.

Normality of residuals: The Shapiro-Wilk W test demonstrated that there was no evidence to reject the hypothesis that the residuals were normally distributed (p=0.34).

Homoscedasticity of residuals: The White's test and Breusch-Pagan test demonstrated no evidence to reject the hypothesis that there was no pattern of the residuals when plotted against fitted values (p=0.45 and p=0.076, respectively).

Multi-collinearity: Variance inflation factors applied to the explanatory variables did not show evidence of collinearity between variables in the model.

Summary

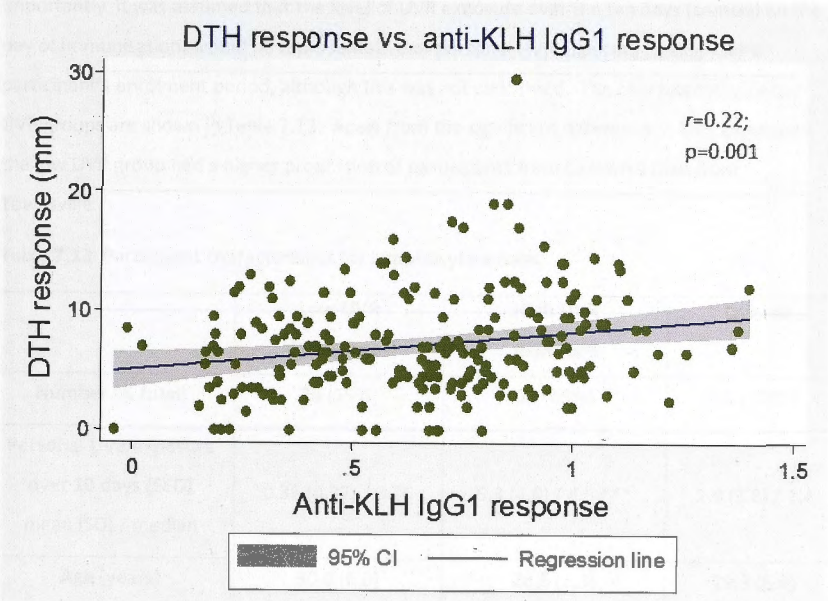
There was moderate to strong agreement between assessors of DTH responses across the two study sites. The DTH distribution was positively skewed and required square-root transformation to allow use of parametric statistical models. On simple linear analysis, females demonstrated significantly higher DTH responses than males. Increased clothing-adjusted personal UVR exposure on Day 5 had a strong linear association with reduced DTH response. Multiple linear regression models confirmed this association, and also an association with aggregated UVR exposure over Days 5 to 8, and 5 to 9 (i.e. spanning the period just before and after immunisation), after adjusting for all other potential immunomodulatory factors. Cumulative UVR exposure, serum 25(OH)D levels and other potential immunomodulatory variables (including sex) were not associated with DTH response in these models.

Diagnostic testing supported the underlying regression model assumptions.

7.1.4 Comparing *in vivo* cell-mediated immune response with humoral responses

As KLH immunisation promoted a T-dependent antibody response that relied on Th1 cell-mediated processes, it was expected that the KLH-specific DTH response would correlate with the KLH-specific IgG1 response. Graph 7.6 shows this to be the case with a positively correlated association (r=0.22; p=0.001).

Graph 7.6 Comparing DTH responses with corresponding KLH IgG1 response at 21 days post-immunisation



7.1.5 *Ex vivo* cell-mediated immune response

Lymphocytes from a selected subgroup (55/222 ~ 25%) of the overall AusUVI Study cohort were analysed to assess whether an association existed between personal UVR exposure and circulating T-helper cell subsets. To maximise the chance of detecting a UVR-associated immunomodulatory effect in this initial analysis, the samples chosen for testing represented the extremes of measured personal UVR exposure (clothing-adjusted aggregated data over ten days). Participants were thus categorised into 'high' or 'low' UVR exposure groups. Importantly, it was assumed that the level of UVR exposure over the ten days (centred on the day of immunisation) would be representative of personal UVR exposure throughout a participant's enrolment period, although this was not confirmed. The characteristics of the UVR groups are shown in Table 7.13. Apart from the significant difference in UVR exposures, the low UVR group had a higher proportion of participants from Canberra than from Townsville.

Table 7.13: Participant characteristics for lymphocyte assays

	Low UVR exposure	**High UVR exposure**	**Overall**
Number (% total)	28 (55%)	27 (45%)	55 (100%)
Personal UVR exposure over 10 days (SED) mean (SD) / median	0.36 (0.32) / 0.26	5.3 (4.0) / 4.5 ***	2.9 (3.8) / 1.4
Age (years) (Mean, SD)	30.0 (6.0)	28.6 (6.3)	29.3 (6.0)
Female (%)	15 (54%)	15 (56%)	30 (55%)
Site: Canberra (%)	25 (89%)	18 (67%)*	43 (78%)

*Significant at $p < 0.05$ by χ^2 test

*** Significant at $p < 0.0001$ by Wilcoxon rank sum test

Cell sorting strategy

As described in Section 4.2.2, *ex vivo* assessment of cell-mediated immunity was undertaken using fluorescent labelled antibodies to identify surface cell markers and intracellular cytokines, followed by flow cytometry to quantify the proportion of Th1, Th2, Th17 and regulatory T cells in PBMC samples isolated at baseline and day 21 post-immunisation. These were not antigen-specific assays. Figures 7.3 and 7.4 illustrate the flow cytometer output and the strategies used for enumerating the T-helper cell subsets.

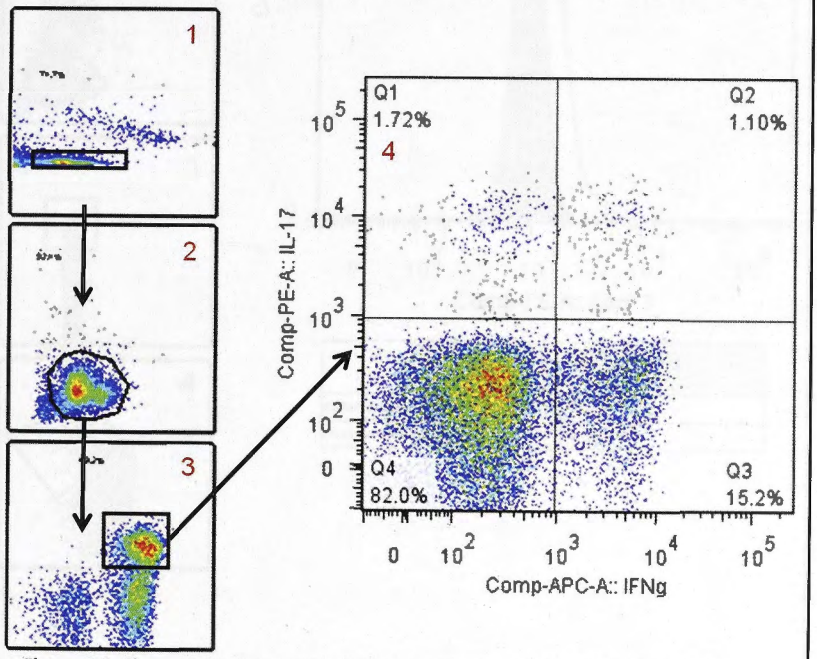
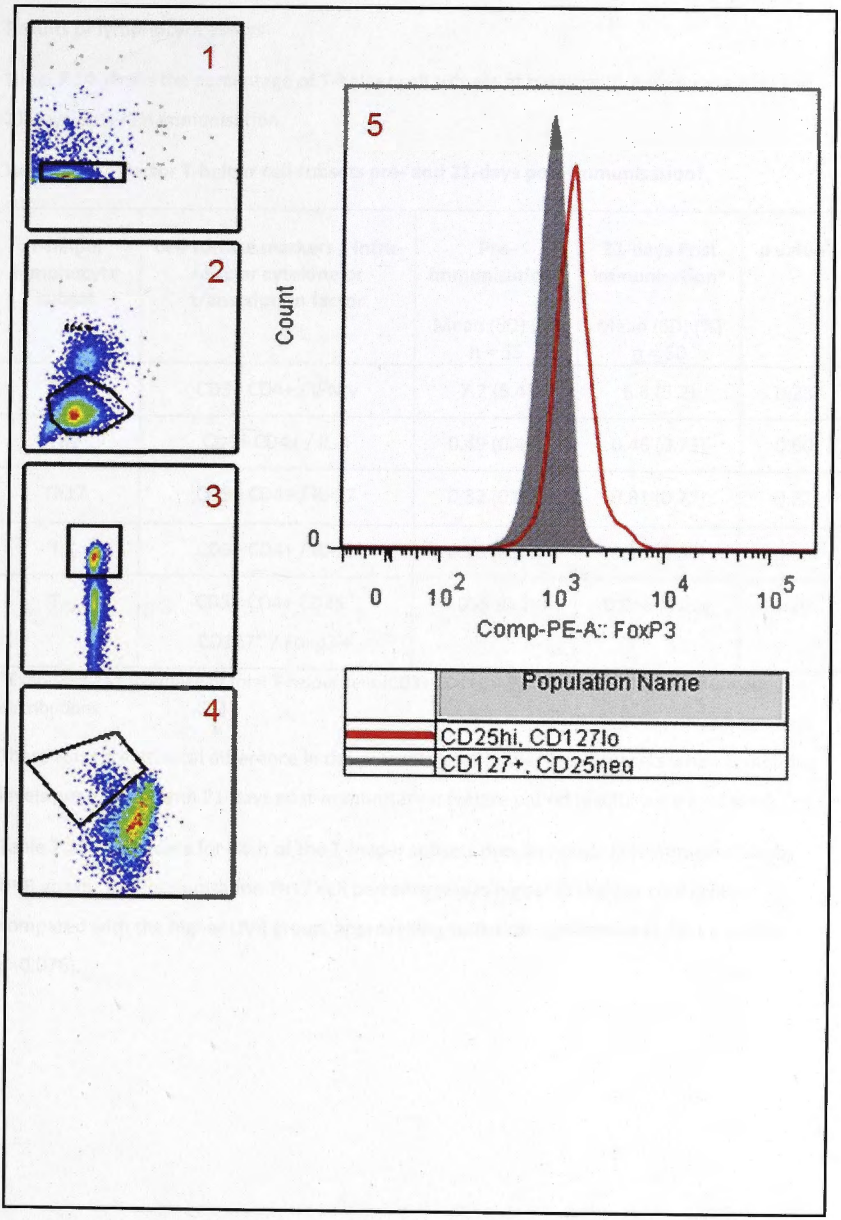


Figure 7.3: Flow cytometer output: Gating strategy to enumerate T-helper cell subsets. ***Panel 1:*** Side scatter *vs.* forward scatter plot to identify and remove debris / non-cellular elements; ***Panel 2:*** Gated to select lymphocytes. ***Panel 3:*** Gated to select CD3+ and CD4+ cell surface markers on lymphocytes (i.e. T-helper cells). ***Panel 4:*** Gated to antibodies against intra-cellular cytokines of interest, in this case IL-17 and IFN-gamma. Q1 represents IL-17 staining T-helper cells only, Q2 represents both IL-17 and IFN-gamma staining cells, Q3 represents IFN-gamma staining cells only, and Q4 represents T-helper cells not staining for IL-17 or IFN-gamma. The percentages in each quadrant reflect the proportion of cells compared with the total number of T-helper lymphocytes. The final number of lymphocytes was determined by subtracting the proportion of cells seen in unstimulated from stimulated samples. A similar strategy was used for enumerating IL-4 and IL-10 staining T-helper cells. Note that each point in the panels represents an individual cell, with colour representing cell density.


1
2
3
4
5
Count
0
0
10^2
10^3
10^4
10^5
Comp-PE-A:: FoxP3
Population Name
CD25hi, CD127lo
CD127+, CD25neg

Results of lymphocyte assays

Table 7.14 shows the percentage of T-helper cell subsets at baseline (pre-immunisation) and 21 days post-KLH immunisation.

Table 7.14: Effector T-helper cell subsets pre- and 21-days post-immunisation*

T-helper lymphocyte subset	Cell surface markers / Intra-cellular cytokine or transcription factor	Pre-immunisation* Mean (SD) (%) n = 55	21-days Post immunisation* Mean (SD) (%) n = 50	p value#
Th1	CD3+ CD4+ / IFN-γ	7.2 (5.4)	6.8 (5.2)	0.23
Th2	CD3+ CD4+ / IL-4	0.49 (0.44)	0.46 (0.73)	0.60
Th17	CD3+ CD4+ / IL-17	0.82 (0.80)	0.81 (0.77)	0.82
T_{reg}	CD3+ CD4+ / IL-10	0.26 (0.39)	0.23 (0.50)	0.55
T_{reg}	CD3+ CD4+ $CD25^{hi}$ $CD127^{lo}$ / Foxp3 +	0.055 (0.18)	0.056 (0.19)	0.26

*Expressed as percentage of total T-helper cells (CD3+ CD4+); # Paired t-test of log-transformed distributions

There was no statistical difference in the percentage of helper T-cell subsets when comparing levels pre-vaccine with 21-days post-immunisation (where paired results were available).

Table 7.15 shows data for each of the T-helper subsets pre- and post- KLH immunisation by UVR group. Pre-immunisation Th17 cell percentage was higher in the low UVR group compared with the higher UVR group, approaching statistical significance (1.0% vs. 0.61%; p=0.076).

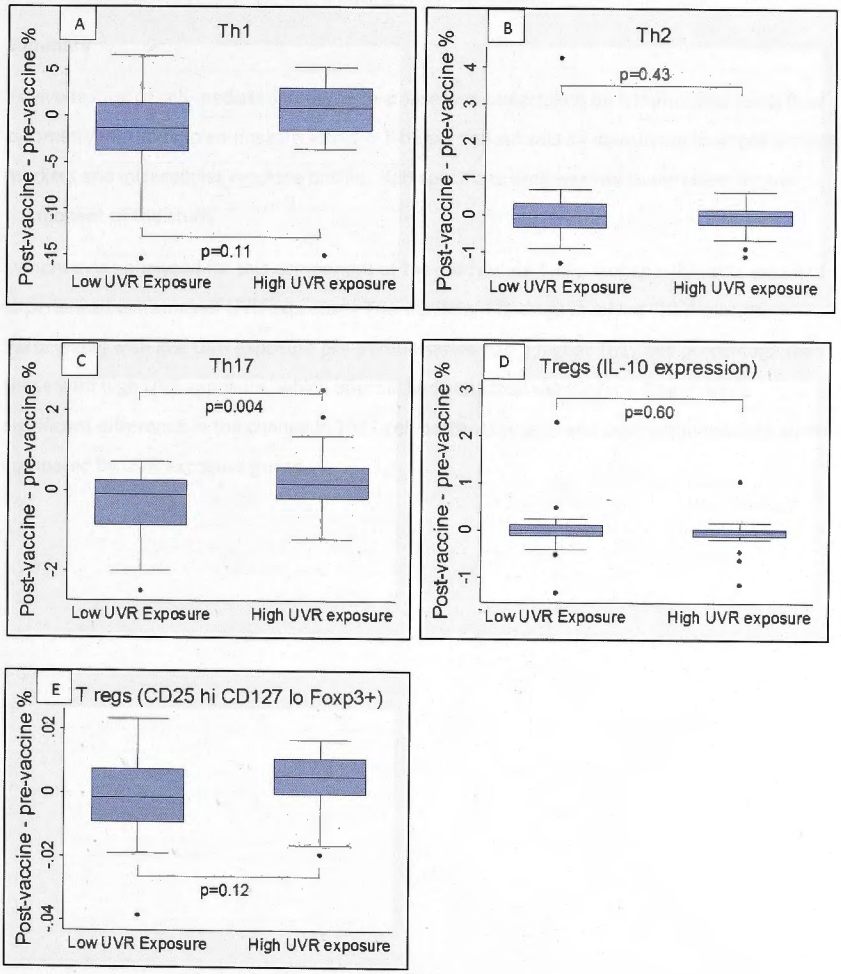
Table 7.15: T-helper cell subsets (%) pre- and post-immunisation by UVR exposure group

T-helper subset	Pre-vaccine Mean (SD)			Post-vaccine % cells (SD)		
	Low UVR (n=27)	High UVR (n=28)	p value*	Low UVR (n=24)	High UVR (n=26)	p value*
Th1	7.2 (6.0)	7.2 (5.6)	0.99	5.8 (4.8)	7.8 (5.5)	0.17
Th2	0.40 (0.38)	0.57 (0.49)	0.15	0.50 (0.96)	0.43 (0.43)	0.76
Th17	1.0 (1.0)	0.61 (0.47)	0.076	0.71 (0.68)	0.90 (0.84)	0.39
Treg (IL-10 staining)	0.27 (0.40)	0.25 (0.38)	0.88	0.31 (0.69)	0.16 (0.23)	0.30
Treg* (Foxp3+)	0.060[a] (0.019)	0.052[b] (0.016)	0.10	0.057[c] (0.019)	0.055[d] (0.019)	0.70

a: n=26; b: n=28; c: n=23; d: n=26; * using t-tests of log-transformed distributions to assess significance
NB: T-helper subsets expressed as percentage of total T-helper cells (CD3+ CD4+)

Following on from the previous data, Graphs 7.12 A→E, illustrate the *difference* in the percentage of T-helper cell subsets pre- and post-vaccine (i.e. using individuals with paired data, pre-vaccine percentage was subtracted from day-21 post-immunisation percentage) comparing low and high UVR exposure groups.

Graph 7.12: Change in proportion of T-helper lymphocytes: by UVR exposure group



The Th17 subsets demonstrated the most significant difference in percentage pre- and post-immunisation time points when comparing low with high UVR exposure (-0.39% *vs.* 0.31%; p=0.004) (Graph 7.12c). The contributions to this difference can again be seen in the data presented in Table 7.15 – in participants with low UVR exposure, the percentage of Th17 cells was significantly higher in the pre-vaccine compared with post-vaccine group (1.0% vs. 0.71%;

p=0.033; paired t-test of log transformed data). Conversely, in the high UVR exposure group, Th17 cell percentage was higher in the post-vaccine group than pre-vaccine, (0.90% vs. 0.61%; p=0.075).

Given that there were no significant differences in Th17 cell percentage between time-points when UVR exposure was not considered, this indicates a possible interaction involving immunisation and UVR exposure influencing Th17 expression.

Summary

Ex vivo testing of cell-mediated immune response was undertaken on lymphocytes using flow cytometry methods to enumerate effector T-helper cell subsets by identifying their cell surface markers and intracellular cytokine profile. KLH specific testing was not undertaken for this component of the study.

Lymphocytes analysed for this component of the study were taken from participants who had experienced extremes of UVR exposure. The significant findings involved Th17 subsets. Participants with low UVR exposure pre-immunisation had a higher Th17 cell percentage than those with high UVR exposure, which approached statistical significance. There was a significant difference in the change in Th17 cell percentage pre- and post- immunisation when compared by UVR exposure group.

7.2 Vaccine-related safety issues

KLH immunisation and skin testing were very well tolerated. Table 7.16 shows the number of adverse events documented amongst AusUVI Study participants. The most common adverse events were transient erythema or pruritus at the site of KLH immunisation.

Table 7.16: Vaccine-associated adverse events

Adverse event classification*	Site		Total
	Canberra	Townsville	
Serious Adverse events:	0	0	0
Unexpected adverse events:	0	1	1
Adverse Events:	4	1	5

*** Definitions:**

Adverse event: Any untoward medical occurrence in a clinical investigation subject administered a pharmaceutical product and which does not necessarily have to have a causal relationship with this treatment.

Unexpected adverse drug reaction: An adverse reaction, the nature and severity of which are not consistent with past clinical experience with the administered agent.

Serious adverse reaction: A serious adverse event (experience) or reaction is any untoward medical occurrence that at any dose results in death or is life threatening. The term "life threatening" refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

An unexpected adverse event occurred in a 20-year old female participant who developed numerous skin lesions (macular, 2-3mm in diameter) bilaterally on the hands and forearms, ten days following immunisation. The lesions resolved after several days with no specific treatment. Her general practitioner performed a skin biopsy which revealed non-specific spongiform dermatitis. This reaction had not previously been noted following KLH administration. The Safety Monitoring Board (SMB) and the Therapeutic Goods Administration were notified of this event. The SMB concluded that a causal relationship between KLH immunisation and the skin reaction remained a possibility in the absence of another explanation, but did not recommend any changes to study protocol. The full details of this adverse event are presented in Appendix L.

There were no serious adverse events experienced by AusUVI participants related to immunisation.

7.3 Chapter summary

Linear regression models were used to determine the association between UVR exposure (both acute and cumulative), other potential immunomodulatory variables and measures of immune function (both antigen and non-antigen specific). Data from participants with high inflammatory markers were excluded from further analyses as inflammation at the time of immunisation may have influenced other baseline and subsequent immune outcome measures.

On multiple regression analyses, higher anti-KLH IgG1 response was only significantly associated with younger age and female sex. There was no association noted between the humoral immune response and cumulative UVR exposure or acute UVR exposure on any day or combination of days before or after immunisation. The simple linear association between higher serum 25(OH)D level (≥50nmol/L) and higher anti-KLH IgG1 titres, did not persist after inclusion of other immunomodulatory variables.

KLH-specific DTH responses were significantly reduced in association with higher personal clothing-adjusted UVR exposure on the day prior to immunisation (Day 5), as well as Days 5 – 8 and Days 5 – 9, which spanned the day prior to immunisation to three to four days following.

KLH-specific assays (DTH and anti-KLH IgG1 at 21 days post-immunisation) were positively correlated, consistent with the assumption that both reflected Th1 cell-mediated processes.

A preliminary study using non-antigen specific *ex vivo* assays to enumerate sub-populations of T–helper cells showed evidence of a possible interaction between UVR exposure, immunisation and Th17 cells.

HMW-KLH was very well tolerated as both a subcutaneous immunisation and an intra-dermal skin test. No severe adverse events were reported, and only minor, transient reactions were noted.

The following chapter will discuss the significance of the findings from this study and how they relate to the overall literature.

Acknowledgements

The Canberra-based laboratory work was conducted by Umang Srivastava (separating and storing PBMCs and serum as they arrived) and Kelvin Groot Obbink (lymphocyte assays). In Townsville, PBMC and serum isolation was performed by Kelly Hodgson , Gareth Rutter and Saffron Bryant. Kelly performed the anti-KLH IgG1 assays on AusUVI participants from both sites at the conclusion of the study.

Chapter 8: Discussion and Conclusions

Synopsis

This chapter discusses the results of the AusUVI Study in light of the current literature, and the study's contributions to the overall understanding of UVR-induced immunomodulation. Limitations of the study are discussed and plans for future work outlined.

8.1 Summary of study

The AusUVI Study is the largest study to date to have assessed the immunomodulatory influence of UVR on immune function in humans. The longitudinal observational study design was carefully planned and conducted, reflecting recommendations of relevant immunotoxicology guidelines and literature (22,23). Unique amongst studies in this field, much effort was made to characterise the participant cohort in terms of demographic, behavioural, physical and physiological attributes. This allowed adjustment for potential immunomodulatory variables apart from UVR exposure that might confound or modify the association of primary interest. Contemporaneous measurement of serum 25(OH)D levels allowed for assessment of an independent immunomodulatory effect of UVR exposure.

Immunisation with the novel protein antigen KLH allowed for a safe, reliable and controlled immune challenge. A range of humoral and cell-mediated immune responses was measured with validated and sensitive assays. Importantly, immunisation was delivered subcutaneously, adjacent to the dermis and epidermis that constitute the relevant biological interface for UVR exposure. Prior immunisation studies in this field have used an intra-muscular route (19,209) and at a high antigen load, thus producing a strong systemic immune response potentially masking the immunomodulatory influence of UVR exposure.

Personal solar UVR exposure was directly measured with electronic dosimeters during usual activities of daily living, an important advance on previous studies that either used surrogates of UVR exposure (e.g. season of vaccination), high UVR doses and/or irradiation with a non-solar UVR spectrum. Additionally, recruitment of participants across all seasons, at sites situated over a large latitudinal gradient and of outdoor workers, allowed for a wide range of measured personal UVR exposures. The narrow participant inclusion and exclusion criteria maximised the potential to detect the immunomodulatory signal of UVR exposure, thereby improving the study's internal validity.

The AusUVI Study successfully recruited the target number of participants in the required time-frame. There was a low rate of participant drop-out after initial enrolment, and this was offset by increased overall recruitment. Immunisation with KLH was well tolerated.

Storage of serum and PBMC samples will enable future genotyping studies (e.g. for cytokine single nucleotide polymorphisms) and immune assays.

8.2 Key findings

8.2.1 Pilot study

A group of young, healthy and fair-skinned adult volunteers were randomised to a UVR-exposure group or control group. Five days of sub-erythemal solar-simulated UVR at the site of subsequent sub-cutaneous KLH immunisation resulted in a relative reduction of KLH-specific DTH response of 56% (p=0.10) compared with the non-irradiated control group. Whilst not a statistically significant finding in this small pilot sample (n=21), the result was consistent with previous work that had used solar-simulated UVR protocols and DTH response outcome measures (albeit with different antigens and testing regimes) (15,185). There were no significant differences in anti-KLH antibody responses between UVR-exposed and control group at 21 days post immunisation. The most differentiating humoral assay was the anti-KLH IgG1 sub-class ELISA. The mineral oil adjuvant used to enhance the immune response to sub-unit KLH was associated with a moderate incidence of local, transient adverse skin reactions and was therefore not used in later studies. Conducting this pilot study allowed trialling and refinement of recruitment strategies, study protocols, immunisation and immune function testing procedures which informed the planning of the larger AusUVI Study.

8.2.2 AusUVI Study

Demographic information

The AusUVI Study recruited 222 healthy adult volunteers, aged between 18 and 40 years, over the period July 2010 to August 2011 from study sites in widely spaced latitudinal locations (Canberra (35°15'S) and Townsville (19°15'S)). The participants were predominantly indoor workers recruited from university campuses and hospital staff. Approximately two thirds of participants were female and three-quarters were of northern European ethnicity. Over 90% of participants reported undertaking moderate to high levels of physical activity in the week prior to enrolment. Just over one-third of participants were classified as overweight or obese according to their BMI. A minority of participants were current smokers.

Vitamin D status

Participants' serum 25(OH)D levels showed a marked seasonal variation over the year, with participants enrolled in winter and spring having significantly lower levels than those enrolled in summer or autumn. Canberra participants and those with non-northern European common parental ethnicity, had a significantly lower seasonally-adjusted 25(OH)D level than their

Townsville and northern European counterparts, respectively. These results are in keeping with previous Australian research that found latitude and season explained a significant proportion of 25(OH)D level variation in study populations of non-elderly adults (490). The addition of a crude categorisation of ethnicity to our regression models increased the explanation of variation in 25(OH)D levels to ~ 37%. It is likely that cultural, gender and age differences in diet, clothing and sun exposure behaviour and protection between participants also contributed significantly to the variation in 25(OH)D levels. This is the subject of ongoing research stemming from this dataset.

Personal UVR exposure

In an innovative measure of personal UVR exposure in immunotoxicological research, participants wore electronic UVR dosimeters on their wrist for ten days and completed comprehensive diaries describing time spent outdoors and clothing items worn over the same period. After identifying and cleaning erroneous data, interpolating missing data (based on corresponding diary data and ambient UVR measurements) and adjusting for body surface area covered by clothing, a daily quantification of personal UVR exposure was obtained. These data again showed seasonal variation in personal UVR exposure at both study sites that was more pronounced amongst Canberra participants. Townsville participants had significantly higher clothing-adjusted personal UVR exposures in the winter months compared with Canberra participants; though at other times of the year, UVR exposures were not statistically different between sites. Participants enrolled in winter in Canberra had significantly lower average adjusted UVR exposures than at other times of the year at this site. Average personal UVR per day was significantly higher on weekends compared with weekdays, reflecting increased recreational time on weekends and consistent with previous research (437). Participants of northern European heritage had significantly higher levels of personal UVR exposure compared with those of non-northern European heritage, after adjustment for season of enrolment. A recently published study (491) showed that south Asian women (aged 20 – 55 years) living in the United Kingdom had markedly low serum levels of 25(OH)D throughout the year (~95% with levels < 50nmol/L), with a main contributory factor being low UVB exposure as measured by polysulphone badges (compared with Caucasian controls). Again, this may reflect cultural differences in outdoor behaviours and physical activity and reluctance for tanning in non-white populations (492). As would be expected, outdoor workers had significantly higher personal UVR exposures compared with indoor workers consistent with previous Australian research (421). Similarly, those categorised in the highest physical activity group by the International Physical Activity Questionnaire, had significantly higher personal UVR exposure than those in the moderate category (and non-significantly

higher than the low activity group). This association between increased physical activity and UVR exposure has been highlighted in many studies (371–373).

UVR exposure over the life course (i.e. cumulative exposure) was measured by analysing the microtopography of skin from the dorsum of the hands obtained by silicone cast impressions. As found in other Australian studies (374,440,493), higher skin cast grades were associated with increasing age, male sex, European heritage and casts taken from the right hand. A significantly increased skin cast score was found in participants from Townsville compared with Canberra likely related to higher year-round ambient UVR exposure. This observation was not found in the only other comparable large multi-centre Australian study (440) that examined skin casts, which may be attributable to older age groups being included in that research (49% over the age of 40 years) with changing regions of residence over the life-course tempering the association. No association between increased skin cast score and cigarette smoking (either current or past smoking) was evident, although this may be due to the low smoking rate and younger participant cohort compared with other comparable studies (374,494).

8.2.3 UVR exposure and immune responses

Humoral response

Anti-KLH IgG1 antibodies were assayed at baseline (day of immunisation), 7 and 21 days post-immunisation. Baseline titres were not significantly different from non-immunised control serum and day 7 post-immunisation titres were minimally raised (consistent with the expected two to three week lag in the generation of an IgG antibody response (134)). Therefore, to determine associations with putative immunomodulatory factors, the antibody responses at day 21 post-immunisation were examined.

Multiple linear regression analysis including all relevant immunomodulatory variables showed that only age and sex were significantly associated with anti-KLH IgG1 response. Age was negatively correlated with antibody response (adjusted regression coefficient: -0.016; p=0.001). A meta-analysis of studies assessing the effect of age on immune response to recombinant DNA hepatitis B vaccination found that 'older age' was significantly associated with a higher rate of serological non-response at least one month following the last dose of vaccination (312). This effect was seen also in included studies where 'older age' was defined as > 30 years of age. In previous studies using KLH vaccine, only Smith et al (358) reported age-stratified humoral responses following immunisation. They found that younger participants (aged 20 -35 years) had higher anti-KLH total IgG and IgM antibody titres compared with older participants (aged 65 – 79 years) up to four weeks post-immunisation. Notably, physical activity status interacted with age for anti-KLH IgG1 titres (i.e. decreasing titres in the following order: physically active older participants > physical active younger participants > sedentary younger participants > older sedentary participants). There was no such interaction between age and physical activity for antibody responses in the present study.

Female AusUVI Study participants demonstrated significantly higher anti-KLH IgG1 titres than males (15% increased titre; p<0.001). Previous vaccination studies have shown differences in serological response by sex, varying by vaccine antigen used and population assessed (326). In previous KLH vaccine studies there has been no reporting of the differences in anti-KLH humoral responses by sex (noting that for several studies, only participants of one sex were recruited (e.g. 11,13)).

UVR exposure and anti-KLH IgG1 response

No association was found on simple or multiple regression analysis between measures of acute personal UVR exposure and anti-KLH IgG1 response at 21 days post-immunisation. This was in keeping with the only other comparable controlled human immunisation study by Sleifjjers et

al (19) which had used hepatitis B vaccination to assess the humoral and cell-mediated immunomodulatory effects of UVB irradiation.

In that Dutch study conducted over two consecutive winter seasons, 191 participants (age range 19 – 52 years, median 21 years) were randomised to either a control group or UVB irradiation group. The irradiated group received 1 personal MED of whole body UVB exposure daily for five days. Both groups were vaccinated intramuscularly with a commercially available recombinant DNA hepatitis B (HBsAg) vaccine to the upper arm. The vaccine contained an aluminium hydroxide adjuvant (known to promote a Th2-mediated humoral response). Vaccination in the irradiated group commenced three days after the last UVB dose. Participants were vaccinated at baseline, one month and six months. Vaccine-specific (anti-HBsAg) IgG antibodies were assayed at seven time points - at baseline, and at various times following the first and second vaccinations (up to 60 days post initial vaccination). There was no statistically significant difference in anti-HBsAg IgG responses between UVB exposure groups at any time points following the first and second vaccinations, although there was a non-significant trend towards a higher antibody response in the irradiated group at the later time points.

A follow-up study which stratified participants according to cytokine polymorphism status found that irradiated participants who were homozygous for the minor allelic variant of IL-1β (n=5) had significantly suppressed anti-HBsAg IgG levels compared with non-irradiated homozygous controls (n=4) (328). This polymorphism variant is known to be associated with an approximately four-fold increase in IL-1β production; stimulation by UVB exposure could result in an immunosuppressive cascade (IL-1β → prostaglandins, IL-4 and IL-10), ultimately leading to suppression of antibody response (7). Notably, participants homozygous for the minor allelic variant of IL-1β also demonstrated higher personal MED values compared with those homozygous for the wild-type variant or with heterozygous profiles. These data suggested that the minimal immunosuppressive dose for UVB was not directly associated with the personal MED for UVB (19).

There have been no controlled clinical studies in humans where antibody responses have been correlated with UVR exposure *following* immunisation. However, Wang and colleagues (178) studied the effect of UVR exposure post-immunisation in mice using various T-cell dependent (TD) antigens (e.g. ovalbumin) and T-cell independent (TI) antigens (e.g. trinitrophenol (TNP)–LPS). Mice were exposed to a single dose of UVB seven days following immunisation, whereas control animals were not irradiated. Antibody response to TD antigen-immunisation was significantly suppressed in UVR-exposed animals compared with controls but immunisation responses to TI antigens were not affected. Further experimentation involving adoptive

transfer of CD4+ T cells from irradiated immunised animals to controls demonstrated that regulatory T cells (IL-10 producing; Foxp3-) were responsible for a reduction in antigen-specific antibody response. Caution is needed in extrapolating from such animal studies, but these striking results warrant further examination in human studies.

Delayed-type hypersensitivity response

In the tradition of many other photoimmunology studies, DTH testing was used to assess the influence of UVR on the *in vivo* cell-mediated (Th1) immune response. Here, 21 days following immunisation (sensitisation) with KLH antigen, an intra-dermal dose of antigen was injected at the same site and subsequent wheal reaction observed 48 hours later. After adjustment for postulated immunomodulatory variables, only UVR exposure at various time-points relative to immunisation was significantly associated with the DTH response. Female sex was associated with a higher DTH response compared with males on simple linear testing but not after multiple linear regression adjustment. Previous studies have shown an association between psychological distress and the DTH response to KLH vaccine (335,342), but this was not seen here, despite similarities in study population and design. The volunteer basis for recruitment in the present study may have affected the prevalence of participants with certain psychological characteristics (i.e. distressed individuals may have been less likely to volunteer for the AusUVI Study).

UVR exposure and DTH response

On multiple linear regression modelling, clothing adjusted-UVR exposure on Day 5 of electronic badge monitoring (i.e. the day prior to immunisation) demonstrated a strong association with subsequent DTH response, as did aggregated UVR exposure over Days 5 to 8 and Days 5 to 9. At these times, higher personal UVR exposure was correlated with reduced DTH response, which supports the hypothesis (and previous research) that solar UVR exposure suppresses the antigen-specific cell-mediated immune response to antigen sensitisation.

Several studies have shown evidence that UVR exposure in the days prior to antigen sensitisation is critical in modifying subsequent measures of *in vivo* cell-mediated immunity (191,193,198). For example, in the study by Kelly et al (198), healthy Caucasian participants aged 18 – 35 years of varying skin phototypes (I to IV) received a single solar-simulated UVR (SS-UVR) exposure of varying dose (0 to 3 MED) to a 5 x 5cm area on the buttock. At 24 hours post UVR exposure, participants underwent sensitization with the contact allergen 2,4-dinitrochlorobenzene (DNCB) at the site of irradiation and elicitation of a subsequent contact hypersensitivity (CHS) response occurred 21 days later. The study found that: i) UVR exposure was associated with reduced CHS responses in a dose-dependent manner; ii) skin phototypes I/II were more sensitive to UVR exposure than phototypes III/IV; iii) sub-erythemal doses of SS-

UVR exposure (including down to 0.25 MED) were associated with significant immunosuppression in Type I/II phototype skin; and iv) there was (unexplained) seasonal variation in CHS responses (with summer > winter responses). Notably, the AusUVI Study did not show evidence of varying sensitivity to immunosuppression of DTH response by season or by skin phototype.

Research by Damian et al (190) showed that the effect of UVR exposure on subsequent CHS response varied by timing of exposure and UVR spectrum. Groups of healthy adult volunteers (predominantly female) of varying skin phototypes (I-V) and with confirmed nickel allergy were exposed to one, two, three, four or five days of sub-erythemal SS-UVR and UVA to separate areas of the back. Nickel patches were placed on the irradiated skin and adjacent non-irradiated skin, immediately after the last UVR dose for groups exposed to three, four or five days radiation, and 24 hours after the last UVR dose for groups exposed to one or two days radiation. The degree of immunosuppression was assessed by comparing reflectance meter measurement of allergic erythemal responses from irradiated skin with non-irradiated skin. The investigators found a significant reduction in erythemal response in irradiated skin after just one day of SS-UVR, reaching maximal levels by two days and sustained suppression with up to five days exposure. In contrast, UVA-associated immunosuppression peaked at three days irradiation and subsided with further exposures.

Most other studies that have used an *in vivo* model for assessing UVR-modulated cell-mediated immune response have used a longer protocol for UVR exposure (i.e. over a number of days or weeks), making it difficult to tease out which are the most critical days of UVR exposure prior to sensitisation. Indeed there is no consensus in the literature for the duration or intensity of UVR exposure prior to immune function testing, and consequently many different study designs have been used (183,184).

The interpretation of the finding that UVR exposure spanning the day before sensitisation to two or three days following (Days 5-8; 5-9, respectively) was associated with reduced DTH response requires consideration. This result could indicate a persistent suppressive effect of the UVR exposure prior to sensitisation, or alternatively, that there was an augmentation of the immunosuppressive effect by post-immunisation UVR exposure (presumably via ongoing impairment of antigen presentation and promotion of a regulatory T-cell environment within the draining lymph node). The lack of a statistically significant association between UVR exposure on Days 6-8 or 6-9 and DTH response lends support to the former, rather than latter, explanation. There is minimal previous research to assist in the interpretation of these results and no relevant human data. In a series of murine experiments (495,496), Nghiem and colleagues found that UVA and SS-UVR exposure seven to nine days following sensitisation

with *Candida albicans* led to maximal reduction in elicitation of a subsequent antigen-specific DTH response. Toda et al (181) showed that mice immunised with a tumour-associated T-dependent antigen (ovalbumin) then irradiated (with predominantly UVB) seven days post-immunisation, had significantly reduced anti-tumour immune response compared to non-irradiated controls when subsequently re-challenge with ovalbumin. The authors ascribed this result to post-sensitisation UVR suppression of antigen-specific cytotoxic T cell response, mediated by IL-10 producing regulatory T cells.

UVR exposure and T-helper cell subsets

Enumeration of helper T lymphocyte populations (Th1, Th2, Th17 and Treg) was undertaken by staining for their signature intracellular cytokines (interferon-γ, IL-4, IL-17 and IL-10, respectively) with subsequent fluorescent antibody cell sorting (FACS) by flow cytometry. A further assay to enumerate regulatory T cells (Foxp3+ $CD25^{hi}$ $CD127^{lo}$) was also conducted. These non-antigen specific assays were performed on peripheral blood mononuclear cells (PBMCs) sampled at baseline (pre-immunisation) and 21 days post-immunisation. Due to the time-consuming nature of these assays, samples from only a subset (n = 55) of the total AusUVI participant group were analysed, selected to represent extremes of personal UVR exposure.

No association was found between personal UVR exposure and the proportions of Th1, Th2 or Treg cells isolated pre- or post-immunisation. Prior research suggests that the effect of UVR exposure on immune responses is an antigen-specific one, so that the findings from the present study are not inconsistent with the bulk of existing literature. However, an interesting recent study (497) suggests that chronic, but not acute, UVR exposure can affect the proportions of T helper cell populations in a non-antigen specific manner. Here, investigators exposed mice to either acute UVR (four consecutive days of 0.5 MED) or chronic UVR (20 days of 0.25 MED), then examined for evidence of local and systemic immunomodulation. The UVR emission spectrum ranged from 280–370 nm (peak 302nm) and included 20–30% UVA. For both UVR groups, there was evidence of local skin changes, with epidermal hyperplasia and reduced epidermal T-cell density. Systemically, acute UVR exposure led to an increase in inguinal lymph node (ILN) T-cell responsiveness to a non-specific T-cell mitogen (ConA), whereas chronic UVR led to a suppressed response. Furthermore, chronic UVR exposure was associated with changes in IL-4, IL-10 and IFN-γ production in ILN and splenic T-cells.

Notable findings in the AusUVI Study, however, were the apparent associations between Th17 cells, UVR exposure and immunisation status. Pre-immunisation, participants with low UVR exposure had a higher Th17 cell percentage than participants with high UVR exposure, approaching statistical significance (p=0.076). Though speculative, this finding would be

consistent with experimental findings showing high UVR exposure suppressed the development of Th17-mediated processes such as experimental autoimmune encephalomyelitis (EAE) (a demyelinating condition of the central nervous system) in mice (498).

Additionally, there was a significant difference in Th17 cell percentage between pre- and post-immunisation when comparing low with high UVR exposure (-0.39% *vs.* 0.31%; p=0.004). The main contributor to this was a fall in Th17 cell percentage post-immunisation in the low UVR group (1.0% vs. 0.71%; p=0.033); however, there was also a similar magnitude but non-significant rise in Th17 cell percentage post-vaccine in the high UVR group (0.61% vs. 0.90%; p=0.075). Given that there were no significant differences in Th17 cell percentage between time-points when UVR exposure was not considered, an interaction involving immunisation and UVR exposure is possible.

The reason for the apparent down-regulation of Th17 cells post-immunisation in the low UVR group and the up-regulation of Th17 cells in the high UVR group is not clear. One potential explanation of the latter phenomenon involves the interaction of UVR-induced breakdown products, the aryl hydrocarbon receptor (AHR) - an important ubiquitous transcription factor, and antigen stimulation. UVR exposure leads to the breakdown of tryptophan (a keratinocyte chromophore), forming intracellular 6-formylindolo[3,2-b]carbazole (FICZ) that acts as a high affinity ligand for the AHR (499). The AHR is a ligand-activated transcription factor expressed in the cytoplasm of many cell types and has been implicated in an increasing number of immunomodulatory functions (500). It has been recently shown that FICZ-mediated AHR activation is an important UVB-induced stress response in epidermal keratinocytes, leading to the expression of numerous genes (499). In mouse and human models, Th17 cells have recently been shown to exhibit high levels of AHR; stimulation by FICZ leads to proliferation of Th17 cells and increased expression of cytokines IL-17 (A and F) and IL-22 (501,502). Th17 cells have been recently implicated in the pathogenesis of an increasing number of autoimmune diseases (503–505). In a landmark paper, Veldhoen et al (504) demonstrated that EAE could be induced experimentally in mice by immunising with myelin oligodendrocyte peptide 35-55 (MOG_{35-55}). In mice also injected with FICZ at the time of MOG_{35-55} immunisation, not only were the numbers of IL-17 staining T-helper cells in spinal cord tissue higher than in control mice (e.g. AHR deficient mice or mice not given FICZ), but progression to disease onset was also significantly faster. This study supports the findings of the AusUVI Study, suggesting that UVR-induced formation of AHR ligands (e.g. FICZ) in the setting of antigen processing (i.e. immunisation) promotes IL-17 production by T helper cells.

The above mechanisms are speculative and cannot be confirmed by the data presented in this thesis. A research design that would better ascertain whether there is an interaction between UVR exposure and immunisation in promoting Th17 cell expression would involve unimmunised and immunised groups exposed to differential levels of UVR (over the duration of the study). In this way, a 2x2 table could be generated, where only those immunised *and* exposed to UVR would be expected to have a change of IL-17 expression over time (see Table 8.1)

Table 8.1: 2 x 2 Table resulting from an experimental design that demonstrated the interaction between UVR exposure and immunisation on Th17 lymphocyte populations

	No UVR	**UVR**
Not Immunised	No change over time	No change over time
Immunised	No change over time	**Change over time**

8.2.4 Vitamin D status and vaccine-associated immune responses

Previous research has demonstrated that calcitriol has a significant immunomodulatory role (largely regulatory) within the adaptive immune system. Low vitamin D status has also been epidemiologically linked to increased rates of various Th1-mediated autoimmune disorders (See Section 2.4). An important research question for the present study, therefore, was to assess how 25(OH)D status related to vaccine-associated immune responses. On simple linear testing, participants with serum 25(OH)D levels ≥50nmol/L had a significantly higher anti-KLH IgG1 titre than participants with 25(OH)D levels <50nmol/L (0.67 *vs.* 0.57; p=0.049). However, the association did not persist when this variable was included (either as a continuous or dichotomous measure) in a multiple regression model. No association was found between serum 25(OH)D level and anti-KLH DTH response.

Studies assessing the relationship between vitamin D status and vaccine-associated antibody response have shown mixed results. In a recent study evaluating responses to T-cell dependent vaccines in post-splenectomy patients, 25(OH)D levels did not differ between 'responders' and 'hypo-responders' to pneumococcal and meningococcal conjugate vaccines (506). However, there was a weak (but significant) negative correlation between 25(OH)D level and *Haemophilus influenzae* Type B antibody titres post-immunisation. Other studies have mainly examined immunosuppressed populations where low 25(OH)D levels have been associated with poor vaccine-specific antibody responses. These include hepatitis B vaccine response in patients with chronic renal failure (507) and influenza vaccine responses in

patients with prostate cancer (508). Low 25(OH)D levels in these groups may also reflect co-existing poor nutrition, physical condition and psychological state which may also lead to suboptimal vaccine response.

The use of serum 25(OH)D levels as a surrogate for the activity of calcitriol in peripheral tissues may have contributed to the inconsistent findings of laboratory, clinical and epidemiological studies in this area. Clinical and epidemiological studies use serum 25(OH)D levels - a stable measure of vitamin D stores (509). However, given the short circulating half-life (6-8 hours) and tight regulation of calcitriol production (by parathyroid hormone, phosphate levels and other molecules independent of 25(OH)D), a linear relationship between serum 25(OH)D and calcitriol levels does not occur (2). Furthermore, extra-renal synthesis of calcitriol by immune cells is likely to play a significant role in immunomodulation via paracrine and autocrine mechanisms (107). Therefore, serum 25(OH)D levels are unlikely to be useful as a calcitriol surrogate where the immunomodulatory effect on vaccine response is postulated to occur cutaneously or within the draining lymph node.

Relatedly, research has been performed assessing the local effect of exogenous calcitriol on immune responses. Animal studies have demonstrated that co-administration of calcitriol with various vaccines (including herpes simplex virus, hepatitis B surface antigen, tetanus toxoid and influenza virus) enhanced the mucosal +/- systemic antibody response to immunisation (510–513). However, a randomised, double-blind clinical trial in humans that evaluated the efficacy of calcitriol co-administered with influenza vaccine, did not reveal similar evidence of local calcitriol-induced immunomodulatory activity (514).

Finally, despite the initial (and ongoing) enthusiasm for vitamin D supplementation for prevention of autoimmune disorders such as multiple sclerosis, the evidence thus far of its effectiveness is weak and conflicting (236). This raises the question of whether low serum 25(OH)D levels simply reflect low UVR exposure, and that it is this latter factor which is most contributory to the development of immune-mediated disease (236). Given that for the majority of the population, vitamin D stores depend on UVR exposure, it is methodologically challenging to identify the independence of effects in epidemiological studies (although it has been attempted (515)).

The results of the AusUVI Study support the hypothesis of a direct UVR suppressive effect on *in vivo* antigen-specific cell-mediated response independent of serum 25(OH)D levels.

8.2.5 Evidence for photoadaptation or photoprotection

As discussed in Section 2.3.9, there is minimal evidence in the literature to suggest that photo-sensitive immune parameters "photo-adapt" (or "photo-protect") with repeated UVR exposure

of skin (reviewed in (8)). Most of the studies reviewed however, made their conclusions based on just several weeks (or months) of repeated UVR exposures, delivered to small areas of skin and/or using non-solar UVR spectrum.

The AusUVI Study offered a unique opportunity to assess whether photoadaptation occurred using measures of acute and cumulative UVR exposure in relation to both DTH and humoral responses. Table 8.2 shows the theoretical changes expected in the setting of no photoadaptation (DTH responses: "A") or photoadaptation (DTH responses: "B").

Table 8.2: Postulated changes in DTH response in the setting of photoadaptation

Acute UVR exposure / Cumulative UVR Exposure	Low	High
Low	**A:** Normal / **B:** Normal	**A:** Decreased / **B:** Decreased
High	**A:** Decreased / **B:** Normal	**A:** Decreased / **B:** Normal

Key - A: No photoadaptation; **B:** Photoadaptation apparent

In the AusUVI Study, skin casts were used as a measure of cumulative UVR exposure over the life course. Participants with higher skin cast scores (≥Grade 4) demonstrated lower DTH responses than lower grade skin grades (≤3.5), however, this was not statistically significant (6.6mm vs. 7.5mm; p=0.20). When adjusted for skin cast grade in the multiple variable analysis, the association between acute UVR exposure (i.e. on Day 5) and DTH response remained significant suggesting that cumulative UVR exposure did not modify the immunomodulatory influence of acute UVR. There was no association between cumulative UVR exposure and anti-KLH IgG1 response. Taken together, the data presented do not support the hypothesis that immune processes of the skin 'photo-adapt' to the immunosuppressive properties of UVR.

8.3 Clinical implications of the AusUVI Study

KLH fulfilled many of the attributes of an ideal vaccine antigen for use in an immunotoxicological study (see Section 3.2.2.3.2). However, unlike the antibody response to a commercial vaccine (e.g. hepatitis B, measles, rubella) where a known minimum titre (threshold) corresponds to likely clinical protection from disease based on longitudinal research (289), there is no level of anti-KLH vaccine response that corresponds with clinical outcome. This is similar for anti-KLH DTH response, in comparison to the established 'cut-offs' for the Mantoux test for example, that gives information regarding prior exposure to tuberculosis (or BCG vaccination) (291). In the AusUVI Study, therefore, anti-KLH humoral and DTH responses were analysed as continuous variables with no attempt to classify as "high" or "low", "protected" or "not-protected", or some other arbitrary grouping.

The influence of UVR on anti-KLH responses following immunisation can be viewed as representative of the effect of UVR on any T-dependent antigen that the skin may encounter. In this way, response to KLH immunisation acts as a 'sentinel' marker of potential immunomodulation by an environmental exposure. Thus the finding of reduced KLH-specific DTH response associated with higher natural solar UVR exposure on the day prior to immunisation, could also indicate reduced host immune response to skin infection where cell-mediated (T-helper cell) processes are important (e.g. secondary to *Candida sp.* (516), herpes simplex virus (517)). Reduced DTH responses in other settings have been shown to be associated with adverse clinical outcomes. In one large study, surgical trauma and intensive-care patients who exhibited anergic (absent or small) DTH responses to ubiquitous antigens pre-operatively (e.g. Trychophyton, tuberculin, *Candida*), had significantly higher sepsis-related mortality following surgery (141). This evidence could be interpreted as showing impaired immune processes (i.e. reduced DTH responses to ubiquitous antigens) increase the susceptibility of the host to subsequent infective complications.

8.4 Epidemiological research and study design considerations

The potential for adverse effects of UVR exposure on the response to vaccines has obvious public health implications (143). As outlined in Section 2.3.8, a number of observational studies have suggested that populations vaccinated in high ambient UVR environments (i.e. either living at low latitudes or vaccinated in the summer months) have lower vaccine-related antibody levels compared with those in low ambient UVR environments (7). Whilst suggestive of an immunosuppressive role for UVR, these types of studies succumb to the ecological fallacy as personal UVR exposures were not taken into account. Similarly, the contributions of other potential confounding factors (e.g. age, sex, nutrition, etc.) were not considered, thereby possibly skewing the association between UVR exposure and immune response. Controlled

clinical studies, such as that conducted by Sleijffers et al (19) where participants were randomised to a UVR exposure group and monitored prospectively for hepatitis B-specific antibody responses, were a significant advance on the afore-mentioned observational studies. Randomisation of study participants nullified the influence of potential confounding factors, although the success of randomisation was uncertain as stratification by immunomodulatory variables was not reported.

The AusUVI Study design represented an improvement on previous studies for a number of reasons summarised in Table 8.2. The conduct of this study should inform future research in this area, particularly in relation to vaccine antigen choice and mode of administration, prospective longitudinal design, direct measurement of personal UVR exposure and comprehensive measurement of potential immunomodulatory variables. The potential for drawing incorrect inferences when confounding factors are not considered was illustrated by the significant simple association noted between serum 25(OH)D level (as a dichotomous variable) and anti-KLH IgG1 vaccine response – the association was no longer apparent when other relevant factors were adjusted for.

The limitations of the AusUVI Study are set out in the next section.

Table 8.3: Immunotoxicological study design issues: The AusUVI Study compared with previous research where vaccines were used

Design issue	Previous research	Disadvantage	AusUVI Study	Advantage
Measuring individual UVR exposure	• Surrogate markers of UVR exposure (e.g. 66,67)	• Ecological fallacy – can't assume all individuals living in a high or low ambient UVR environment experience high or low personal exposure	• Direct UVR measures via electronic dosimeters & diaries • Skin casts – cumulative UVR	• Quantify personal UVR exposure, with day-to-day variation, adjusted for clothing • Can assess seasonal change • Life-time UVR measurement with skin casts • Missing data replaced with interpolated values
Using biologically relevant UVR spectrum and dose	• Artificial UVR sources (i.e. UV lamps) (19) • Low ambient UVR environment (209)	• Non-solar UVR spectrum or only an approximation of solar UVR • UVR doses may not be relevant to day-to-day living • +/- Only small areas of skin exposed • Low range of personal UVR doses	• Solar UVR exposure	• Measurement of biologically relevant, solar UVR • At levels relevant to day-to-day living • Wide range of personal UVR doses
Immunisation dose, route and type	• Commercial vaccines (e.g. Hepatitis B) (209,518) • Childhood vaccinations (e.g. measles, polio, rubella) (17,207,519,520)	• Large dose (high antigen load) – aims to generate a very large antibody response • Use of adjuvant (e.g. alum) which can skew immune response to Th2 processes (19) • Multiple vaccinations required (e.g. hepatitis B) • Non-cutaneous route of administration (e.g. IM) • May not be primary immunisation or TD antigen	• KLH antigen	• TD antigen • Reliable immune response to single dose • Low dose – doesn't "swamp" the immune system with antigen • No adjuvant required • Skin administration – at site of UVR interaction
Accounting for potential confounding variables	• Minimal or no attempt to measure potential confounding or effect modifying variables	• Any association found between UVR exposure and immune outcome may be misleading due to non-consideration of other variables	• Careful measurement of potential immunomodulatory variables	• Allows for adjustment for other immunomodulatory variables • ↑ accuracy of the estimate of association between UVR & immune outcome
Measurement of immune outcome	• Non-comprehensive evaluation of immune outcome measures (e.g. 66)	• Narrow set of immune tests limits analysis of the association between UVR and immune response	• Measurement of a range of humoral and cell-mediated immune responses	• Able to quantify humoral and cell-mediated immune response to immunisation • *Ex vivo* and *in vivo* testing, including functional assays (i.e. DTH)

TD = T-cell dependent

8.5 Limitations

Despite the robustness of study design, and improvements on previous human research in the area of UVR-induced immunotoxicology, there were a number of limitations identified.

A strength but also limitation of the AusUVI Study was the set of strict inclusion and exclusion criteria for participant recruitment. They were designed to increase the internal validity of the study (i.e. the chance of detecting the signal of UVR-induced immunomodulation) but at the possible expense of generalisability to the wider population. Indeed, excluded individuals may have been more susceptible to UVR-induced immunomodulation. These groups might include children, elderly, malnourished and immunosuppressed individuals (e.g. organ transplant recipients appear to be highly sensitive to UVR exposure with documented increased rates of skin cancers and mucocutaneous infections).

Some tools used for measuring potential immunomodulatory variables were likely not reliable enough to accurately and discriminately measure the variable of interest. The International Physical Activity Questionnaire (IPAQ-SF) has been used extensively as a measure of physical activity in epidemiological studies, but a recent systematic review concluded that the IPAQ-SF tended to overestimate physical activity and/or not correlate with objective measures of physical activity (e.g. pedometer, accelerometer) to an acceptable standard (446). The use of a pedometer may have been a better choice for the AusUVI Study.

Body mass index (BMI) and albumin were used as crude measures of nutritional status of participants. Though convenient to measure and widely used, BMI does not discriminate between mass derived from lean muscle compared with fat. There are also caveats related to gender and leptin levels (521). However, other anthropometric measures such as waist-to-hip ratio and skinfold thickness also have limitations and more sophisticated measures (e.g. electrical bio- impedance, DXA scans) have cost and/or logistic implications. Additionally, although most measures of body fat quantify total body fat percentage, it may be the *distribution* of fat which is more important for abnormal physiological processes and disease (522).

Immune responses were measured out to 21 days from immunisation, but this length of follow-up may not have demonstrated the full extent of UVR-induced immunomodulation. However, longer periods of observation would have been logistically challenging with higher number of study visits and blood testing potentially leading to reduced numbers of volunteers or more participants dropping out of the study. Furthermore, personal UVR exposure was measured directly (by electronic dosimeter) only for five days either side of KLH immunisation; thus the study of UVR-induced immunomodulation was restricted to the antigen sensitisation

phase of the immune response. Comment therefore cannot be made on the influence of UVR exposure on the elicitation phase of the DTH response, for example. Nevertheless, extending the duration of wearing of the electronic dosimeter and/or completing of the sun diary would have been an additional participant burden with the likely consequence being a fall-off in the reliability of data recording.

Although sunscreen worn during the days of direct UVR monitoring was recorded on the sun and physical activity diaries, this information has yet to be integrated into the personal UVR dataset. Inclusion of this additional information would clearly influence participants' personal received UVR dose and possibly the association with immune outcomes. However, adjusting for the UVR protection afforded by sunscreen is problematic. Sunscreen protection varies by thickness and distribution of application, sun protection factor and decay in effectiveness over time (e.g. towelling after swimming) (30). There is currently no validated method for correcting UVR exposure for sunscreen use based on questionnaire-derived data.

Personal UVR exposure, as measured by the electronic UVR badge, was adjusted for the body surface area covered by clothing based on information obtained in the sun and activity diary. This adjustment was crude at best as it assumed that all clothing types provided complete UVR protection regardless of fabric type, weave, thickness and colour – variables known to affect the UVR protective effectiveness of clothing (62). It also depended on accurate completion of the diary – unreliable details regarding clothing worn could lead to significant inaccuracies in personal UVR dose determinations.

The clothing-adjusted personal UVR dose also did not take into account the different planes at which UVR arrives at the skin surface. The UVR electronic dosimeter was calibrated in a horizontal position, whereas anatomic regions positioned in a vertical plane (e.g. the wrist, trunk) can receive higher levels of radiation (523). Again, the personal UVR dose measured by the dosimeter and adjusted for clothing should be taken only as an approximation of the actual dose. However, as all participants wore the badge in the same position, there should not be any bias in the way the measurements were undertaken.

To maximise the chance of detecting an immunomodulatory influence from UVR exposure, it was important to immunise at an anatomic site that was reliably exposed to sunlight. To this end, the anterior aspect of the forearm was chosen with the additional benefits of ease of access and ample subcutaneous tissue suitable for injection. However, this site was often covered by long-sleeved clothes particularly in the colder months. Therefore, for a (likely sizable) proportion of the participant cohort, the immunomodulatory effect of UVR was secondary to a systemic rather than a local effect (i.e. UVR delivered remote to the site of immunisation). Given that the local UVR-induced immunomodulatory effects are stronger

than systemic effects, the likely consequence for this study is an underestimation of the effects of UVR exposure on vaccine response.

Overcoming this limitation in future studies could be achieved by insisting that clothing not cover the vaccinated area for a designated period before and after immunisation (challenging for the forearm) or instead, choosing a different site for immunisation. Possible sun-exposed immunisation sites include the dorsum of the hand or at the wrist - however, minimal subcutaneous tissue at these sites and density of important structures (e.g. nerves) would make injection difficult.

Due to limitations in resources, a full array of immune outcome measures was not possible in time for reporting in this thesis. Anti-KLH IgG1 was chosen as the antibody to be first assayed as it showed the greatest response in the pilot study (additionally noting that IgG1 is the predominant IgG sub-class and the primary antibody product of Th1 cell-mediated processes in humans. However, antigen-specific assays of IgGfc (full complement of sub-IgG sub-classes) and IgG2 would be additionally informative particularly in relation to UVR-induced promotion of Th2 processes.

Antigen-specific *ex vivo* lymphocyte assays were not conducted for this study but would complement the DTH response assays. Nevertheless, antigen-specific cellular assays are problematic due to the very small frequencies of circulating memory T-cells and also the lack of MHC-complexes to present antigen to T cells in the *ex vivo* setting.

As part of the study protocol, paracetamol was recommended for participants to take if they experienced fever or discomfort post-immunisation or skin test. Paracetamol administration has been associated with reduced antibody response post-immunisation in children (524). Information regarding paracetamol ingestion during the study was not collected. However, there was no reason to suspect that paracetamol ingestion was more prevalent in any particular UVR exposure group or associated with another immunomodulatory variable.

8.6 Future research

Planned or underway

In response to some of the limitations noted above, a method for integrating the detailed records of participant's sunscreen coverage during the period of UVR exposure measurement with the clothing-adjusted electronic dosimeter data will be developed. In addition, further laboratory work is planned using stored serum and lymphocyte samples. Antigen-specific antibody testing (anti-KLH IgGfc and IgG2) will be performed. To build on the preliminary results of the lymphocyte assays already performed, preparatory work has commenced on developing antigen-specific cellular assays (i.e. incubating PBMCs with KLH for various periods of time, and then quantifying the ratios of the various CD3+ CD4+ T lymphocyte populations present).

Previous work has shown that the immunomodulatory effects of UVR exposure are greater in the presence of specific cytokine gene polymorphisms. This will be explored further within the AusUVI Study, using a commercial gene array kit or chip and residual PBMCs as the source of participant DNA. Along the lines of the work by Sleiffjers et al (328), a re-analysis of the relationship between personal UVR exposure and the immune response to immunisation, stratified by specific genotypic sub-populations, will then be undertaken.

A study is currently underway using the AusUVI Study database to better understand the variability of serum 25(OH)D amongst different ethnic populations. The database offers a unique opportunity to assess this important question as it includes information on physical activity, clothing coverage, skin complexion, sun protection and vitamin D supplementation as well as personal UVR exposure.

Potential

Given the results of the lymphocyte assays performed for this study where changes in Th17 cell populations have been observed related to UVR exposure and immunisation, further work is warranted to confirm and extend these findings. A study design to assess this question has already been outlined in Section 8.3.3.5 where the same or a different vaccine (e.g. influenza) could be used.

UVR-induced immunomodulation is likely to be most influential to the immune cells and processes of the epidermis and dermis. This may be relevant for Nanopatch^{TM} technology, a promising vaccine delivery system under development that uses micro-projection arrays (>20,000/cm^2) to topically deliver vaccine antigen and adjuvant to the skin's immune system (525). Determining the extent to which UVR modulates the Nanopatch^{TM} response would be of considerable interest.

Conducting studies involving potentially susceptible populations (e.g. elderly, infants, immunosuppressed) that may be more sensitive to UVR-induced immunomodulation may provide valuable information. For example, research into the effect of UVR on the immune response to routine childhood immunisation (e.g. measles, BCG) in a high ambient UVR environment (e.g. indigenous communities of northern Australia; peri-equatorial regions of the developing world) should be given strong consideration. Given that vaccination programs are already established in most parts of the developing world and reliance on vaccine effectiveness is paramount, particularly in these less affluent settings, this constitutes an excellent opportunity to extend UVR-immunotoxicology research in a context where the results are clinically translatable.

8.7 Conclusions

Since Kripke's landmark experiments in the 1970s, the emerging field of photoimmunology has demonstrated that UVR exposure is an important environmental immunotoxin to animal and human immune systems. However, much of the experimental and observational research conducted in this area has not allowed for extension of conclusions to the broader population under "real world" conditions due to limitations in the spectrum or dose of UVR studied; measurement and / or control of relevant immunomodulatory variables; and / or immune function tests used. The AusUVI Study was carefully designed and conducted using immunotoxicology research principles to avoid or minimise these potential pitfalls.

Ultimately, the AusUVI Study set out to answer the following question – "Does solar UVR exposure, at levels relevant to normal daily activity, influence the immune response to primary immunisation in humans?" At the completion of the study, the following conclusions are made:

- Acute solar UVR exposure at levels relevant to day-to-day activity suppresses *in vivo* measures of cell-mediated immune response to immunisation with a T-cell dependent antigen, with UVR exposure on the day prior to immunisation most important.
- Acute solar UVR exposure, in conjunction with immunisation, modulates the expression of T-helper lymphocytes expressing IL-17, a cytokine known to be important in the development of inflammation and autoimmune diseases.
- Acute solar UVR exposure at the time of immunisation does not affect the production of vaccine-specific IgG1 antibody.
- UVR exposure over the life course does not influence vaccine-specific cell-mediated or humoral immune responses. There was no evidence to support the contention that long-term UVR exposure leads to photo-adaptation to the immunomodulatory effects of acute UVR exposure.
- There was no evidence that vitamin D status influenced antigen-specific cell-mediated or humoral immune responses.

The clinical significance of the observed processes is unclear, although any degree of UVR-induced immunomodulation could be expected to alter the susceptibility of a population to common infections, cancers and/or to the pathogenesis of autoimmune disease. This research contributes to our understanding of the optimal range of UVR exposure that individuals and populations should have to balance the positive and negative health outcomes of sun exposure.

Further analyses will be conducted on the data thus far obtained in the AusUVI Study, and experimental work is planned to strengthen or extend the findings discussed. This study adds to the growing literature in this important area and, hopefully, lessons learnt from this research will be applied to future photoimmunology studies.

Seeking to better understand the influence of ubiquitous environmental exposures on human physiological functions should remain an important scientific research priority. This is already apparent for populations subject to extremes of exposure, but will take on increased import in the context of global climate and environmental change.

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Appendix A: Anti-KLH IgG ELISA optimisation and protocol

ELISA optimisation strategies

The protocol for the ELISA assay described below was developed after consideration of published protocols that had successfully quantified the anti-IgG response to KLH immunisation in humans (342, 387). Much effort was undertaken by laboratory researchers following the pilot study (Canberra laboratory) and the main AusUVI Study (Townsville laboratory) to optimise the assay output. Differences in assay output (e.g. reduced labelled-antibody signal or increased background 'noise') were noted between laboratories that may reflect varying reagents, characteristics of antibodies, technique and equipment.

The protocol described was finalised following much experimentation including varying the concentration of initial serum concentration (e.g. 1/10 to 1/100) (Step 7.ii), varying concentration of biotin-labelled secondary antibody (Step 10), changing the time to reading well absorbance (e.g. 15 – 30 minutes) (Step 18), and/or use of stopping agent (Step 19). The main difference between the pilot study and AusUVI Study assays was that a stopping agent was not used in the latter protocol.

Anti-KLH IgG ELISA protocol

Stage One – Coat with KLH

1. 96 well NUNC plates coated with 1ug of KLH (Hemocyanin from Keyhole Limpets, Sigma No. H 7017) per 100ul carbonate coating buffer
   i. pipette 100ul using 200tips
2. Refrigerate overnight in cold room (should always leave overnight)

Stage Two - Block

3. Wash plates x4 with PBS-Tween 20 (Phosphate Buffered Saline with Tween 20, Sigma No. P3563)
4. Block with 200ul of 1% skim milk
5. Incubate at room temp 2 hours or 45 mins in 37°C

Stage Three – Serum (Primary Antibody)

6. Wash plates x4 with PBS-Tween 20
7. Undertake a half log dilution
   i. Pipette 100ul 1% skim milk into all wells
   ii. Pipette 2ul of serum into the first row of wells (i.e. concentration 1/50)

iii. Mix and Transfer 46.2ul of solution from 1st row to 2nd row

iv. Discard pipette tips

v. Mix and Transfer 46.2ul of solution from 2nd row to 3rd row etc.

8. Incubate at 37°C for 45mins or leave at room temperature for 2 hours or incubate overnight (preferred)

Stage Four – Secondary Biotin-labelled Antibody

9. Wash plates x4 with PBS-Tween 20

10. IgGFc (Sigma, Monoclonal Anti-human IgGFc Biotin Conjugate, No. B3773), Dilution 1/50,000 in skim milk; IgG1 (Sigma, Monoclonal Anti-human IgG1 Biotin Conjugate, No. B6775), Dilution 1/2000 in skim milk; IgG2 (Sigma, Monoclonal Anti-human IgG2 Biotin Conjugate, No. B3398), Dilution 1/25,000 in skim milk

11. Pipette 100µl of diluted antibody in each well

12. Incubate at 37°C for 45mins or leave at room temp for 2 hours

Stage Five – Immuno-enzyme to bind to Biotin-labelled antibody

13. Wash plates x4 with PBS-Tween 20

14. Add 100ul of 200:1 skim milk:Avidin Peroxidise (Sigma, No.A3151, Reconstituted with 1000ul PBS)

15. Incubate at 37°C for 45mins or leave at room temperature 2 hours

Stage Six – Detecting labelled antibody and Reading Results

16. Wash plates x4 with PBS-Tween 20

17. Add 100ul ABTS (3,3,5,5 Tetramethylbenzidine Liquid substrate system, Sigma No. A3219) solution to each well

18. Leave for 15 minutes

19. Add 100ul 1M phosphoric acid in distilled water to each well *

20. Read on spectrophotometer plate reader (at wavelength 450nm)

* This step was performed in the Canberra laboratory for the pilot study assays but not for the main AusUVI Study assays conducted in Townsville (see below)

Appendix B: AusUVI Study Promotions

Poster

THE AUSTRALIAN ULTRAVIOLET RADIATION & IMMUNITY STUDY

Needs your body!

Does sun exposure affect your immune system?

Do you get too much sun? Or not enough?

What are ultraviolet rays doing to your skin?

If you are healthy and aged between 18 and 40, why not get

sure affect your
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much sun?
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FURTHER INFORMATION

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JAMES COOK UNIVERSITY
AUSTRALIA

THE AUSTRA
ULTRAVIOLE
RADIATION
& IMMUNIT

Needs your bod

What is the sun

:eded?

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is important in helping
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ticipating?

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itive digital badges worn

a harmless protein (KLH)
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t participants, aged 18 to

What are the benefits of participating?

We will provide feedback on your measurements taken for the study, including blood pressure, random blood glucose level and useful information regarding your natural skin pigmentation.

At your request, we can also inform you of your blood test results (including full blood count, vitamin D level, kidney and liver function) and skin cast result (which reflects the cumulative degree of solar skin damage). A study doctor will help interpret the test results for you, if required.

Information collected for this study will help researchers better understand the effect of UV radiation on the human immune system, and assist in the development of appropriate 'sun smart' public health messages for the community.

Are there any ris

Vaccination with KLH
no reports of severe
humans, despite exten
40 years.

Participation will inv
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anaesthetic ointment
to reduce the level of

Reimbursements

Any reasonable travel
in participating in this

In addition, at the end
you will receive a $50
your valuable time.

Who is conducti

This research is bein
The Australian Natio
and James Cook Univ
experienced in studyin

JAMES COOK UNIVERSITY AUSTRALIA

THE AUSTRALIAN NATIONAL UNIVERSITY

INFORMATION SHEET

TITLE: **The Australian Ultraviolet Radiation and Immunity Study**

INVESTIGATORS: **Dr Ashwin Swaminathan**
Dr Robyn Lucas
Dr Simone Harrison
A/Prof Natkunam Ketheesan
A/Prof Matthew Cook
Professor Michael Kimlin
Professor Tony McMichael

You are invited to take part in a **research study**. The following information is provided to help you make a decision about whether or not you would like to participate in the study.

This consent form may contain words that you do not understand. **Please read the information carefully** and ask the study officer to explain anything you do not understand. You may take home a copy of this consent form to think about or talk about with family or friends before making your decision.

This is a **research study** and you **do not** have to be involved.

Once you understand what the study is about and if you agree to take part, you will be asked to sign the Consent Form. Signing the Consent Form indicates that you understand the information and that you give your consent to participate in the research study.

1. Background and Purpose of this Study

Scientific studies show that sun exposure may suppress human immune function. This could affect how we fight infection, respond to vaccination and defend against skin cancer, amongst other things.

The purpose of our research is to find out whether exposure to ultraviolet (UV) radiation received via sunlight and in doses relevant to activities of normal daily living, is sufficient to suppress the human immune system. We do not know the answer to this question currently, but it is clearly important in helping to understand the effects of sun exposure on human health. This is particularly relevant for people living in very sunny countries such as Australia.

For this study, we will measure personal UV exposure through use of special UV absorbent badges attached to a wrist band that is worn daily for 10 days. We will also measure sun exposure with the use of activity diaries. We will use vaccination with a harmless protein (KLH) as a way of generating an immune response that can be accurately and reliably measured.

2. Study Procedures

We aim to recruit **healthy adult participants**. At the time of recruitment, you will be asked some simple screening questions to ensure that you do not have any conditions or are taking any medications that would make you unsuitable for this study.

The following table details the procedures and requirements of each visit.

JAMES COOK UNIVERSITY AUSTRALIA

ANU THE AUSTRALIAN NATIONAL UNIVERSITY

STUDY TIMELINE

Pre-Study	After you have expressed interest in participating in the study, a convenient time will be organised for you to attend for the first visit.
Day 1	• Questionnaires: On general health, medications, sun exposure history and physical fitness • Brief Physical exam: Height, Weight; Skin, Eye and Hair Colour • Skin cast: a silicone rubber impression of a small part of the back of your hand to estimate chronic sun damage • Assessment of degree of tanning • Instruction on use of UV sensing digital badge and sun diary • Time: **Approx 50 – 60 mins**
Day 3 - 7	• Wear UV sensing badge on wrist band daily • Use Sun Diary to document sun exposure and use of sun protection
Day 8	• Blood test: this will be sent for immune function tests, Vitamin D level, full blood count and blood glucose level. • Vaccination - a sub-cutaneous injection of KLH to the forearm • Questionnaire: On general psychological well-being (as it also can affect immune function) • Time: **40 minutes**
Day 8 - 12	• Continue to wear wrist band daily; and fill in Sun Diary
Day 15	• Blood Test: for immune function testing • Time: **5 minutes**
Day 29	• Blood test – for immune function testing • Skin test – involves an injection of KLH with a very fine needle just under the skin of the forearm; May require shaving of a small area of skin • Time: **15 minutes**
Day 31	• Read result of Skin tests • Feed back of interim results • Thank-you gift • Time **10 minutes**

3. Possible Benefits

Although you will not receive any direct medical benefit from participating in this study, there are a number of other potential indirect benefits. We will feedback information regarding your **blood pressure, resting heart rate, random blood glucose level** and useful information about your **natural skin pigmentation**.

We can also tell you the **results of your blood tests** (including **full blood count, liver and kidney function).** At the end of the overall study, we can inform you of your **vitamin D level and skin cast result** (which reflects the cumulative level of solar skin damage).

A study doctor can counsel you with regard to the interpretation of these results.

The information collected from this study will also help researchers understand the effect of UVR on the human immune system, and therefore assist in the development of appropriate "sun smart" public health messages for our community.

4. Risks and Discomforts

Vaccination with the protein "Keyhole Limpet Haemocyanin" (KLH) is very safe. There have been **no reports of severe reaction to the use of KLH in humans**, despite extensive use in research for over 40 years.

Studies have shown that the side effects that can occur with KLH are: transient and localised discomfort and/or redness and/or swelling at the site of injection and/or transient fever. Rarely have more severe reactions been reported such as skin breakdown or nodules at the site of vaccination, although these have tended to occur with much higher doses of KLH than we are using in this study.

You will be observed for 30 minutes for any side effects following vaccination as an added safety measure.

You will also be having blood tests which may cause some mild discomfort and/or bruising. You will be offered a local anaesthetic ointment prior to blood tests to reduce the level of discomfort.

We also request completion of a brief questionnaire asking about recent stressful events as this can also impact on immune function.

5. Privacy, Confidentiality and Use of Information

Any identifying information about you which is collected as a result of your participating in this study will remain confidential and will only be used for the purpose of this research study. Your information will only be disclosed with your permission, or as required by law.

Blood taken for this study will also be tested for specific genes that are associated with immune function. The results of these genetic tests will not be released to any third parties.

The results of this study may be published in medical journals or presented at scientific meetings. In any information which is presented, you will not be identified.

If you choose to withdraw from the study you may elect to have your personal data destroyed or kept by the researchers for use in their analysis.

6. Physical Injury Resulting from Participation

You should report any discomfort, problems, or research related injuries to the study doctor. If you are injured as a direct result of your participation in this study, appropriate medical care will be provided at no cost to you.

7. Costs and Reimbursements

Any reasonable travel cost that has been borne by you in participating in this study will be reimbursed.

In addition, at the end of the study you will receive a **$50 gift voucher** in appreciation of providing your valuable time.

8. Ethical Review

The ethical aspects of this research study have been considered by the Human Research Ethics Committees of the Australian National University, James Cook University and ACT Health.

This study will be carried out according to the National Statement on Ethical Conduct in Human Research (2007) produced by the National Health and Medical Research Council of Australia (NHMRC). This document has been developed to protect the people who agree to participate in human research studies.

The investigators do not have any financial conflicts of interest in conducting this study.

This study is funded by a National Health and Medical Research Council grant (Project No: 585489).

Appendix D: List of medications and medical conditions that preclude AusUVI Study enrolment

Immunosuppressive Medications

Medication	Trade Name	Class of Agent	Minimum exclusion period
Aclomethasone dipropionate		Topical steroids	7
Aspirin	*Astrix*	Anti-pyretic	3
Azathioprine	*Imuran*	Systemic immunosuppressant	30
Beclomethasone Dipropionate	*Becotide Qvar*	Inhaled corticosteroid	30
Betamethasone	*Diprosone*	Topical Steroids	7
Budenoside	*Symbicort Pulmicort*	Inhaled corticosteroid	30
Cyclophosphamide	*Cycloblastin, Endoxan*	Systemic immunosuppressant	30
Cyclosporin	*Neoral, Cicloral, Sandimmun*	Systemic immunosuppressant	30
Fluticasone	*Flixotide seretide*	Inhaled corticosteroid	30
Hydrocortisone (Acetate)		Topical Steroids	7
Ibuprofen	*Brufen Nurofen*	Anti-inflammatory (NSAID)	3
Indomethacin	*Indocid*	Anti-inflammatory (NSAID)	3
Interferon		Systemic immunosuppressant	30
Ketorolac		Anti-inflammatory (NSAID)	3
Leflunamide	*Arava*	Systemic immunosuppressant	30
Methotrexate	*Methoblastin*	Systemic immunosuppressant	30

Methylprednisolone		Topical Steroids	7
Mometasone Furoate	*Elocon*	Topical Steroids	7
Mycophenolate	*Cellcept*	Systemic immunosuppressant	30
Naprosyn	*Neurofen*	Anti-inflammatory (NSAID)	3
Prednisolone	*Solone, Panafcortelone, Predsone*	Corticosteroid	30
Sulphasalazine	*Salazopyrine*	Systemic immunosuppressant / anti-inflammatory	30
Tacrolimus	*Prograf*	Systemic immunosuppressant	30

Participants with the following conditions were excluded from the study:

- Cancer (not in remission)
- Cardiac failure
- Chronic liver disease
- Chronic renal (kidney) disease
- Diabetes
- HIV / AIDS
- Inflammatory bowel disease (i.e. Crohn's disease, ulcerative colitis) (NB - irritable bowel disease not excluded)
- IgA deficiency
- Lupus (Systemic lupus erythematosis (SLE)
- Lymphoma or Leukaemia
- Renal impairment or failure
- Splenectomy (no spleen)

Appendix E: Certificate of Analysis for Biosyn Keyhole Limpet Haemocyanin

Autorisierte Person AA

Certificate of Analysis

Native KLH, 0.6 mg, GMP-Grade

Batch No.: 015503.A

No.: CA-KL699210-01
Effective date: 04.05.2010

biosyn

Date of manufacture: 30.04.2010
QC-ID-No.: 12232

Examination / Method	Specification	Result
General Properties		
Extractable volume / Ph. Eur. 2.9.17	≥ 0.6 ml	0.7 ml
appearance of solution / visual test	Slightly bluish liquid	Slightly bluish liquid
pH-value / Ph. Eur. 2.2.3	7.0 – 7.4	7.2
osmolality / Ph. Eur. 2.2.35	400 - 500 mOsmol/kg	434 mOsmol/kg
absolute density / electronic measurement of the duration of oscillation	1.0000 - 1.1000 g/cm³	1.0140 g/cm³
Identity		
distribution of molecular weight/ MPLC-SEC	retention time of hemocyanin 30.0 - 40.0 min	retention time of hemocyanin 32.5 min
identity KLH / SDS PAGE	characteristic band pattern with typical band near 370 kDa	characteristic band pattern with typical band near 370 kDa
copper / AAS	clear signal at 324.8 nm	clear signal at 324.8 nm
UV-spectra / photometric	E_{max} (345 - 349 nm) E_{max} (277 - 281 nm)	E_{max} (345 nm) E_{max} (279 nm)
Purity		
purity KLH / MPLC-SEC	> 98.0 %	100.0 %
nucleic acids / electrophoretic	DNA < 300 pg/µl	DNA < 300 pg/µl

Autorisierte Kopie

Certificate of Analysis

Native KLH, 0.6 mg, GMP-Grade

No.: CA-KL699210-01
Effective date: 04.05.2010

biosyn

Batch No.: 015503.A

Date of manufacture: 30.04.2010
QC-ID-No.: 12232

Examination / Method	Specification	Result
Content		
protein content / photometric / 280 nm	0.9 – 1.1 mg/ml	1.0 mg/ml
Biological Safety		
bacterial endotoxins / Ph. Eur. 2.6.14, E	≤ 11.7 I.U./mg	< 0.2 I.U./mg
sterility / Ph. Eur. 2.6.1	sterile	sterile

Additional testing on active ingredient bulk solution (Batch No.: 015503)

abnormal toxicity / Ph. Eur. 2.6.9	no abnormal toxicity	no abnormal toxicity
Viruses / PCR	HAV no genome of HAV detectable	HAV no genome of HAV detectable
	HBV no genome of HBV detectable	HBV no genome of HBV detectable
	HCV no genome of HCV detectable	HCV no genome of HCV detectable
	Enteroviruses no genomes of enteroviruses detectable	Enteroviruses no genomes of enteroviruses detectable
	Noroviruses no genomes of Noroviruses detectable	Noroviruses no genomes of Noroviruses detectable
	Rotaviruses no genomes of Rotaviruses detectable detectable	Rotaviruses no genomes of Rotaviruses detectable detectable

biosyn Arzneimittel GmbH · Schorndorfer Straße 32 · 70734 Fellbach · Deutschland · Telefon 07 11 5 75 32 00 · Fax 07 11 5 75 32 99

Appendix F: Questionnaires used in the AusUVI Study

Questionnaire A: General health and demographic information

27232

THE AUSTRALIAN ULTRAVIOLET RADIATION & IMMUNITY STUDY

THE AUSTRALIAN NATIONAL UNIVERSITY

JAMES COOK UNIVERSITY AUSTRALIA

Part A: General Health & Information Questionnaire

Thank you for agreeing to take part in the AusUVI study. We are conducting this study to assess how sunlight-derived ultraviolet radiation (UVR) exposure affects the human immune system.

The AusUVI study relies on the willingness of participants to share information about their lives and their experiences. This will assist researchers in understanding the positive and negatives impacts of sun exposure on our health.

Participation is completely voluntary and you may withdraw from the study at any time.

Study Participant Code: ☐☐☐

Today's Date: ☐☐ / ☐☐ / ☐☐☐☐

There are no right or wrong answers to these questions. It is very important that you answer **ALL** of the questions relevant to you. To help us read your answers, please write as clearly as possible using a **BLACK or BLUE pen.**

Please put a **tick or cross** in the small box beside each answer that is right for you, or write in the big box or on the line as appropriate.

All of the information you provide to us will remain strictly confidential

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THE AUSTRALIAN NATIONAL UNIVERSITY

JAMES COOK UNIVERSITY AUSTRALIA

SECTION 1: GENERAL QUESTIONS ABOUT YOU

1. What gender are you? (Please cross the appropriate box) ☐ Male ☐ Female

2. What is your date of birth? ☐☐ / ☐☐ / ☐☐☐☐

3. In what country were you born?

3a. If you were **NOT** born in **Australia**, in what year did you come to live in Australia? ☐☐☐☐

3b. In what state/territory do you live? (Please cross one box)

☐ Queensland	☐ Tasmania	☐ ACT
☐ New South Wales	☐ South Australia	☐ Northern Territory
☐ Victoria	☐ Western Australia	

3c. For how many years have you been living in this state/territory? ☐☐ years

4. What is your **PARENT'S ETHNIC ORIGIN** (that is, the place where most of their ancestors came from) and the **COUNTRY THEY WERE BORN IN.**

Parent	Country of birth:	Ethnic Origin (Please cross the ONE that best describes your mother)		
Mother		☐ Australian	☐ English	☐ Irish
		☐ ATSI	☐ Vietnamese	☐ Croatian
		☐ Chinese	☐ Italian	☐ Greek
		☐ Scottish	☐ German	☐ Lebanese
		☐ Dutch	☐ Maltese	☐ Polish
		☐ Filipino	☐ Indian	☐ Other, please describe:
		☐ Don't know	☐ Prefer not to answer	
Parent	**Country of birth:**	**Ethnic Origin (Please cross the ONE that best describes your father)**		
Father		☐ Australian	☐ English	☐ Irish
		☐ ATSI	☐ Vietnamese	☐ Croatian
		☐ Chinese	☐ Italian	☐ Greek
		☐ Scottish	☐ German	☐ Lebanese
		☐ Dutch	☐ Maltese	☐ Polish
		☐ Filipino	☐ Indian	☐ Other, please describe:
		☐ Don't know	☐ Prefer not to answer	

5. What is the **highest** technical, professional or academic qualification that you have completed?
(Please cross one of the boxes below)

☐ Did not complete primary school	☐ Trade/Apprenticeship
☐ Primary school	☐ Certificate or Diploma
☐ Some high school (Year 11 or under)	☐ Bachelor's degree
☐ Year 12 Senior Certificate (or HSC)	☐ Postgraduate degree

5a. Which of the following **best describes** the occupation you had for the longest period?
(Please cross one of the boxes below)

☐ Student
☐ Manager or administrator
☐ Professional (e.g. engineer, doctor, teacher, nurse, police, technical officer)
☐ Tradesperson (e.g. carpenter, electrician, plumber, mechanic, etc.)
☐ Clerk (e.g. typist, receptionist, data processor, etc.)
☐ Salesperson or personal service worker (e.g. sales rep, teller, insurance rep, etc.)
☐ Plant or machine operator or driver (e.g. taxi driver, bus driver)
☐ Farmer
☐ Labourer or related worker (e.g. trade assistant, factory hand, agricultural labourer, construction, mining)
☐ Member of defence forces (army, navy, air forces)
☐ Other (please state) _____

5b. Which of the following **best describes your current employment status?**
(Please cross one of the boxes below)

☐ Unemployed
☐ Home duties
☐ Part-time work - employed/self-employed
☐ Full-time work - employed/self-employed
☐ Student
☐ Sole parent pension
☐ Disability pension
☐ Retired
☐ Other (please state) _____

5c. Which of the following best describes your **current, main occupation?**
(Please cross one of the boxes below)

☐ Mainly indoors (e.g. office worker)
☐ Half indoors and half outdoors (e.g. physical education teacher)
☐ Mainly outdoors (e.g. gardener)

SECTION 2: SMOKING/ALCOHOL CONSUMPTION

The following questions refer to smoking and alcohol consumption.

6. Were you ever a regular smoker of cigarettes or cigars or pipes? (A regular smoker is one who smoked daily, or at least 7 times per week, for at least 3 months.)

☐ Yes (Please go to Question 7a)

☐ No (Please go to Question 8)

7a. Are you currently a regular smoker of cigarettes or cigars or pipes? ☐ Yes ☐ No

7b. Over the past month, on average, how many cigarettes and/or cigars and/or pipes did you smoke each day? ☐☐ Per day

7c. Over the past month, which brand and type have you smoked most often?

Brand (eg. Horizon/Craven A/Winfield) ☐

Type (eg. Plain/Super Mild 8/Extra mild (Blue)) ☐

Did they have a filter? ☐ Yes ☐ No

These next questions ask about your smoking history.

7d. How old were you when you first became a regular smoker? ☐☐ Years of age

7e. If you are no longer a smoker, at what age did you stop? ☐☐ Years of age

☐ I did not stop

7f. If you have stopped being a regular smoker in the past, and then started being one again (perhaps doing this several times) for how long in total have you stopped being a regular smoker? ☐☐ Years

☐ I did not start and stop

7g. On average, during the years that you smoked, how many cigarettes, cigars or pipes did you smoke each day? ☐☐ Per day

8. About how many alcoholic drinks do you have each week?

☐☐ number of standard alcoholic drinks each week (one standard drink = a glass of wine, middy of beer, or nip of spirits) Please put "0" if you do not drink, or have less than one drink each week.

9. On how many days each week do you usually drink alcohol? ☐ Days each week

SECTION 3: GENERAL HEALTH INFORMATION

The following questions refer to your general health and to prescription medications (medications prescribed by your doctor) and any over-the-counter vitamin / mineral supplements that you take.

10. Overall, how would you rate your general health over the **PAST MONTH?** (please cross one box)

☐ Excellent ☐ Fair
☐ Very good ☐ Poor
☐ Good ☐ Very Poor

11. In the past 3 months, have you suffered from an infection/s? (Please tick the appropriate boxes below, if you haven't experienced any infections, please select 0 for each.)

	Frequency			
	0	1	2	3+
Cold or upper respiratory tract infection	☐	☐	☐	☐
Gastrointestinal infection	☐	☐	☐	☐
Skin or nail infection	☐	☐	☐	☐
Urinary or genitourinary infection	☐	☐	☐	☐
Other infection (please specify) _____	☐	☐	☐	☐

12. Do **YOU** suffer from any **chronic illnesses?** (Please tick the appropriate boxes below, if you do not suffer from any chronic illnesses, please select No for each.)

Liver disease	☐ Yes	☐ No
Kidney disease	☐ Yes	☐ No
Heart disease	☐ Yes	☐ No
Skin condition	☐ Yes	☐ No
Respiratory / lung disease	☐ Yes	☐ No
Immune system disorder; Please specify _____	☐ Yes	☐ No
Other; Please specify _____	☐ Yes	☐ No

13. Does anyone in your family have a history of an immune system related illness or "autoimmune" disorder? (Please tick the appropriate boxes for each item below.)

Rheumatoid arthritis	☐ Yes	☐ No
Lupus or SLE	☐ Yes	☐ No
Thyroid disease	☐ Yes	☐ No
Type I diabetes	☐ Yes	☐ No
Scleroderma	☐ Yes	☐ No
Multiple sclerosis	☐ Yes	☐ No
Other; Please specify _____	☐ Yes	☐ No

14. Do you take any regular medications? (including prescription medicines from your doctor and "over-the-counter" medicines). ☐ Yes ☐ No

If Yes, please specify the medications below.

Medication 1 _____

Medication 2 _____

Medication 3 _____

Medication 4 _____

Medication 5 _____

15. Do you take any medications or supplements which may contain Vitamin D?

Vitamin D supplement alone	☐ Yes	☐ No
Vitamin D supplement with calcium	☐ Yes	☐ No
Multivitamin tablet	☐ Yes	☐ No
Cod liver oil / tablets	☐ Yes	☐ No

SECTION 4: VACCINATIONS

This section relates to vaccinations you have had and any problems you might have had with them. Also this section will inform us whether there are any issues with you taking part in our vaccination study.

16. Have you suffered any side effects from vaccination?

☐ No

☐ Yes	Vaccine	Year	Please describe the reaction
Please list			

17. Are you allergic to shellfish?

☐ No

☐ Yes	Type of Shellfish	Year	Please describe the reaction
Please list			

18. Have you ever had an "Anaphylactic reaction" to shellfish or a medication? That is have you ever suffered from breathing problems, swelling of lips or throat or required immediate medical attention after consuming shellfish or a medication?

☐ No

☐ Yes	Shellfish / Medication	Year	Please describe the reaction
Please list			

The following two questions are to be answered by women only. They are to identify women who could be or are pregnant or are breastfeeding, as they will be excluded from the study.

19. Is there any possibility that you could be pregnant?

☐ No
☐ Yes

20. Are you currently breastfeeding an infant?

☐ No
☐ Yes

SECTION 5: GENERAL INFORMATION

22. What is your usual **PERSONAL** income before tax, from all sources? (Please include benefits, pensions, superannuation, etc.) (Please tick one box only)

☐ less than $5000 per year	☐ $50,000 - $69,000 per year
☐ $5,000 - $9,999 per year	☐ $70,000 - $89,999 per year
☐ $10,000 - $19,999 per year	☐ $90,000 - $109,999 per year
☐ $20,000 - $29,999 per year	☐ $110,000 or more per year
☐ $30,000 - $49,000 per year	☐ I would prefer not answer

You have now completed Part A of the AusUVI study questionnaire!

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THE AUSTRALIAN ULTRAVIOLET RADIATION & IMMUNITY STUDY

THE AUSTRALIAN NATIONAL UNIVERSITY

JAMES COOK UNIVERSITY AUSTRALIA

Part B: SUN & SKIN QUESTIONNAIRE

Thank you for agreeing to take part in the AusUVI study. We are conducting this study to assess how sunlight-derived ultraviolet radiation (UVR) exposure affects the human immune system.

The AusUVI study relies on the willingness of participants to share information about their lives and their experiences. This will assist researchers in understanding the positive and negatives impacts of sun exposure on our health.

Participation is completely voluntary and you may withdraw from the study at any time.

Study Participant Code: ☐☐☐

Today's Date: ☐☐ / ☐☐ / ☐☐☐☐

There are no right or wrong answers to these questions. It is very important that you answer **ALL** of the questions relevant to you. To help us read your answers, please write as clearly as possible using a **BLACK or BLUE pen.**

Please put a **tick or cross** in the small box beside each answer that is right for you, or write in the bib box or on the line as appropriate

All of the information you provide to us will remain strictly confidential

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SECTION 1: YOUR SKIN AND THE SUN

The following questions ask about your skin and how it reacts to the sun.

1. How does your skin react when you sit in the sun in your current area of residence, in the middle of the day, for the first time in summer, without sunscreen? (please cross one box)

- ☐ Never Burn
- ☐ Burn after more than 2 hours sun exposure
- ☐ Burn after 1-2 hours
- ☐ Burn after 1/2-1 hour
- ☐ Burn within half an hour

2. At the end of summer or after a two week holiday in the sun, what kind of tan would you have? (please cross one box)

- ☐ A dark tan
- ☐ A medium tan
- ☐ Light tan
- ☐ Practically no tan

3a. How does your skin react when you go out in the sun in your current area of residence, for one hour in the middle of the day, for the first time in summer, without sunscreen? (please cross one box)

- ☐ Burn then peel
- ☐ Burn then tan
- ☐ Tan only

3b. How **frequently** did you get **sunburnt** in the last 12 months?
Please indicate the frequency as a number in the boxes provided

	Summer (Dec - Feb)	Autumn (Mar - May)	Winter (June - Aug)	Spring (Sept - Nov)
Frequency	☐☐	☐☐	☐☐	☐☐

3c. When was the last time you got sunburnt? Please complete the following statement:

The last time I was sunburnt was ☐ ○ Days ○ Weeks ○ Months ago.

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SECTION 1: YOUR SKIN AND THE SUN

The following questions ask about your skin and how it reacts to the sun.

1. How does your skin react when you sit in the sun in your current area of residence, in the middle of the day, for the first time in summer, without sunscreen? (please cross one box)

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- ☐ Burn after 1/2-1 hour
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2. At the end of summer or after a two week holiday in the sun, what kind of tan would you have? (please cross one box)

- ☐ A dark tan
- ☐ A medium tan
- ☐ Light tan
- ☐ Practically no tan

3a. How does your skin react when you go out in the sun in your current area of residence, for one hour in the middle of the day, for the first time in summer, without sunscreen? (please cross one box)

- ☐ Burn then peel
- ☐ Burn then tan
- ☐ Tan only

3b. How **frequently** did you get **sunburnt** in the last 12 months?
Please indicate the frequency as a number in the boxes provided

	Summer (Dec - Feb)	Autumn (Mar - May)	Winter (June - Aug)	Spring (Sept - Nov)
Frequency	☐☐	☐☐	☐☐	☐☐

3c. When was the last time you got sunburnt? Please complete the following statement:

The last time I was sunburnt was ☐ ○ Days ○ Weeks ○ Months ago.

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SECTION 2: TIME SPENT IN THE SUN

The following questions ask about your indoor and outdoor activities and your sun exposure.

4. Have you worked a night shift at least once in the past month?

☐ Yes

☐ No *(Please go to question 5)*

4a. Please tell us approximately how many night shifts you have worked in the past month? (Please cross one box)

☐ 1 Night Shifts	☐ 8-10 Night Shifts
☐ 2 Night Shifts	☐ 11-13 Night Shifts
☐ 3 Night Shifts	☐ 14-16 Night Shifts
☐ 4 Night Shifts	☐ 17-19 Night Shifts
☐ 5-7 Night Shifts	☐ 20+ Night Shifts

5. Thinking about the **PAST MONTH**, we would like to know the **TIMES OF DAY** as well as the **USUAL LENGTH OF TIME** that you spend **OUTSIDE** between sunrise and sunset on:

5a. A typical MONDAY in the PAST MONTH (Cross one response for EACH time period)

	Length of time spent outside				
	Never	<15 minutes	15-30 minutes	30-45 minutes	45-60 minutes
Morning (am)					
5-6	☐	☐	☐	☐	☐
6-7	☐	☐	☐	☐	☐
7-8	☐	☐	☐	☐	☐
8-9	☐	☐	☐	☐	☐
9-10	☐	☐	☐	☐	☐
10-11	☐	☐	☐	☐	☐
11-12	☐	☐	☐	☐	☐
Afternoon (pm)					
12-1	☐	☐	☐	☐	☐
1-2	☐	☐	☐	☐	☐
2-3	☐	☐	☐	☐	☐
3-4	☐	☐	☐	☐	☐
4-5	☐	☐	☐	☐	☐
5-6	☐	☐	☐	☐	☐
6-7	☐	☐	☐	☐	☐

5b. A typical TUESDAY in the PAST MONTH (Cross one response for EACH time period)

	Length of time spent outside				
	Never	<15 minutes	15-30 minutes	30-45 minutes	45-60 minutes
Morning (am)					
5-6	☐	☐	☐	☐	☐
6-7	☐	☐	☐	☐	☐
7-8	☐	☐	☐	☐	☐
8-9	☐	☐	☐	☐	☐
9-10	☐	☐	☐	☐	☐
10-11	☐	☐	☐	☐	☐
11-12	☐	☐	☐	☐	☐
Afternoon (pm)					
12-1	☐	☐	☐	☐	☐
1-2	☐	☐	☐	☐	☐
2-3	☐	☐	☐	☐	☐
3-4	☐	☐	☐	☐	☐
4-5	☐	☐	☐	☐	☐
5-6	☐	☐	☐	☐	☐
6-7	☐	☐	☐	☐	☐

5c. A typical WEDNESDAY in the PAST MONTH (Cross one response for EACH time

	Length of time spent outside				
	Never	<15 minutes	15-30 minutes	30-45 minutes	45-60 minutes
Morning (am)					
5-6	☐	☐	☐	☐	☐
6-7	☐	☐	☐	☐	☐
7-8	☐	☐	☐	☐	☐
8-9	☐	☐	☐	☐	☐
9-10	☐	☐	☐	☐	☐
10-11	☐	☐	☐	☐	☐
11-12	☐	☐	☐	☐	☐
Afternoon (pm)					
12-1	☐	☐	☐	☐	☐
1-2	☐	☐	☐	☐	☐
2-3	☐	☐	☐	☐	☐
3-4	☐	☐	☐	☐	☐
4-5	☐	☐	☐	☐	☐
5-6	☐	☐	☐	☐	☐
6-7	☐	☐	☐	☐	☐

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6. Is the PATTERN of sun exposure that you have described FOR THE PAST MONTH, similar to your exposure over the previous months?

☐ Yes *Go to Question 7*
☐ No

6a. Do you usually spend::

☐ MORE time in the sun?
☐ LESS time in the sun?

6b. Please tell us WHY your pattern of sun exposure has been DIFFERENT over the PAST MONTH?

Were you ill?	☐ Yes ☐ No	If yes, what was the duration of your illness? _____
Were you on holidays?	☐ Yes ☐ No	If yes, was your sun exposure while on holidays: ☐ More than usual ☐ Less than usual

Did you change jobs and are now working:

More outdoors ☐

Less outdoors ☐

More shift work ☐

Less shift work ☐

Other, please describe:

SECTION 3: SUN-PROTECTIVE BEHAVIOUR

The following questions ask about how you protect yourself from the sun.

7. We are interested in the CLOTHING AND SUN PROTECTION that you have worn in the PAST MONTH. During the past month, when outside, did you.....

	Never/Rarely	Less than half the time	More than half the time	Always/almost always
7a. Wear a broad-brimmed hat?	☐	☐	☐	☐
7b. Wear a cap?	☐	☐	☐	☐
7c. Wear any other head covering?	☐	☐	☐	☐
7d. Wear a shirt with long sleeves?	☐	☐	☐	☐
7e. Wear long trousers or clothing that covers all or most of your legs?	☐	☐	☐	☐
7f. Wear sunglasses?	☐	☐	☐	☐

8. Have you used sunscreen in the PAST MONTH?

☐ Yes

☐ No *Go to Question 9*

8a. What is the sun-protection factor (SPF) of the sunscreen that you have used most often? (eg. SPF30+) Consider any product you may use that have an SPF, including daily moisturiser or make-up products.

Sunscreen SPF ☐☐

Daily Moisturiser SPF ☐☐

Make-up SPF ☐☐

8b. Over the PAST MONTH, how often on average have you used any of the products described in 8a?

☐ Every day

☐ 5-7 days a week

☐ 3-4 days a week

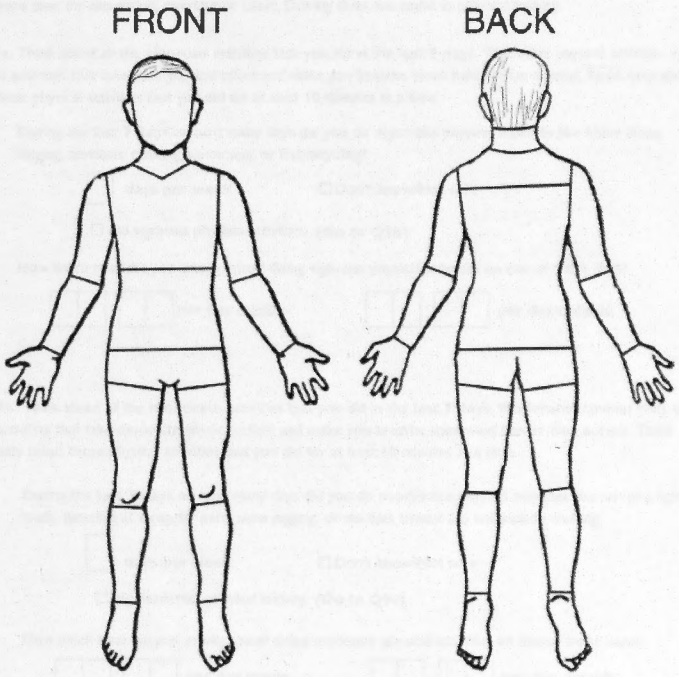
☐ 1-2 days a week

☐ Less than once a week

8c. On days that you have used sunscreen in the **PAST MONTH**, how often did you apply it?

☐☐ Times per day

8d. Please look at this diagram and tell us over the **PAST MONTH**, when you used sunscreen, **WHERE** did you usually apply it? (Remember to include any products you included in 8a.)



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SECTION 4: PHYSICAL ACTIVITY

The following questions are about the amount of exercise that you do as part of your everday life.

The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport. Driving does not count as physical activity.

9a. Think about all the **vigorous** activities that you did in the **last 7 days. Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

During the **last 7 days** on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, running, swimming, or fast bicycling?

☐ **days per week** ☐ Don't know/Not sure

☐ No vigorous physical activities. **(Go to Q9b)**

How much time did you usually spend doing vigorous physical activities on one of those days?

☐☐ : ☐☐ **per day inside** ☐☐ : ☐☐ **per day outside**

9b. Think about all the **moderate** activities that you did in the **last 7 days. Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

During the **last 7 days** on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, slow jogging, or doubles tennis? Do not include walking.

☐ **days per week** ☐ Don't know/Not sure

☐ No moderate phsyical activity. **(Go to Q9c)**

How much time did you usually spend doing moderate physical activities on one of those days?

☐☐ : ☐☐ **per day inside** ☐☐ : ☐☐ **per day outside**

9c. Think about all the time you spend **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and walking that you might do solely for recreation, sport, exercise, or leisure.

During the **last 7 days** on how many days did you **walk** for at least 10 minutes at a time?

☐ days per week ☐ Don't know/Not sure

☐ No walking. **(Go to Q9d)**

How much time did you usually spend walking on one of those days?

☐☐:☐☐ **per day inside** ☐☐:☐☐ **per day outside**

9d. The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

During the **last 7 days**, how much time did you spend **sitting** on a week day?

☐☐:☐☐ **per day inside** ☐☐:☐☐ **per day outside**

☐ Don't know/Not sure

10a. Is the PATTERN of physical activity that you described above **for the past 7 days**, typical of your physical activity **over the past month**?

☐ Yes **(Go to Q11)**

☐ No

10b. Do you usually spend: ☐ MORE time being physically active?

☐ LESS time being physically active?

10c. Please tell us WHY your pattern of physical activity has been DIFFERENT over the past 7 days, compared with the past month?

Were you ill?	☐ Yes ☐ No	If yes, what was the duration of your illness? _____
Were you on holidays?	☐ Yes ☐ No	If yes, was your sun exposure while on holidays: ☐ More than usual ☐ Less than usual
Did you change jobs and are now working:		☐ In a more physically active occupation ☐ In a less physically active occupation
Other, please describe:		

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SECTION 5: MENSTRUAL CYCLE (FEMALES ONLY)

Hormonal changes occurring through the menstrual cycle are known to affect immune function. These questions relate to the timing of menstruation.

11a. With regard to your menstrual cycle, do you currently:

Have regular periods?	☐	Go to Quesion 11b
Have irregular periods?	☐	Go to Quesion 11b
Not have menstrual periods?	☐	

11b. When was the first day of your most recent menstrual period?

☐☐ Days ago

You have now completed Part B of the AusUVI Questionnaire!
Thank you for your time and assistance.

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THE AUSTRALIAN ULTRAVIOLET RADIATION & IMMUNITY STUDY

THE AUSTRALIAN NATIONAL UNIVERSITY

JAMES COOK UNIVERSITY AUSTRALIA

Part C: PSYCHOLOGICAL PROFILE QUESTIONNAIRE

Thank you for agreeing to take part in the AusUVI study. We are conducting this study to assess how sunlight-derived ultraviolet radiation (UVR) exposure affects the human immune system.

The AusUVI study relies on the willingness of participants to share information about their lives and their experiences. This will assist researchers in understanding the positive and negatives impacts of sun exposure on our health.

Participation is completely voluntary and you may withdraw from the study at any time.

Study Participant Code: ☐☐☐

Today's Date: ☐☐ / ☐☐ / ☐☐☐☐

There are no right or wrong answers to these questions. It is very important that you answer **ALL** of the questions relevant to you. To help us read your answers, please write as clearly as possible using a **BLACK or BLUE pen.**

Please **fill in the circle** beside each answer that is right for you.

All of the information you provide to us will remain strictly confidential

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SECTION 1: MOODS

We know that our psychological state can affect how our immune system functions. The following surveys will ask structured questions assessing your current psychological state and moods. It is important that you be as honest as possible with your answers.

Directions: Describe **HOW YOU FEEL RIGHT NOW** by **filling in the correct circle** after each of the words listed below.

FEELING	Not at all	A little	Moderately	Quite a bit	Extremely
Lively	O	O	O	O	O
Forgetful	O	O	O	O	O
Unhappy	O	O	O	O	O
Active	O	O	O	O	O
Confused	O	O	O	O	O
Tense	O	O	O	O	O
Angry	O	O	O	O	O
Worn-out	O	O	O	O	O
Sad	O	O	O	O	O
Unable to concentrate	O	O	O	O	O
Restless	O	O	O	O	O
Annoyed	O	O	O	O	O
Exhausted	O	O	O	O	O
Blue	O	O	O	O	O
Cheerful	O	O	O	O	O
Bewildered	O	O	O	O	O
Nervous	O	O	O	O	O
Grovely	O	O	O	O	O
Weary	O	O	O	O	O
Discouraged	O	O	O	O	O
Full of pep	O	O	O	O	O
Uncertain about things	O	O	O	O	O
Anxious	O	O	O	O	O
Resentful	O	O	O	O	O
Bushed	O	O	O	O	O
Helpless	O	O	O	O	O
Bitter	O	O	O	O	O
Vigorous	O	O	O	O	O
Worthless	O	O	O	O	O
Furious	O	O	O	O	O
Fatigued	O	O	O	O	O
Miserable	O	O	O	O	O
Peeved	O	O	O	O	O
Energetic	O	O	O	O	O
On edge	O	O	O	O	O
Uneasy	O	O	O	O	O
Hopeless	O	O	O	O	O

SECTION 2: RECENT STRESSFUL LIFE EVENTS

This section assesses the number and severity of recent stressors you may have encountered.
Directions: Please completely fill in the circle next to one or more statements which describes an experience that has happened to **YOU** in the last **6 MONTHS.**

Health	
An illness or injury that kept you in bed for more than a week or sent you to the hospital	○
An illness or injury that was less serious than above	○
Major dental work	○
Major change in eating habits	○
Major change in sleeping habits	○
Major change in your usual type and/or amount of recreation	○
Work	
Change to a new type of work	○
Change in your work hours or conditions	○
More work responsibilities	○
Fewer work responsibilities	○
A promotion	○
A demotion	○
A transfer	○
Trouble with your boss	○
Trouble with your co-workers	○
Trouble with those you supervise	○
Other work troubles	○
Major business readjustment	○
Retirement	○
Laid off	○
Fired	○
Took a course to help your work	○
Home and family	
Move within same city or town	○
Move to different town, city, or state	○
Major change in living conditions	○
Change in family get-togethers	○
Major change in health or behavior of a family member	○
Marriage	○
Pregnancy	○
Miscarriage or abortion	○
Birth of a child	○
Adoption of a child	○
Relative moves in with you	○
Spouse begins or stops work	○
Child leaves home to attend college or for marriage	○
Child leaves home for other reasons	○
Change in arguments with spouse	○
Problems with relatives or in-laws	○

SECTION 2: RECENT STRESSFUL LIFE EVENTS

Directions: Please completely fill in the circle next to one or more statements which describes an experience that has happened to **YOU** in the last **6 MONTHS.**

Parents divorce	○
A parent remarries	○
Separation from spouse due to work	○
Separation from spouse due to marital difficulties	○
Divorce	○
Birth of grandchild	○
Death of spouse	○
Death of child	○
Death of parent	○
Death of a brother or sister	○
Personal and social	
Change in personal habits	○
Beginning or ending school	○
Change of school or college	○
Change in political beliefs	○
Change in religious beliefs	○
Change in social activities	○
Vacation	○
New, close personal relationship	○
Engagement to marry	○
Girlfriend or boy friend problems	○
Sexual difficulties	○
An accident	○
"Falling out" of a close personal relationship	○
Minor violation of the law	○
Being held in jail	○
Major decision about immediate future	○
Major personal achievement	○
Death of a close personal friend	○
Financial	
Major loss of income	○
Major increase in income	○
Investment and/or credit difficulties	○
Loss/damage to personal property	○
Major purchase	○
Moderate purchase	○
Foreclosure on mortgage or loan	○

SECTION 3: EMOTIONS

These next questions are about how you have felt over the past month.

Directions: Please read each question and **fill in the circle** by the **one** statement that **best describes** how things have been for you during the past month. There are no right or wrong answers.

1. How happy, satisfied, or pleased have you been with your personal life during the past month?

- O Extremely happy, could not have been more satisfied or pleased
- O Very happy most of the time
- O Generally, satisfied, pleased
- O Somtimes fairly satisfied, sometimes fairly unhappy
- O Generally dissatisfied, unhappy
- O Very dissatisfied, unhappy most of the time

2. How much of the time have you felt lonely during the past month?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

3. How often did you become nervous or jumpy when faced with excitement or unexpected situations during the past month?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

4. During the past month, how much of the time have you felt that the future looks hopeful and promising?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

5. How much of the time, during the past month, has your daily life been full of things that were interesting to you?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

SECTION 3: EMOTIONS

These next questions are about how you have felt over the past month.

Directions: Please read each question and **fill in the circle** by the **one** statement that **best describes** how things have been for you during the past month. There are no right or wrong answers.

1. How happy, satisfied, or pleased have you been with your personal life during the past month?

- O Extremely happy, could not have been more satisfied or pleased
- O Very happy most of the time
- O Generally, satisfied, pleased
- O Somtimes fairly satisfied, sometimes fairly unhappy
- O Generally dissatisfied, unhappy
- O Very dissatisfied, unhappy most of the time

2. How much of the time have you felt lonely during the past month?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

3. How often did you become nervous or jumpy when faced with excitement or unexpected situations during the past month?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
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- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

5. How much of the time, during the past month, has your daily life been full of things that were interesting to you?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

SECTION 3: EMOTIONS

6. How much of the time, during the past month, did you feel relaxed and free from tension?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

7. During the past month, how much of the time have you generally enjoyed the things you do?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

8. During the past month, have you had any reason to wonder if you were losing your mind, or losing control over the way you act, talk, think, feel or of your memory?

- O No, not at all
- O Maybe a little
- O Yes, but not enough to be concerned or worried about
- O Yes, and I have been a little concerned
- O Yes, and I am quite concerned
- O Yes, and I am very much concerned about it

9. Did you feel depressed during the past month?

- O Yes, to the point that I did not care about anything for days at a time
- O Yes, very depressed almost every day
- O Yes, quite depressed several times
- O Yes, a little depressed now and then
- O No, never felt depressed at all

10. During the past month, how much of the time have you felt loved and wanted?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

11. How much of the time, during the past month, have you been a very nervous person?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

SECTION 3: EMOTIONS

12. When you have got up in the morning, this past month, about how often did you expect to have an interesting day?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

13. During the past month, how much of the time have you felt tense or "highly strung"?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

14. During the past month, have you been in firm control of your behaviour, thoughts, emotions and feelings?

- O Yes, very definitely
- O Yes, for the most part
- O Yes, I guess so
- O No, not too well
- O No, and I am somewhat disturbed
- O No, and I am very disturbed

15. During the past month, how often did your hands shake when you tried to do something?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

16. During the past month, how often did you feel that you had nothing to look forward to?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

17. How much of the time, during the past month, did you feel calm and peaceful?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

18. How much of the time, during the past month, have you felt emotionally stable?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

SECTION 3: EMOTIONS

19. How much of the time, during the last month, have you felt downhearted and blue?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

20. How often have you felt like crying, during the past month?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

21. During the past month, how often have you felt that others would be better off if you were dead?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

22. How much of the time, during the past month, were you able to relax without difficulty?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

23. How much of the time, during the past month, did you feel that your love relationships, both loving and being loved, were full and complete?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

24. How often, during the past month, did you feel that nothing turned out for you the way you wanted it to?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

25. How much have you been bothered by nervousness, or your 'nerves' during the past month?

- O Extremely so, to the point where I could not take care of things
- O Very much bothered
- O Bothered quite a bit by nerves
- O Bothered sometimes, enough to notice
- O Bothered just a little by nerves
- O Not bothered at all by this

SECTION 3: EMOTIONS

26. During the past month, how much of the time has living been a wonderful adventure for you?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

27. How often, during the past month, have you felt so down in the dumps that nothing could cheer you up?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

28. During the past month, did you think about taking your own life?

- O Yes, very often
- O Yes, fairly often
- O Yes, a couple of times
- O Yes, at one time
- O No, never

29. During the past month, how much of the time have you felt restless, fidgety or impatient?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

30. During the past month, how much of the time have you been moody or brooded about things?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

31. How much of the time, during the past month, have you felt cheerful, light hearted?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

32. During the past month, how often did you get rattled, upset or flustered?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

SECTION 3: EMOTIONS

33. During the past month, have you been anxious or worried?

- O Yes, extremely to the point of being sick or almost sick
- O Yes, very much so
- O Yes, quite a bit
- O Yes, some, enough to bother me
- O Yes, a little bit
- O No, not at all

34. During the past month, how much of the time were you a happy person?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

35. How often during the past month did you find yourself trying to calm down?

- O Always
- O Very often
- O Fairly often
- O Sometimes
- O Almost never
- O Never

36. During the past month, how much of the time have you been low or in very low spirits?

- O All of the time
- O Most of the time
- O A good bit of the time
- O Some of the time
- O A little of the time
- O None of the time

37. How often, during the past month, have you been waking up feeling fresh and rested?

- O Always, every day
- O Almost every day
- O Most days
- O Some days, but usually not
- O Hardly ever
- O Never wake up feeling rested

38. During the past month, have you been under or felt you were under any strain, stress or pressure?

- O Yes, almost more than I could stand or bear
- O Yes, quite a bit of pressure
- O Yes, some - more than usual
- O Yes, some, but about normal
- O Yes, a little bit
- O No, not at all

You have now completed Part C of the AusUVI Questionnaire!
Thank you for your time and assistance.

Appendix G: Examination data collection sheet and sunburn history

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JAMES COOK UNIVERSITY AUSTRALIA

AusUVI Data Collection Sheet

LRO Initials: ☐☐ **Participant ID:** ☐☐☐

VISIT 1

Date: ☐☐ / ☐☐ / ☐☐☐☐

Natural Hair Colour at 18 years: ☐ Black ☐ Brown ☐ Blonde ☐ Red

Eye colour: ☐ Brown ☐ Hazel ☐ Blue or Grey ☐ Green

Recent use of tanning products:
Have you used any tanning products in the past month that may have affected the colour of your skin?

	YES	NO
1. Left inner upper arm	☐	☐
2. Your face	☐	☐

Skin Reflectance:

	Flash Number	400nm	420nm
Left Inner Upper Arm	☐☐☐ ☐☐☐ ☐☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐
		Expected values 11.0 - 33.6%	
Left Cheek	☐☐☐ ☐☐☐ ☐☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐
		Expected values 6.0 - 26.4%	

Blood Pressure: ☐☐☐ **Systolic** ☐☐☐ **Diastolic**

Weight: ☐☐☐.☐ **kg** **Height:** ☐☐☐.☐ **cm**

Body Mass Index:
Weight (kgs) / (height (m))^2 ☐☐.☐

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JAMES COOK UNIVERSITY AUSTRALIA

VISIT 2:

Date: ☐☐ / ☐☐ / ☐☐☐☐

LRO Initials: ☐☐ **Participant ID:** ☐☐☐

Recent use of tanning products:
Have you used any tanning products in the past month that may have affected the colour of your skin?

	YES	NO
1. Left inner upper arm	☐	☐
2. Your face	☐	☐

Skin Reflectance:

	Flash Number	400nm	420nm
Left Inner Upper Arm	☐☐☐ ☐☐☐ ☐☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐
		Expected values 11.0 - 33.6%	
Left Cheek	☐☐☐ ☐☐☐ ☐☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐
		Expected values 6.0 - 26.4%	

9756

THE AUSTRALIAN NATIONAL UNIVERSITY

JAMES COOK UNIVERSITY AUSTRALIA

VISIT 3:

Date: ☐☐ / ☐☐ / ☐☐☐☐

LRO Initials: ☐☐ **Participant ID:** ☐☐☐

Recent use of tanning products:
Have you used any tanning products in the past month that may have affected the colour of your skin?

	YES	NO
1. Left inner upper arm	☐	☐
2. Your face	☐	☐

Skin Reflectance:

	Flash Number	400nm	420nm
Left Inner Upper Arm	☐☐☐ ☐☐☐ ☐☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐
		Expected values 11.0 - 33.6%	
Left Cheek	☐☐☐ ☐☐☐ ☐☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐	☐☐.☐☐ ☐☐.☐☐ ☐☐.☐☐
		Expected values 6.0 - 26.4%	

9758

THE AUSTRALIAN NATIONAL UNIVERSITY

JAMES COOK UNIVERSITY AUSTRALIA

VISIT 4

Date: ☐☐ / ☐☐ / ☐☐☐☐

LRO Initials: ☐☐ Participant ID: ☐☐☐

Skin test: Site: _____ Arm: ☐ Right ☐ Left

Comments: _____

_____

Administered by: _____ Time: ☐☐:☐☐

9756

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VISIT 5

Date: ☐☐ / ☐☐ / ☐☐☐☐

LRO Initials: ☐☐ Participant ID: ☐☐☐

Result of Skin Test

Horizontal Axis ☐☐ mm Time: ☐☐:☐☐

Long Axis ☐☐ mm

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AusUVI Sunburn Data Sheet

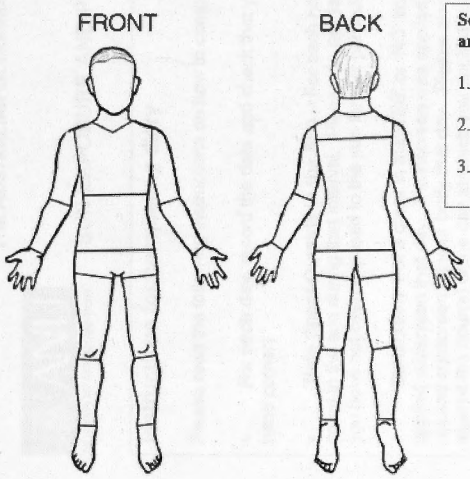
Study Number: ☐☐☐

Study Visit: 2 3 4 5 Circle

Date/s of Sunburn: ☐ ☐ ☐

Day of study: (1 – 30) ☐☐ ☐☐ ☐☐

Site/s of Sunburn / redness / windburn (Shade all affected areas)



Severity of *worst* affected area (circle)

1. Redness without peeling
2. Burn with peeling
3. Very painful with Blistering

THE AUSTRALIAN ULTRAVIOLET RADIATION AND IMMUNITY STUDY

THE AUSTRALIAN NATIONAL UNIVERSITY

JAMES COO UNIVERSIT AUSTRALIA

SUN EXPOSURE AND PHYSICAL ACTIVITY DIARY

Instructions for completing diary

Please read the following instructions on how to complete your sun exposure and physical activity diary.

- For **each day**, record the date and check that your participant study number is entered on each page (tc hand corner)

- **Time Spent Outside in the Sun** – For each hour interval, put a cross in the box under the amount of tir spent in the sun during that interval. Please **do not leave** any hour interval(s) not crossed; ie. Cross '0 minute: you have not been exposed to the sun for that particular hour interval.

- **Sunscreen**: Put a cross in the 'YES' or 'NO' box on the top right of the page to indicate whether you hav applied sunscreen that day. If sunscreen was applied, shade in the area(s) on the diagram to reflect where yo applied sunscreen on your body that day. Please also put a box under the "**Did you apply sunscreen**" columi against the hour(s) of the day at which you applied sunscreen. If you did not apply sunscreen that day, do not cross in any of the boxes under this column.

- **Clothing worn**: Use the '**Sun diary clothing guide**' to fill in the type of clothing you wore for each hour interval that day. Insert the relevant number that matches the picture for upper and lower body, headwear and footwear. Please make sure that you have specified all clothing worn at each hour interval, and that you have **written a number in every box** in this section. Please put a cross against the hour(s) that gloves have been v that day. If no gloves have been worn, do not put a cross in any of the boxes in the gloves column.

- **Level of physical activity**: Referring to the coding guide (0 = No activity; 2=moderate activity; 3 = Vigor activity), specify your level of physical activity for every hour interval throughout the day. If you have complete indoor and outdoor activities within an hour interval, for example, 30 minutes of moderate physical activity bet 5-6am, in which 10 minutes was spent indoors warming-up, and 20 minutes outdoor jogging; write '2' in both in and outdoors box.

* NB: Please do not include driving as a physical activity.

er to the following **EXAMPLE as a guide to filling out the Sun Exposur**
ıctivity diary

– 01 – 2009

t ID – 103 (Will be assigned to you once enrolled)

ı: applied at 7am on face, neck, upper and lower limbs (excluding hands ar
: noon

ıt outside in the sun and type of physical activity:

40 minutes of slow jogging, outdoor
10 minutes of walking to the bus stop and to work
10 minutes of walking to restaurant, outdoor
10 minutes of walking to work place; outdoor
10 minutes of walking to the bus stop and to home
30 minutes swimming; indoor

othing worn:

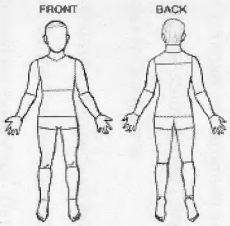
Short sleeved top with shorts, cap, enclosed shoes and gloves
Long sleeved top with long trousers, no headwear, enclosed shoes worn u

SUN DIARY CLOTHING GUIDE

DY	NO CLOTHING ON UPPER BODY						
	0 No upper clothing	1 Bikini	2 Swimsuit	3 Crop top	4 Singlet top	5 Short-sleeved top	Lo
DY	NO CLOTHING ON LOWER BODY						
	0 No lower clothing	1 Speedos/briefs	2 Shorts or short skirt	3 Medium shorts or ¾ pants	4 Long trousers/ jeans	5 Medium skirt	
R	NO HEADWEAR						
	0 No headwear	1 Beanie	2 Cap	3 Legionnaire's cap	4 Bucket hat	5 Wide-brimmed hat	
R	NO FOOTWEAR/ HANDWEAR						
	0 No footwear	1 Thong/open sandals	2 Semi-enclosed shoes	3 Enclosed shoes	4 Workboots	5 Long socks	

DAY 1

Date ☐☐ / ☐☐ / ☐☐☐☐



Participant ID: ☐☐☐

30684

Did you apply any sunscreen today?

☐ Yes ☐ No

IF YES, SHADE THE DIAGRAM ON THE LEFT TO SHOW THE PARTS OF THE BODY THAT YOU APPLIED SUNSCREEN TO TODAY

DID YOU MAKE SURE THAT YOUR WRISTBAND WAS NOT COVERED BY CLOTHING TODAY?

☐ Yes ☐ No

	TIME OUTDOORS (Cross the box which best represents the amount of time you spent outdoors during each one hour interval shown below)					DID YOU APPLY SUNSCREEN	INSERT NUMBER DENOTING TYPE OF CLOTHING WORN (You must write one number in each box)					SELECT NUMBER DENOTING LEVEL OF PHYSICAL ACTIVITY 0 = No physical activity 1 = Mild physical activity 2 = Moderate physical activity 3 = Vigorous physical activity
	0 minutes	<15 minutes	15-30 minutes	30-45 minutes	45-60 minutes	Cross as many as applicable	Upper body	Lower body	Head wear	Footwear	Gloves (cross all that apply)	Indoors / Outdoors
Morning												
5 - 6 am	☐	☐	☐	☐	☐	☐					☐	
6 - 7 am	☐	☐	☐	☐	☐	☐					☐	
7 - 8 am	☐	☐	☐	☐	☐	☐					☐	
8 - 9 am	☐	☐	☐	☐	☐	☐					☐	
9 - 10 am	☐	☐	☐	☐	☐	☐					☐	
10 - 11 am	☐	☐	☐	☐	☐	☐					☐	
11 - 12 am	☐	☐	☐	☐	☐	☐					☐	
Afternoon												
12 - 1 pm	☐	☐	☐	☐	☐	☐					☐	
1 - 2 pm	☐	☐	☐	☐	☐	☐					☐	
2 - 3 pm	☐	☐	☐	☐	☐	☐					☐	
3 - 4 pm	☐	☐	☐	☐	☐	☐					☐	
4 - 5 pm	☐	☐	☐	☐	☐	☐					☐	
5 - 6 pm	☐	☐	☐	☐	☐	☐					☐	
6 - 7 pm	☐	☐	☐	☐	☐	☐					☐	

Please document any problems you had with the UV badge (e.g. The badge was covered by clothing for approx 30 minutes while I was outside at midday; the badge fell off at 3pm; the badge got wet etc).

_____

Appendix I: Electronic UVR dosimeter error checking

This important phase of data cleaning was designed and conducted by James Liley who was based at the National Institute for Water and Atmospheric Research, New Zealand. This section is an adaptation of the methods described by him.

1. Construction of a maximum-UVR model

This part of the analysis sought to identify badge UVR readings which exceeded the maximum possible UVR available. Abnormally high readings of this type can occur due to electrical interference or similar phenomena, and can cause a falsely high value of aggregate UVR to be calculated. To identify such readings, a model of the UVR level attainable under a clear sky at a given hour was produced for the Canberra and Townsville sites. Individual dosimeter badge readings were then compared to this theoretical maximum, and badge readings exceeding twice the clear-sky UVR were marked as noise.

The estimate of maximum available UVR assuming a clear sky was made for each daylight hour during the study period using a database of column ozone levels over each city and solar zenith angles for each time.

An estimate of maximum available UVR under cloud cover was also made for each daylight hour during the study period using a composite of two databases and clear-sky available UVR adjusted for cloud data. Data from the first and second (non-overlapping) databases were used preferentially, with the third being used in areas where data was absent.

Both the maximum potential clear-sky UVR and maximum potential cloudy-sky UVR were plotted along with data for each participant, although only clear-sky UVR was used for filtering data. This was due to the possibility that the contributing sensors (outdoor spectroradiometers) may have been exposed to clouds while the participants of the study were not (i.e. the participants were not assumed to always be close enough to the sensor site to experience cloud cover at exactly the same time as the sensor).

2. Removal of repeats, oscillations, and baseline errors

This part of the analysis located and removed known types of errors in data. These errors included 'repeat sequences', in which identical series of data readings of significant length were found to occur at more than one time, 'oscillations', in which readings would change erratically but remain bounded at a low value for a period of time, and 'baseline drift', in which the badge returns to a non-zero steady level.

A repeat sequence in a sequence of badge readings {*time*[i], *data*[i]} was formally defined as the presence of indices a, b with a<b, and integer n>30 such that

1. $data[a+j]=data[b+j]$ for all $0 \le j < n$

2. At least 10 of $\{data[a], data[a+1]...data[a+n-1]\}$ are nonzero

2. At least 5 of

$$\{|data[a+1]-data[a]|, |data[a+2]-data[a+a]|...|data[a+n-1]-data[a+n-2]|\}$$

are non-zero.

An exhaustive search was conducted for repeat sequences. Only one sequence of significant length was found to be present. An example is shown in figure 1.1.

A baseline drift was deemed to be present in a sequence of badge readings {time[i], data[i]} if the moving median of the values of order 100 was nonzero; that is

$median(data[i-50], data[i-49]....data[i+50]) > 0$ for all *i* in the sequence

Several instances of baseline drift were found that were logged in an 'Error Log' file.

Oscillations were not formally defined but were found easily by observation. Several instances were found, which are logged in the 'Error Log' file.

Appendix J: Development of the interpolation algorithm for missing or erroneous UVR dosimeter data

James Liley (based at NIWA, NZ) developed the interpolation algorithm to replace missing or erroneous data obtained from the electronic dosimeters. This section is an adaptation of the methods described by him.

Participant-specific data obtained from UVR dosimeters was sometimes unreliable for periods of time. Removal of such unreliable data meant that data series for each participant frequently had 'holes' during which data was unavailable. An interpolant function was constructed for each participant to estimate the amount of UVR to which they were exposed each hour. This interpolant could be used to 'fill' the holes, with the interpolant value being used as a substitute to the measured value when the latter was unavailable.

The interpolant estimated individual UVR exposure for a participant at a given hour from the maximum cloud-adjusted UVR available during that hour, a diary entry recording the amount of time the participant spend outside during that hour, and the participant's valid data measurements from other hours. When diary entries or maximum cloudless UVR measurements were unavailable, they were estimated. This meant that some interpolant values were more 'reliable' than others, in that they were more likely to represent the true measurement. The way in which each interpolant value was calculated was logged in order to quantify this.

The calculation of the interpolant was based on the approximation that the proportion of maximum available cloud-adjusted UVR attained by a participant while outside (denoted F, for factor) was constant at a given hour. To illustrate this, assume a participant *P* had a measured UVR dose of u_3 between 10:00 and 11:00 on Day 3, having recorded spending 15 minutes outside, during which the maximum available UVR was m_3. If *P* recorded spending 30 minutes outside between 10:00 and 11:00 on Day 5 during which the maximum available UVR was m_5, the assumption is that u_5, the actual UVR attained by *P* on Day 5, satisfies:

$$\frac{u_3}{m_3}\frac{60}{15} = \frac{u_5}{m_5}\frac{60}{30}$$

This is justified by the assumption that, had *P* spent 60 minutes outside on Day 3 instead of 15, the amount of UVR attained would be four (60/15) times the amount measured for the time (u_3). The LHS of the above equation is thus the proportion of the maximum UVR available during that hour which would have been attained had *P* spent the whole hour outside, which is assumed to be consistent for all Days during the hour 10:00-11:00.

The above equation gives:

$$u_5 = m_5 \frac{30}{60} \frac{u_3}{m_3} \frac{60}{15} = m_5 \frac{30}{60} F$$

where *F* is assumed to be constant for a given participant at a given hour.

In this case

$$F = \frac{u_3}{m_3} \frac{60}{15}$$

In practice, however, *F* is defined as an average of values for at least four days; that is

$$F = \sum_{\{i|u_i defined\}} \frac{u_i}{m_i} \frac{60}{15 d_i} \quad \text{where } d_i \text{ is the time spent outside on day i}$$

A similar strategy was used in the case where the participant recorded spending no time outside, with the equivalent of *F* (*G*) as the ratio of recorded UVR to maximum UVR while diary records were 0.

As the interpolant required a complete diary record, in the sense of a workable estimate of time outside for each participant during each hour they were (meant to be) measuring UVR exposure, a 'complete' diary *D'* was constructed from recorded diary entries *D*. Each missing diary entry $D'(p_0, d_0, h_0)$ for participant p_0 on Day d_0 at hour h_0, was estimated from known values $D(p,d,j)$ according to the formula

$$D'(p_0,d_0,h_0) = \left\{ \begin{array}{l} \mu(D(p_0,d,h_0)) \text{ if at least four such values were available} \\ \mu(D(p,d,h_0)) \text{ if less than four values as above were available} \end{array} \right\}$$

where $\mu(D(p0,d,h0))$ denotes the mean of $D(p0,d,h0)$ overall values of d for which it is defined (that is, the diary entries for that participant during that hour of the day) and $\mu(D(p,d,h0))$ denotes the mean of $D(p,d,h0)$ overall values of *p,d* for which it is defined (that is, the diary entries for any participant during that hour of the day).

Broadly, missing diary values were estimated from diary entries at similar times to those missing; if possible, from that participant during that hour on other days; if not, from all participants during that hour of the day.

The interpolant also required a complete record *M'* of maximum UVR available for each hour during which participants were (meant to be) measuring UVR exposure, which was estimated from measured records *M*. The value $M'(d_0,h_0)$ for Day d_0 at hour h_0 was estimated if unknown as

$$M'(d_0,h_0)=\begin{cases}\mu(M(d_0-5,h_0),M(d_0-4,h_0)...M(d_0+5,h_0)) & \text{if at least four such values were available}\\ M(d_0\pm 365,h_0) & \text{if less than four values as above were available}\end{cases}$$

where $\mu(...)$ denotes the mean over available values, as before. The restriction of the contributing values to five days either side of d_0 ensured that estimations of maximum UVR came from a similar time of year.

Missing hourly values of maximum available cloud-adjusted UVR were estimated from values several days around it if possible, and if not, from those at a similar time and date on the previous year.

The interpolant for participant p_0 on Day d_0 at hour h_0, $I(p_0,d_0,h_0)$, was constructed in one of two ways according to whether the 'complete' diary entry $D'(p_0,d_0,h_0)$ was zero or nonzero. It required the use of the measured UVR values $U(p_0,d_0,h_0)$ where defined, as well as the arrays *D'* and *M'*. $I(p_0,d_0,h_0)$ was defined as:

If $D'(p_0,d_0,h_0)\neq 0$ then

$$I(p_0,d_0,h_0)=F\cdot\frac{M'(p_0,d_0,h_0)}{D'(p_0,d_0,h_0)}$$

$$\text{where } F=\begin{cases}\text{(a) } \mu\left(\dfrac{U(p_0,d,h_0)}{M'(p_0,d,h_0)D'(p_0,d,h_0)}\right) & \text{if at least four such values are available}\\ \text{(b) } \mu\left(\dfrac{U(p_0,d,h)}{M'(p_0,d,h)D'(p_0,d,h)}\right) & \text{if at least four such values are available and (a) was undefined}\\ \text{(c) } \mu\left(\dfrac{U(p,d,h_0)}{M'(p,d,h_0)D'(p,d,h_0)}\right) & \text{if (a) and (b) were undefined}\end{cases}$$

If $D'(p_0,d_0,h_0)=0$ then

$$I(p_0,d_0,h_0)=G\cdot M'(p_0,d_0,h_0)$$

$$\text{where } G=\begin{cases}\text{(a) } \mu\left(\dfrac{U(p_0,d,h_0)}{M'(p_0,d,h_0)}\right) & \text{if at least four such values were available}\\ \text{(b) } \mu\left(\dfrac{U(p_0,d,h)}{M'(p_0,d,h)}\right) & \text{if at least four such values were available and (a) was undefined}\\ \text{(c) } \mu\left(\dfrac{U(p,d,h_0)}{M'(p,d,h_0)}\right) & \text{if (a) and (b) were undefined}\end{cases}$$

Broadly, the interpolant was set as a proportion of the maximum cloud-adjusted UVR according to the diary record of how much time was spent outside. The value was adjusted according to what proportion of maximum UVR was usually obtained when outside. If possible,

the adjustment factor was calculated to be specific for each participant at each hour. If this was not possible, it was calculated to be specific for each participant but non-specific for that hour. If this, too, was impossible, it was calculated to be specific for the hour, but non-specific for the participant (i.e. the average proportion attained by all participants at that hour).

The final dataset, $M'(p_0,d_0,h_0)$, was then simply defined as:

$$M'(p_0,d_0,h_0)=\begin{Bmatrix} M(p_0,d_0,h_0) & \text{if available} \\ I(p_0,d_0,h_0) & \text{if M is unavailable} \end{Bmatrix}$$

This form of interpolant has several useful features and several drawbacks. This interpolant has the advantage of being linear in M and 1/D. Thus for two different days on which the maximum available UVR was identical at a given hour and a participant recorded spending 15 minutes outside on the first day and 30 on the second, the interpolant would have twice the value on the second day as on the first. Likewise, if the participant spent 15 minutes outside on both days but the maximum available UVR was twice as high on the second, the value on the second day would be twice as high.

Where possible, it is only assumed that a constant proportion of available UVR was attained by each participant during a given hour of the day, rather than consistently over the entire duration they were measuring. This allowance was made based on empirical evidence that the proportion of maximum available UVR attained while outside changes through the day.

It is not assumed that when a participant recorded a diary entry of 0 they were exposed to no UVR during that hour. This was based on empirical evidence from existing measurements. Possibly this could be attributed to participants going outside for short periods (e.g. 30s – 1 min) such that the badge recorded a non-zero value, but the total length was not long enough for them to have considered they were going outside for 15 min.

Diary values of 1-4 were individually too sparse to be treated in the same way as '0' values (that is, the interpolant formed by averaging over normalised measured values during which the diary entry was 0). Likewise, the method which was used to interpolate hours with a nonzero diary entry degenerated when the diary entries were 0 (as it involved dividing by the diary entry).

Because the interpolant was constructed from measured data, interpolant values cannot be simply compared to measured values against a null hypothesis of being unrelated

The interpolant tends to be more uniform in appearance than measured data, although average values over most indices are similar. 'Spikes' in measured data are very difficult to predict, and for this reason the interpolant tends to be much lower than measured values at

points where the measured value is very high, and slightly higher when the measured value is at a 'baseline' level.

The interpolation procedure when the diary value is non-zero assumes that attained UVR over one hour is proportional to time spent outside during that hour. However, this assumption would lead to expecting the interpolant value at 0 whenever the diary entry was 0, which did not hold empirically. This meant that two different assumptions were used for the relation of attained UVR to time spent outside, according to whether or not the time outside was recorded as 0.

Appendix K: Peripheral blood mononuclear cell separation, storage and lymphocyte assays

This section provides the standardised protocols used to collect, isolate, freeze and thaw peripheral blood mononuclear cells (PBMCs). Assays to delineate T-helper cell sub-populations by characteristic cytokine markers and cell surface markers are also described here. These protocols were developed from existing laboratory protocols and / or manufacturers guidelines (e.g. PBMC separation using Ficoll gradient (397)).

Separation of PBMCs from whole blood

Commence separation procedures within 12 hours (maximum of 16 hours) of blood being taken from the study participant.

Preparation of Sample:

1. Prepare sample at room temperature (18 –20°C)

2. Ensure blood samples (in Acid-citrate-dextrose tubes; Interpath services Cat No: 455055) are clearly labelled

3. Label 10 mL glass test tubes (2 for every blood sample)

4. Add 2mL of blood sample to a 10mL test-tube with a transfer pipette

5. Add an equal amount of RPMI (Invitrogen Cat. No 21870-092) (final volume 4mL)

6. Mix by drawing the blood and the buffer in and out with transfer pipette. Avoid bubbles. Gently swirl mixture.

Procedure for isolation of Lymphocytes:

1. Label centrifuge tubes (2 for each blood sample)

2. Remove blue cap on the bottle of Ficoll Paque PLUS (GE Healthcare Cat No: 17-1440-03) (Fig 1)

3. Invert the Ficoll bottle several times to ensure mixing

4. Using aseptic technique, pierce the septum of the Ficoll bottle with syringe & attached needle; withdraw 3mL for each centrifuge tube from the inverted bottle (Fig 2).

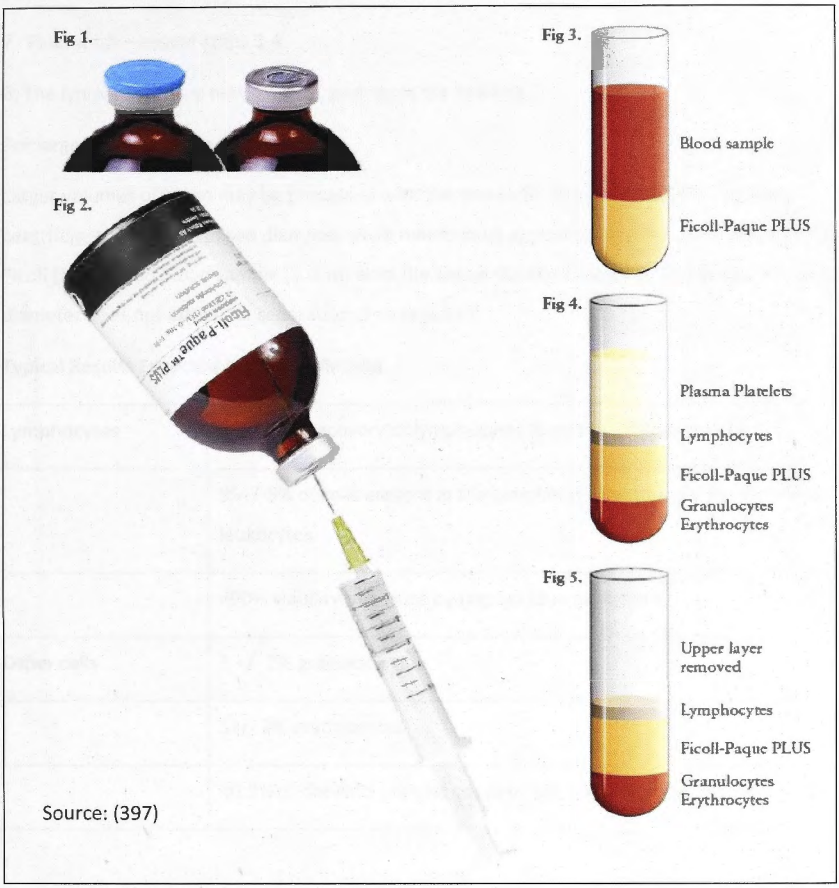
5. Add 3mL Ficoll to each centrifuge tube

6. With Pasteur pipette, carefully layer diluted blood sample (4mL) onto the Ficoll. DO NOT mix the Ficoll and the diluted blood sample. Screw caps on securely. (Fig 3)

7. Centrifuge at $400g_{av}$ for 30 min at 18 – 20°C. No brake.

8. Draw off the upper layer (plasma) using a clean Pasteur pipette leaving the lymphocyte layer undisturbed at the interface. Care should be taken not to disturb the lymphocyte layer. (Fig 4 and 5)

Figure: Steps involved in step-gradient separation of PBMCs from whole blood



Source: (397)

Washing lymphocytes free of platelets

1. With a clean Pasteur pipette with pipette bulb, transfer the lymphocyte layer to a clean centrifuge tube. It is critical to remove all the material at the interface but in a minimal volume. Removing excess Ficoll causes granulocyte contamination; removing excess supernatant results in platelet contamination.

2. Suspend the lymphocytes in 6 – 8mL of RPMI by gently drawing in and out of a transfer pipette.

3. Centrifuge at $300g_{av}$ for 10 minutes at 18 – 20°C.

4. Remove from centrifuge. Carefully tip out and discard supernatant.

5. Repeat steps 2 – 4.

6. Suspend the lymphocytes in 6-8mL of RPMI by gently drawing in and out of a transfer pipette. Prior to last spin, remove 250µL and place in a micro-tube. Perform cell count.

7. Final wash – repeat steps 3-4

6. The lymphocytes are now washed, and ready for freezing.

For larger blood sample volumes

Larger volumes of blood may be processed with the same efficiency of separation by using centrifuge tubes of increased diameter while maintaining approximately the same heights of Ficoll (2.4cm) and blood sample (3.0cm) as in the above standard method. Increasing the tube diameter does not affect the separation time required.

Typical Results Expected from this Method

Lymphocytes	60+/-20% recovery of lymphocytes from the original sample
	95+/-5% of cells present in the lymphocyte fraction are mononuclear leukocytes
	>90% viability (measure by tryptan blue exclusion)
Other cells	3 +/- 2% granulocytes
	5+/- 2% erythrocytes
	<0.5% of the total platelet count of the original blood sample

Protocol for cell freezing

Preparing the freezing medium

The freezing medium is made at least several hours before it is required and is placed in the fridge. Usually there is a bottle already prepared waiting in the fridge. The freezing medium is made up as follows.

1. Use a 50ml Falcon tube as a measuring cylinder, and transfer 70ml of RPMI into a 100 ml glass bottle.

2. Add 20ml of heat inactivated foetal calf serum (Serana Cat No: FCS-500). Mix well by swirling.
3. Add 10 ml of DMSO. It is important to add the DMSO drop by drop whilst gently swirling the FCS medium at the same time. This is because there is an exothermic reaction, generating heat which can precipitate protein from the FCS.
4. When above steps complete, cover glass cylinder with foil; Label with today's date; Place in fridge. Ensure that there is always a stock of freezing solution ready.

Freezing lymphocytes

1. Place ice in an Esky™
2. Label the cryovials with all the details of the cells – cell number, study code, date.
3. Place the required number of cryovials on ice.
4. Tip the required amount of freezing medium from the stock bottle of freezing medium into an appropriate size tube and place on ice.
5. Place the centrifuge tube containing lymphocyte pellet on ice. Cells are to be frozen in aliquots of 2×10^6.
6. Allow everything to pre-cool on ice for 15 mins.
7. Place the ice Esky under the biohazard hood. Turn off the gas flame – the pipette is not to be flamed and the gas generates heat.
8. Place no more than 5 vials in the rack at any one time. Loosen the lids.
9. Rub up the cell pellet in the centrifuge tube, and then add pre-cooled freezing mixture (1 ml per vial) with a 3mL transfer pipette and mix gently.
10. Twist off the loosened lid of a cryovial, add cell suspension and immediately recap the vial. When all vials are filled and capped, transfer them back onto ice.
11. Immediately take the ice Esky with cryovials and isopropanol freezing container (Mr Frosty (Sigma)); Isopropanol (Sigma Cat No: I9516) to the -80°C freezer in the liquid nitrogen freezer room.
12. Transfer the cryovials into the Mr Frosty and place in the -80°C freezer.
13. Leave overnight, or over the weekend before transferring the vials to liquid nitrogen.

Protocol for recovering frozen cells from liquid nitrogen

1. Remove the cryovial containing the frozen cells from liquid nitrogen storage and immediately place it into a 37°C water bath.
2. Quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37°C water bath until there is just a small amount of ice left in the vial.
3. Transfer the vial into a laminar flow hood. Before opening, wipe the outside of the vial with 70% ethanol.
4. Transfer thawed cells into a labelled 15ml tube and while agitating the cell suspension (swirling) add 10mL of cold RPMI (made up as 500mL RPMI + 50mL FCS + 10mL Hepes + 5mL Pen Strep/Glutamine solution) drop-wise.
5. Spin cell suspension at 4 degrees, 300g for 5mins and then decant.
6. Resuspend the cell pellet and repeat steps 4 and 5
7. Gently resuspend the cells in 2mL of RPMI, and transfer into a 10mL test tube. Count the cell concentration using a Tryptan Blue stain.

Lymphocyte assays

Intracellular cytokine staining for Interferon-γ, IL-4, IL-10 and IL-17

1. On a 24 well plate (BD Biosciences; Cat No 351147), label one well 'unstimulated' and one well 'stimulated'. Place $1x10^6$ thawed participant cells into each of these wells and top up to 1mL with RPMI.
2. Thaw a pre-aliquoted vial of Leukocyte Activation Cocktail (LAC) solution with BD Golgi Plug (BD Biosciences Cat No: 550583) and transfer into the well that is labelled stimulated.
3. Incubate for 4hrs at 37degrees, 100% humidity, 5%CO2.
4. In a 96 well plate (BD Biosciences; Cat No 353227) label 2 wells each for Th1/17 unstimulated, Th1/17 stimulated, Th2 unstimulated and Th2 stimulated. These are the experiment wells. Also label a well for unstained control and one well for each single stained control (PerCP, FITC, PE and APC). These act as the control wells.
5. After incubation, add 100µL of unstimulated cells to the four unstimulated labelled wells in the 96 well plate. Do the same for the stimulated cells.
6. Add 40µL of stimulated cells to each of the control wells.
7. Top up each well with 100µL of cold FACS buffer (PBS + 2% FCS + Sodium Azide) and spin at 300g for 5mins.
8. Decant the supernatant, vortex, and wash again with 100µL FACS buffer.
9. Decant, vortex and add 25µL of surface staining antibody cocktail (See below) to all of the experimental wells.
10. Add 25µL of single dilutions of the surface antibodies (see below) to the appropriate control well and 25µL of FACS buffer to the unstained well.
11. Incubate on ice for 20mins in the dark.
12. Add 150µL of FACS buffer to all of the wells and spin at 300g for 5mins.
13. Decant, vortex, and spin again in 100µL FACS buffer.
14. Decant, vortex, add 100µL of CytoFix/CytoPerm solution (BD Biosciences; Cat No 554714) to every well.
15. Incubate for 20mins on ice.
16. Add 100µL of Perm wash (BD Biosciences, 1:10 in MQ water) and spin at 300g for 5mins.
17. Decant, vortex and repeat step 16.

18. Decant, vortex, and add 25µL of Th1/17 intracellular staining antibodies (see below) to each of the experimental wells. The control wells can be left without re-suspending.
19. Incubate on ice for 20mins in the dark.
20. Repeat steps 12 and 13.
21. For the experiment well resuspend cell pellets in 100µL of FACS buffer and combine the duplicate experiment wells into a labelled FACS tube for a final volume of 200µL. For the control wells, resuspend cell pellets in 150µL of FACS buffer and transfer into labelled FACS tubes. Samples are now ready for FACS analysis.

Antibody cocktail

Cell surface staining antibodies

In FACS buffer make up the following dilution of antibodies. Calculate the volume needed by multiplying the number of wells by 25µL.

- CD3 FITC (BD Biosciences; Cat No 555339) 1:100
- CD4 PerCP (BD Biosciences; Cat No 347324) 1:20

For example: 8 wells x 25µL = 200µL, therefore add 2µL of CD3 FITC and 10µL of CD4 PerCP to 178µL of FACS buffer.

Intracellular cytokines staining antibodies

In Permwash, make up the following dilutions of antibodies. Calculate the volume needed by multiplying the number of wells by 25µL.

- IL-17 PE (BD Biosciences; Cat No 560436) 1:6
- IFNγ APC (BD Biosciences; Cat No 341117) 1:50
- IL-4 PE (Miltenyi; Cat No 130-091-647) 1:30
- IL-10 APC (BD Biosciences; Cat No 554707) 1:100

Treg surface staining antibody cocktail

In FACS buffer make up the following dilution of antibodies. Calculate the volume needed by multiplying the number of wells by 25µL

- CD4 PerCP (BD Biosciences; Cat No 347324) 1:50
- CD25 APC (BD Biosciences; Cat No 340939) 1:6
- CD127 FITC (eBioscience; Cat No 11-1278-42) 1:20

Staining for T-helper cells by Foxp3 status

1. In a 96 well plate add $1x10^5$ thawed cells into 2 wells.
2. Add 100µL of FACS buffer and centrifuge for 5mins at 300g.
3. Decant and vortex gently.
4. Add 25µL surface staining antibodies (see below) to each well.
5. Incubate on ice for 30mins in the dark.
6. While incubating prepare fix and perm buffers. Fix buffer is made by diluting FoxP3 buffer A (BD Bioscience) 1:10 in MQ water. Perm buffer is made by diluting FoxP3 buffer B (BD Bioscience; Cat No 560098) 1:50 in Fix buffer. For one patient 200µL of Perm buffer and 400µL of fix buffer is required.
7. After incubation add 150µL of FACS buffer and spin at 300g. Decant, vortex, and wash again with 100µL of FACS buffer.
8. Decant, vortex and then fix the cells by adding 100µL of fix buffer to each well and incubate for 10mins in the dark at room temperature.
9. Repeat step 7 only using 100µL of FACS each time.
10. Add 50µL of Perm buffer to each well and incubate for 30mins in the dark at room temperature.
11. Repeat step 7.
12. Add 25µL of FoxP3 PE antibody (made as a 1:6 dilution in Perm buffer) to each well.
13. Incubate for 30mins in the dark at room temperature.
14. Wash twice with 150µL of FACS buffer.
15. Resuspend in 100µL of FACS buffer and combine into a labelled FASC tube.

Appendix L: KLH vaccine-associated adverse event report

The following report was sent to the Australian Therapeutic Goods Administration as notification of a possible adverse reaction related to the KLH vaccination.

Participant Details

Gender: Female

Date of Birth: 17 Nov 1990 **Age:** 20 years

Ethnicity: Caucasian

Occupation: University student (physiotherapy)

Medical History: Coeliac disease, IgA deficiency, seasonal allergic rhinitis

Medications: 1. Juliet Oral contraceptive pill

Previous Adverse Events to Vaccination: Nil

Study Participation Details

Study Number: 509

Study Site: Townsville

Date of First Visit: 10th August 2010

Date of Vaccination: 17th August 2010 **Site:** L forearm

Date of Skin test: 7th September 2010 **Site:** L forearm

Completed Study: Yes

Details of Adverse event

On day 10 (Aug 27) post-vaccination, participant 509 describes developing lesions over the dorsal aspect of her fingers bilaterally (R>L). She describes the lesions as small (<3mm) that appeared like an "oil splashback burn – red outside with a white raised middle".

By day 15 (Sept 2) post vaccination, she describes over 20 lesions over her fingers and knuckles, with spread to her elbows. Her palms were involved by this stage with 'blisters' up to 5-6mm in diameter. Pain felt on applying pressure to lesions.

On day 21(Sept 7) post vaccination, the blisters were resolving though she consulted a general practitioner at this stage. A punch biopsy was conducted.

On day 24 (Sept 10) post vaccination, the skin biopsy revealed "spongiform dermatitis". No treatment was initiated.

On day 28 (Sept 14) post vaccination, participant reports resolution of lesions on the dorsum of her hands, with the palmar lesions also resolving. AusUVI Study doctor (AS) notified by Townsville Local Research Officer.

On day 29 (Sept 15) post vaccination, participant was contacted by AusUVI Study doctor to review events. Participant did not describe use of new medications, soaps, cosmetics, hand rubs, animal exposures, new work or home environment. She denies having had a fever or systemic symptoms associated with blistering rash. There were no mouth or mucosal involvement and feet were spared. She denies having this type of rash previously. Rash had completely resolved.

AusUVI Study Doctor's Impression

The skin lesions followed the vaccination by 10 days. The lesions occurred only on the hands and forearms and were bilateral. The rash self-resolved without need for treatment. A biopsy revealed non-specific spongiform dermatitis.

Spongiform dermatitis is a non-specific histological finding characterized by inter- and intra-cellular oedema within the epidermis, elongation of the intercellular bridges and inflammatory cells within the dermis. This reaction can occur secondary to primary dermatological conditions, atopic precipitants or contact irritant, infection or drugs.

Individuals with IgA deficiency are at increased risk of atopic and allergic reactions.

This participant did not list any known drug allergies or previous reactions to vaccination.

This reaction has not been previously described in the literature describing KLH use and is not currently listed in the Investigator's Brochure. No similar reaction has occurred to others in the AusUVI Study (currently 44 enrolled – as of 19 October 2010), or in the Pilot study (conducted in 2009, n=21). The vaccine has been very well tolerated – transient mild redness at injection site, the only reported adverse effect in 2 participants in the AusUVI Study.

Classification of Adverse Event: Unexpected adverse drug reaction

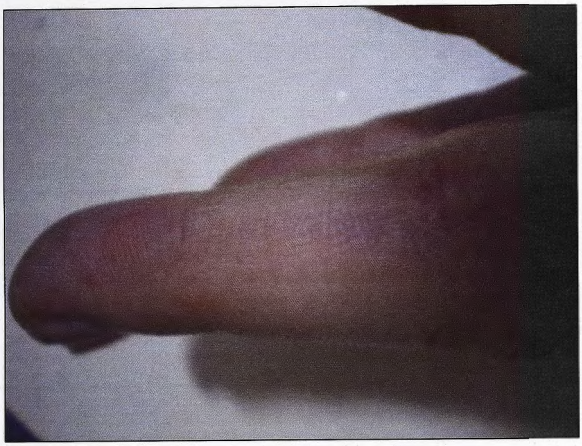
Conclusion: Skin reaction is temporally related to vaccination – a causal relationship between KLH vaccination and skin reaction remains a possibility in the absence of another explanation.

Action:

- Therapeutic Goods Administration notified – ADRAC blue card submitted
- Human Research Ethics Committee and Safety Monitoring Board notified
- IgA deficiency to be added to list of exclusion criteria

- Importance of timely notification of study medical officer re-affirmed when a participant presents with potential vaccine-associated adverse reactions

Picture taken September 14, 2010 (Day 28 post vaccination)



Safety Monitoring Board comment

From Report 1 (July 2010 – 14 October 2010)

"There has been one non-serious unexpected adverse event, a self-resolving non-specific rash (spongiotic dermatitis) 10 days post vaccination, not at the site of vaccination but distally affecting the hands and forearms bilaterally. In reviewing the clinical and histological details of this case, we agree with the classification of this event as 'mild' and the relationship to the vaccination as 'possible'. We recommend that the AusUVI Study continue according to the current ethically-approved protocol."