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# THE MEASUREMENT, TREATMENT AND IMMUNOPATHOLOGY OF LEPROSY TYPE 1 REACTIONS

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# ABSTRACT

Leprosy is a chronic granulomatous condition principally affecting the skin and peripheral nerves. It is caused by infection with the obligate intracellular pathogen *Mycobacterium leprae*. The host immune response of an infected individual determines the disease phenotype. The borderline states of the disease are complicated by immunologically mediated Type 1 reactions in up to 30% of people. Type 1 reactions cause inflammation of the skin and peripheral nerves and can lead to permanent nerve function impairment. The treatment of Type 1 reactions is with oral corticosteroids but there are few data concerning the optimal dose and duration of corticosteroid treatment. Clinical trials have been hampered due to a lack of a valid measure of disease severity.

A clinical severity scale was developed and tested in Bangladesh and Brazil. It was shown to be valid and able to discriminate between mild and moderate and moderate and severe Type 1 reactions. It was also shown to be reliable with excellent inter-observer agreement.

A double blind randomized controlled clinical trial of high dose intravenous methylprednisolone and prednisolone (total dose equivalent to 6.15 g of prednisolone) was compared to placebo infusion and prednisolone (total dose 2.52 g of prednisolone). There were no significant differences in the rate of adverse effects between the two study groups. A large proportion, almost 50%, of individuals in both arms required additional prednisolone. Only 20% of individuals with nerve function impairment completely recovered although another 50% did improve.

Skin biopsies were taken from participants before and at two time points during corticosteroid therapy. These biopsies were stained with monoclonal antibodies directed against toll-like receptors 1, 2, 4 and 9. Toll-like receptor 2 is highly expressed in skin lesions of Type 1 reaction but high expression of toll-like receptor 1, 2 and 4 was found in non-reactional patients with borderline lepromatous and lepromatous leprosy. The expression in the skin of the toll-like receptors 1, 2 and 4 fell during corticosteroid therapy. The gene expression of toll-like receptor 2 and 4 fell during treatment and this change in gene expression was associated with disease outcome. The human acidic ribosomal protein P0 was validated as a control gene in PCR assays in this group of patients.

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For my mother
Joan Walker
13<sup>th</sup> May 1927 – 22<sup>nd</sup> August 1985

# ACKNOWLEDGEMENTS

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

**Appendix 5**

# ABBREVIATIONS

| | |
|---|---|
| AFB | Acid-fast bacilli |
| AGE | Advanced glycation endproducts |
| AMFES | ALERT MDT Field Evaluation Study |
| ANOVA | Analysis of variance |
| APC | Antigen presenting cell |
| ART | Antiretroviral therapy |
| BANDS | Bangladesh Acute Nerve Damage Study |
| BB | Borderline borderline |
| BCG | Bacille Calmette-Guerin |
| BI | Bacterial index |
| BL | Borderline lepromatous |
| BT | Borderline tuberculoid |
| CBP | CREB binding protein (CBP) |
| CD | Cluster of differentiation |
| CIL | Corticosteroid-induced lipodystrophy |
| CMI | Cell mediated immunity |
| CREB | Cyclic AMP response element binding |
| $C_T$ | Cycling threshold |
| DAB | 3, 3' diaminobenzidine |
| DAMP | Damage-associated molecular pattern |
| DC-SIGN | Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin |
| EMBL | European Molecular Biology Laboratory |
| ENL | Erythema nodosum leprosum |
| FITC | Fluorescein isothiocyanate |
| FROM | Full range of movement |
| GILZ | Glucocorticoid-induced leucine zipper |
| GR | Glucocorticoid receptors |
| GRE | Glucocorticoid response elements |
| hARP-P0 | Human Acidic Ribosomal Phosphoprotein P0 |
| HAT | Histone acetyltransferase |
| HIV | Human immunodeficiency virus |
| HLA | Human leucocyte antigens |
| HMGB1 | High-mobility group box 1 |
| hsp | Heat shock protein |
| ICH | International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use |
| IFNγ | Interferon-γ |
| Ig | Immunoglobulin |
| IHC | Immunohistochemistry |
| IKK | IκB kinase-2 |
| IL | Interleukin |
| ILEP | International Federation of Anti-Leprosy Associations |
| INFIR | ILEP Nerve Function Impairment and Reaction |
| iNOS | Inducible nitric oxide |
| IQR | Inter-quartile range |
| IRAK | IL1R-associated kinase |
| IRF | Interferon regulatory factors (IRF) |
| IRIS | Immune reconstitution inflammatory syndrome |
| IV | Intravenous |
| JNK | c-Jun N-terminal kinase |
| LL | Lepromatous leprosy |
| LRR | Leucine-rich repeat |
| LSAB | Labelled streptavidin-biotin |
| LSHTM | London School of Hygiene and Tropical Medicine |
| MAP | Mitogen-activated protein |
| MB | Multibacillary |
| MDT | Multi-drug therapy |

| | |
|---|---|
| MHC | Major histocompatibility complex |
| MID | Minimally important difference |
| MKK 6 | MAP kinase kinase 6 |
| MP | Methylprednisolone |
| MRC | Medical Research Council |
| MS | Multiple sclerosis |
| Mtb | *M. tuberculosis* |
| MUD | Mycobacterium ulcerans disease |
| MyD88 | Myeloid differentiation primary response gene (88) |
| NFI | Nerve function impairment |
| OMEGA | Oral Megadose Corticosteroid Therapy of Acute Exacerbations of Multiple Sclerosis |
| OR | Odds ratio |
| P | Prednisolone |
| PAMP | Pathogen-associated molecular pattern |
| PAP | Peroxidise-anti-peroxidase |
| PASI | Psoriasis Area Severity Index |
| PB | Paucibacillary |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PGL | phenolic glycolipids |
| PMN | Peripheral morphonuclear neutrophils |
| PNL | Pure neuritic leprosy |
| PRR | Pattern recognition receptor |
| QST | Quantitative sensory testing |
| RA | Rheumatoid arthritis |
| RAGE | Receptor for advanced glycation endproducts |
| ROC | Receiver operating characteristic |
| SCORAD | Severity Scoring of Atopic Dermatitis index |
| SD | Standard deviation |
| SN | Silent neuropathy |
| SNP | Single nucleotide polymorphism |
| SPNS | Subjective Peripheral Neuropathy Screen |
| SPSS | Statistical Package for the Social Sciences |
| ST | Sensory testing |
| SWM | Semmes-Weinstein monofilaments |
| T1R | Type 1 reaction |
| TAK1 | TGF$\beta$ -activating kinase 1 |
| TAP | Transporter associated with antigen-processing |
| TB | Tuberculosis |
| TBM | Tuberculous meningitis |
| TGF$\beta$ | Transforming growth factor-$\beta$ |
| $T_h$ | T helper |
| TIR | Toll-IL-1R |
| TIRAP | TIR-domain-containing-adaptor |
| TLR | Toll-like receptor |
| $T_m$ | Melting temperature |
| TNF | Tumour necrosis factor |
| TRAF6 | TNF receptor-associated factor 6 |
| TRAM | TRIF-related adaptor molecule |
| TRIF | TIR-domain-containing adaptor protein inducing IFN$\beta$ |
| TRIPOD | Trials In Prevention of Disability |
| TT | Tuberculoid (leprosy) |
| VDR | Vitamin D receptor |
| VEGF | Vascular endothelial growth factor |
| VMT | Voluntary muscle testing |
| WHO | World Health Organization |

# CHAPTER ONE

## Introduction and literature review

## 1.1 Introduction

Leprosy is a disease recorded in the writings of ancient civilisations in Egypt and India (Rastogi and Rastogi, 1984). It was and remains a highly stigmatizing disease. The first effective antimicrobial agent for the infection dapsone was introduced in the 1940s. Dapsone resistance became an increasing problem in the 1960s but the emergence of rifampicin and clofazimine resistance has not been a clinically significant problem.

The surgeon Paul Brand drew attention to the deleterious effects of the neuropathy of leprosy and how this resulted in the disability and deformity of the disease (Brand, 1952). One aspect of leprosy that contributes to the deterioration in nerve function is the immunological reactions.

The research contained in this thesis has three components each of which improves our ability to understand and study leprosy Type 1 reaction (T1R) and nerve function impairment (NFI). The three themes of the research are:

- the quantification of the clinical severity of T1Rs
- the treatment of T1Rs and NFI with corticosteroids
- the expression of toll-like receptors (TLR) in the skin during T1R

A detailed review of the literature is used to provide context and the rationale for this research.

## 1.2 Literature review of leprosy

Leprosy is a chronic granulomatous infection principally affecting the skin and peripheral nerves caused by the obligate intracellular organism *Mycobacterium leprae* (Lockwood, 2004). The disease causes skin lesions and neuropathy. Complications secondary to the neuropathy can result in deformity and disability. Leprosy remains a stigmatising disease. The early detection of the disease and treatment with multidrug therapy (MDT) which cures the infection is the goal of leprosy control programmes (Britton and Lockwood, 2004). In many individuals leprosy can be effectively treated before disability develops (Britton and Lockwood, 2004).

### 1.2.1 Epidemiology

249 007 new cases of leprosy were diagnosed and reported to World Health Organization (WHO) in 2008 (WHO, 2009). It continues to be an important health problem worldwide with 121 countries reporting cases to WHO. The highest number of new cases detected are

in India, Brazil, the Democratic Republic of Congo, Bangladesh, Nepal, Myanmar and Ethiopia. The disease burden in India is 53.9% of all new cases worldwide. 134 184 cases were reported from India, 38 914 cases from Brazil and 4708 from Nepal in 2008 (WHO, 2009).

The epidemiological indices of leprosy used are the number of new cases detected and registered prevalence (WHO, 2009). The registered prevalence of leprosy is the number of patients receiving MDT during the reported year per 10000 of population. An accurate estimate of the actual prevalence of the disease is not possible because of the prolonged incubation period. The incubation period for tuberculoid leprosy (TT) disease  varied between 2.9 and 5.3 years and between 9.3 and 11.6 years for lepromatous disease in United States military personnel exposed for relatively short periods of time (Noordeen, 1994). However the incubation period has been as long as 30 years in other individuals.

The number of cases reported to WHO is dependent on operational factors and the political will of governments. The operational factors include the ascertainment and registration of cases, the appropriate training and deployment of staff  (Fine, 2008). The reported number of cases increases when active case detection strategies are employed. In 2007 in Maharashtra 254 active cases of undetected leprosy were diagnosed during one survey. The new case detection rate for the surveyed areas ranged from 1.9-9.42 per 10 000 population. This is much greater than the 0.9 cases per 10 000 reported by health posts. Children represented 35% of these cases indicating that active transmission was occurring (Shetty *et al.*, 2009). The registered prevalence is reduced if the duration of treatment is reduced or if people are given a complete course of treatment at the time of diagnosis and discharged. The decline in the number of cases reported to WHO is dramatic from 719 219 in 2000 to 249 007 in 2008 (WHO, 2002; WHO, 2009). This decline has been attributed to the lack of ascertainment and reporting of cases rather than a biologically plausible decrease in the actual number of cases (Fine, 2008; Penna and Penna, 2007).  The eradication of leprosy has not been achieved despite over 25 years of MDT.

The proportion of women diagnosed with leprosy reported to WHO in 2008 varied widely between different geographical regions. The reported range is from 12% in the Philippines to 64.5% in Congo but mostly there is a male preponderance (WHO 2009). The male to female ratio of registered cases was 2:1 in Nepal in 2008. The imbalance has been attributed to social rather than biological factors (Varkevisser *et al.*, 2009).
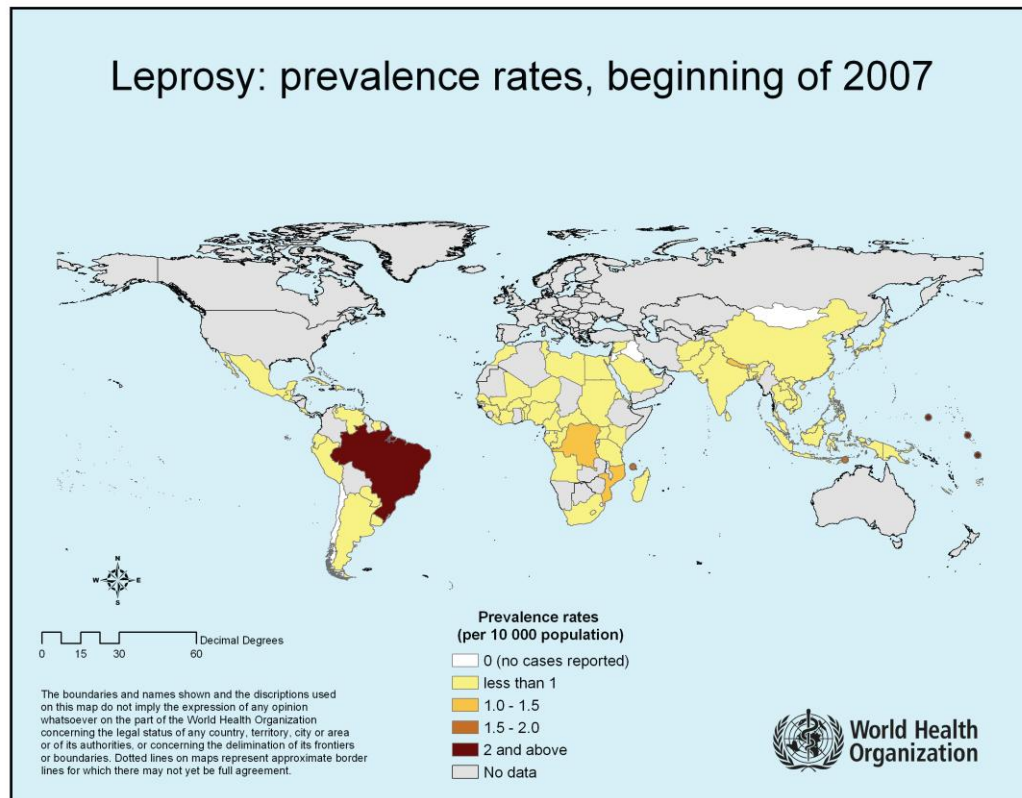
**Figure 1.01. The reported prevalence rates of leprosy 2007 (http://www.who.int/lep/situation/PrevStart2007a.pdf)**

## 1.2.2 Transmission

Transmission of *M. leprae* is thought to be from untreated lepromatous patients to healthy individuals via inhalation of the organism. Immunosuppressed mice can be experimentally infected with *M. leprae* via the airborne route (Rees and McDougall, 1977). 48% of patients with lepromatous leprosy compared with 3% of borderline patients have nasal discharge containing *M. leprae*. The number of acid-fast bacilli (AFB) in a single nasal blow ranged from 1.4 x $10^6$ to 4.3 x $10^8$ (mean 1.1 x $10^8$) in a study of 17 of these patients (Davey and Rees, 1974). Contacts of leprosy patients are at higher risk of developing the disease that the general population. The risk for household contacts of multibacillary (MB) patients in Malawi is as much as eight times that of the general population and for household contacts of paucibacillary (PB) patients approximately two-fold. The risk was greatest for those household contacts residing in rather than simply visiting the household of MB patients. There was no such difference in the risk for the contacts of PB patients suggesting that the PB cases may not be the source of infection (Fine *et al.*, 1997).Nasal carriage of *M. leprae* DNA was found in 8% of healthy subjects in a leprosy endemic region of Indonesia (Hatta *et al.*, 1995). In Ethiopia the rate of *M. leprae* DNA carriage was 5.7% in healthy subjects (Beyene *et al.*, 2003). After entry via the nose *M. leprae* then

spreads to the skin and nerves via the circulation. It is hypothesised that in endemic areas most people have encountered *M. leprae* and have mounted an immune response against it (Hatta *et al.*, 1995).

There are case reports of leprosy occurring following presumed inoculation through the skin during surgical procedures, tattooing or accidental trauma (Brandsma *et al.*, 2005). The organism can persist outside the body under various environmental conditions for up to five months (Desikan and Sreevatsa, 1995). *M. leprae* was demonstrated in the stratum corneum in 60% of untreated patients with borderline lepromatous (BL) leprosy and lepromatous leprosy (LL) in a small study from India. 17% of household contacts of untreated patients had *M. leprae* DNA detectable in washings from their skin (Job *et al.*, 2008).

### 1.2.3 *Mycobacterium leprae*

Armauer Hansen first identified *M. leprae* in 1873 in unstained tissue from the nodules of Norwegian patients (Hansen, 1874). *M. leprae* is the only bacterium that invades and multiplies in Schwann cells and this neurotropism is one of the hallmarks of the organism (Job, 1994). *M. leprae* is an obligate intracellular pathogen. Attempts to culture it in axenic media have failed. It can be obtained following prolonged growth in the mouse foot pad and from the nine-banded armadillo (*Dasypus novemcinctus*) which is a natural reservoir of the organism. In the footpad of the mouse *M. leprae* has a very slow doubling time of approximately 11 days (Levy, 1976).

The organism is an acid-fast bacillus. It is best visualised using carbol-fuchsin based stains. The mycolic acids in the cell wall cause the retention of the dye and prevent decolourisation by acid. *M. leprae* has a peptidoglycan cell wall similar to other mycobacteria. The lipid rich capsule contains phenolic glycolipids (PGL) which are unique to *M. leprae* (Mehra *et al.,* 1984). PGL-1 is the major surface glycolipid and binds complement (Schlesinger and Horwitz, 1991)

In 2001 the genome of an armadillo derived *M.leprae* was sequenced (Cole *et al.*, 2001). The organism appears to have undergone extensive reductive evolution with considerable downsizing of its genome compared to *M. tuberculosis* (Mtb). *M. leprae* has 1604 protein coding genes (1439 of which are common to both organisms). The superoxide dismutases are encoded in the genome of *M. leprae* and these allow it to combat reactive oxygen species produced within the macrophage. Almost half of the genome is occupied by pseudogenes which have intact counterparts in Mtb. *M. leprae* lacks the mbt operon which is required for the production of the mycobactin siderophore which chelates iron. It retains

many genes for haem and iron based proteins. The enzymes formate dehydrogenase, nitrate and fumarate reductase have been lost impairing anaerobic and microaerophilic electron transfer systems. The lack of intrinsic essential components of key biochemical pathways underlies the inability of the organism to grow in culture. It requires the host cell to provide these essential metabolic requirements.

### 1.2.4 Genetics of susceptibility

The host response to *M. leprae* is important in determining the nature of the disease. This has prompted investigators to examine potential genetic factors that predispose to or protect against developing clinical disease following exposure. There have been studies demonstrating higher concordance rates for leprosy among monozygotic compared to dizygotic twins (Chakravartti and Vogel, 1973).

Various genes and regions in the human genome have been linked to or associated with susceptibility to leprosy per se or with a particular type of leprosy. The human leucocyte antigens (HLA) encoded by both class I and class II genes of the major histocompatibility complex (MHC) have been studied in a wide variety of populations. HLA DR2 has been associated with TT leprosy in patients from Thailand (n=32), Brazil (n=32) and India (n=28) (Schauf *et al.*, 1985; Visentainer *et al.*, 1997). The Indian patients had the DRB1*15 allele (Rani *et al.*, 1993). In the same Indian study the association of lepromatous disease (BL (n=25) and LL (n=41)) and the same allele was even stronger. HLA DR2 was also associated with BL leprosy and LL in a study of 50 Turkish patients (Cem Mat *et al.*, 1988). Sixty-nine Chinese multibacillary (MB) patients had a lower frequency of the HLA I genes HLA-B46 and MHC Class I chain-related A than 112 healthy controls (Wang *et al.*, 1999). The transporter associated with antigen-processing (TAP) is formed by two polypeptides TAP1 AND TAP2. The genes encoding these two polypeptides are located between HLA-DP and HLA-DQ in the HLA class II region on chromosome six. A study of 50 patients with TT leprosy from north India showed that these individuals were more likely to have a variant of the *TAP2* gene than healthy controls (Rajalingam *et al.*, 1997).

Mira et al identified that certain alleles in the *PARK2* and *PACRG* region on chromosome 6 are associated with susceptibility to leprosy in 205 Vietnamese patients from 86 families and 587 unrelated Brazilian patients (Mira *et al.*, 2004; Mira *et al.*, 2003). The analysis did not examine any association with clinical type of leprosy. PARK2 is expressed by both Schwann cells and macrophages. It is an ubiquination E3 ligase and is involved in the delivery of polyubiquinated proteins to the proteosome complex involved in protein

degradation (Ciechanover, 2006). However this finding was not reproduced in a study of six single nucleotide polymorphisms (SNPs) in these regions in 286 Indian patients compared to 350 controls (Malhotra *et al.*, 2006).

An Indian cohort of 107 TT leprosy and 124 LL patients and 166 healthy controls were studied for the *Taq1* polymorphism of the Vitamin D receptor (*VDR*) gene. TT leprosy patients were more likely to be homozygous for the polymorphism whereas the LL patients were less likely to have the *Taq1* allele (Roy *et al.*, 1999). This study suggests that the VDR gene may be implicated in the type of leprosy an individual develops. Upregulation of the *VDR* gene on macrophages is associated with increased intracellular killing of Mtb (Liu *et al.*, 2006).

The inflammatory cytokine tumour necrosis factor (TNF) α is essential for granuloma formation. The SNP 308 G → A of the *TNF* promoter region was shown to be associated with increased susceptibility to LL (Roy *et al.*, 1997) in a study of 121 patients. However in a cohort from southern Brazil the same allele was protective against leprosy per se (Santos *et al.*, 2002). A study of Malawians who had predominantly PB disease did not find any association of this *TNF* promoter with leprosy (Fitness *et al.*, 2004).

Interleukin (IL) -10 is associated with the inhibition of TNFα and other T helper ($T_h$) 1 cytokines (Kontoyiannis *et al.*, 2001). 297 Brazilian leprosy patients were compared to 283 healthy controls for five SNPs in the *IL10* promoter. No single SNP was associated with leprosy or leprosy type however one haplotype of three SNPs was protective (Moraes *et al.*, 2004). An extended haplotype in the *IL10* promoter was more frequent in 266 healthy Indian controls than 282 leprosy patients (Malhotra *et al.*, 2005). Another Brazilian study by the same group but using a different cohort of 300 patients showed that homozygosity for the SNP 819 C → T in the *IL10* promoter was significantly more frequent in the patients than controls (Santos *et al.*, 2002).

Two *TLR4* gene SNPs are associated with a lower risk of having leprosy in Ethiopians. SNP 896 G → A and SNP 1196 C → T result in a substitution of an aspartic acid with a glycine and a threonine with an isoleucine respectively (Bochud *et al.*, 2009b).

The differing and sometimes conflicting results of genetic studies may be attributed to differences in study design and sample size. It is also possible that different populations have distinct genetic susceptibilities (Fitness *et al.*, 2002).

**1.2.5 Pathology**

The pathology of leprosy is studied on biopsies taken from affected tissue, most commonly the skin. The tissue is fixed and embedded in paraffin. Standard staining with haematoxylin and eosin is performed along with a stain for AFB. The Fite-Faraco modification of the carbol-fuchsin stain is preferred to the standard Ziehl-Neelsen as it causes less decolourisation. The use of immunohistochemical stains such as anti-BCG and anti-S100 antibodies may also aid histological diagnosis (Gupta *et al.*, 2006).

In biopsies the presence of granulomatous inflammation associated with infiltration and destruction of nerve fibres is characteristic of lesions of TT leprosy. The granulomas extend into the papillary dermis. AFB are not seen and fibrinoid necrosis or caseation are rare phenomena.

The histology of indeterminate skin lesions does not show evidence of granuloma formation. There is a non-specific inflammatory infiltrate around skin appendages.

The biopsies of skin lesions in LL have an atrophic epidermis with loss of the rete ridges histologically. The papillary dermis appears as a clear band (Grenz zone) (Martens and Klingmuller, 1984) whilst the deeper dermis is diffusely infiltrated with macrophages, lymphocytes and plasma cells. The macrophages have a granular cytoplasm but with increasing chronicity they become more foamy and vacuolated. There are abundant AFB both singly or in clumps (Job, 1994).

The formation of small granulomas is characteristic of borderline leprosy. The granulomas becoming more diffuse from borderline tuberculoid (BT) to BL disease. AFB may not be visualised in BT leprosy but are seen in increasing numbers in borderline borderline (BB) leprosy and BL disease.

The pathology of peripheral nerves associated with leprosy starts distally and affects more proximal parts of the nerve as it progresses. Inflammation is both intraneural and perineural. Demyelination and axonal degeneration occur (Scollard, 2008).

In biopsies of peripheral nerves from patients with TT leprosy inflammation of a fascicle may be isolated or all the fascicles may be involved. The granuloma consists of epithelioid cells, lymphocytes and Langhan's giant cells. There is a reactive proliferation of perineural cells. Nerve abscess formation is a well recognised complication of TT disease and is characterised by caseous necrosis which usually contains AFB (Shetty and Antia, 1997).

In LL the intracellular proliferation of the organism is associated with foamy degeneration of Schwann cells. The Schwann cells and axons degenerate. There is marked perineural thickening which is thought to contribute to ischaemia of the already damaged cells. The dead Schwann and axons are replaced by fibrous tissue (Job, 1994).

*M. leprae* infects both Schwann cells and intraneural macrophages. The macrophages and possibly Schwann cells present antigen to T lymphocytes (Krutzik *et al.*, 2005; Spierings *et al.*, 2000). The macrophages secrete inflammatory cytokines such as TNFα.

### 1.2.6 Immunology of leprosy

Infection with *M. leprae* is followed by a subclinical phase during which an unknown proportion of individuals (but probably the majority) will clear the infection without ever showings signs of the disease (Harboe, 1994). Healthy contacts of leprosy patients have lymphocytes that in vitro are more greatly stimulated by *M. leprae* antigen than those of non-exposed controls. This suggests that these individuals have encountered the organism and mounted a successful response to it (Closs *et al.*, 1982).

Another group of individuals will pass through the subclinical phase and develop indeterminate leprosy. This can either heal spontaneously or progress to established clinical leprosy. The immunological response mounted by the host dictates the clinical phenotype that develops. People with leprosy show a spectrum of clinical types. The polar forms of the disease are said to conform to an immunological paradigm. Tuberculoid disease being the result of high cell mediated immunity (CMI) with a largely $T_h1$ type immune response. These individuals who have strong CMI have none or very few organisms in the skin or nerves. Lepromatous disease however is characterised by an anergic response to *M. leprae* with a humoral $T_h2$ response (Modlin, 1994). This lower CMI is associated with large numbers of proliferating bacilli.

The macrophage is the predominant host cell for *M. leprae*. Murine macrophages infected with *M. leprae* are less responsive to IFNγ measured by their ability to restrict the growth of intracellular *Toxoplasma gondii* in culture *in vitro* (Sibley and Krahenbuhl, 1987). *M. leprae* is taken up by macrophages and dendritic cells. Phagocytosis of *M. leprae* by macrophages and other antigen presenting cells (APCs) is facilitated by C3 which is avidly fixed to PGL-1in a dose-dependent fashion *in vitro* (Schlesinger and Horwitz, 1991). Other surface components of *M. leprae* do not fix complement. In murine macrophages phagocytosis of *M. leprae* is regulated by protein kinases. The phagocytosis is blocked *in vitro* by protein kinase inhibitors (Prabhakaran *et al.*, 2000).
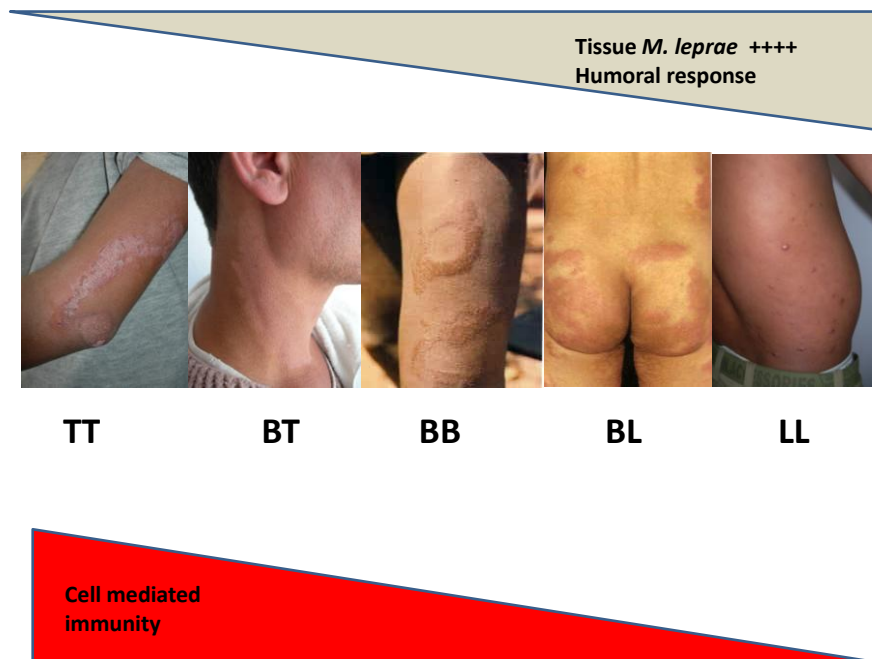
**Figure. 1.02. Ridley-Jopling Classification and features of the host immune response. (BB and BL images reproduced from www.aifo.it)**

In the phagosome *M. leprae* evades immune surveillance mechanisms and in individuals with lepromatous disease is able to proliferate in a lipid-rich environment. The survival of *M. leprae* within the macrophage is facilitated by components of the cell wall which inhibit the macrophage's inherent killing mechanisms such as oxidative stress. APCs present mycobacterial antigen to T cells resulting in activation and proliferation of the T cells. Inflammatory cytokines are produced which further activate the APCs. *M. leprae* infected dendritic cells express PGL-1 on their cell surface. If this expression is blocked *in vitro* then there is increased T cell activation (Hashimoto *et al.*, 2002).

*M. leprae* peptide antigens are presented by either MHC I or II complexes. Antigen presentation also occurs via CD1 molecules which bind lipid or glycolipid antigens and pattern recognition receptors (PRR) of the innate immune system. Intracellular pathogens such as *M. leprae* are initially recognised by the innate immune system. The highly conserved toll-like receptors (TLRs) on the surface of monocytes and macrophages recognise mycobacterial lipoproteins and can lead to the production of IL12 and the expression of inducible nitric oxide (iNOS) (Brightbill *et al.*, 1999). In the case of *M. leprae* this appears to takes place mainly through the TLR1/2 heterodimer and leads to

monocyte differentiation into macrophages and dendritic cells (Krutzik *et al.*, 2003; Krutzik *et al.*, 2005). The latter present antigen and cause the activation of naïve T-cells by IL12 secretion (Demangel and Britton, 2000). The IL12βR2 portion of the IL12 receptor is expressed more on $T_h1$ lymphocytes, preferentially shifting the immune response further towards a $T_h1$ response. TLR stimulation also activates the nuclear transcription factor NFκB which modulates the transcription of many immune response genes (Texereau *et al.*, 2005).

The C-type lectin Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) also known as CD209 is expressed on macrophages in the skin lesions of leprosy patients. This receptor recognises mannose containing carbohydrates including those found in the cell wall of *M. leprae*. DC-SIGN is upregulated following activation of the TLR1/2 heterodimer *in vitro* (Krutzik *et al.*, 2005). Soilleux demonstrated that DC-SIGN was highly expressed in cells in the skin of patients with LL but only sparsely in the skin of some individuals with BT disease (Soilleux *et al.*, 2006). These authors also showed that DC-SIGN was expressed on nearly all cells infected with *M. leprae*. DC-SIGN expressing HeLa cells bind fluorescent *M. leprae*, Mtb and *M. smegmatis* with greater affinity than HeLa cells which do not express this lectin (Barreiro *et al.*, 2006). The binding of *M. leprae* is increased eight-fold. However polymorphisms in the *DC-SIGN* gene did not appear to alter susceptibility to leprosy in 194 Pakistani patients compared to 78 matched controls.

The receptor for advanced glycation endproducts (RAGE) which is a member of the immunoglobulin superfamily binds advanced glycation endproducts (AGE) which are non-enzymatically altered proteins and also damage-associated molecular patterns (DAMPs) (Sparvero *et al.*, 2009). This receptor and an associated ligand EN-RAGE (S100A12) have been shown (using polyclonal rabbit and goat antibodies) to be expressed on the surface of macrophages and endothelial cells in the skin of patients with PB leprosy and patients with MB leprosy (Kim *et al.*, 2006). Activation of RAGE leads to increased expression of inflammatory cytokines and vascular endothelial growth factor (VEGF).

The $T_h1$ /$T_h2$ model of CD4 lymphocyte subsets is used to explain the occurrence of the polar forms of leprosy (TT and LL). The first work to support this was performed on eight patients with TT leprosy and eight with LL (Yamamura *et al.*, 1991). Using PCR of cDNA reverse transcribed from RNA extracted from skin lesions they were able to show that the products when electrphoresed on 2% agarose gel were different in the two groups. The TT patients had bands of greater intensity for IL2, IFNγ and lymphotoxin compatible with a $T_h1$ cytokine pattern. The LL patients had bands for IL4, IL5 and IL10 but these were

much weaker in intensity than the $T_h1$ products. Yamamura *et al* also showed that mRNA expression for TNFα and TGFβ was greater in TT patients. The group had previously shown that in patients with TT leprosy IFNγ and IL2 protein expressing cells are more abundant in the granulomas of skin lesions than in patients with LL (Modlin *et al.*, 1984).

The borderline part of the spectrum is immunologically dynamic and movement between the two polar forms occurs. These shifts in the immunological response underlie the T1Rs that are a feature of the borderline states. The $T_h1$ /$T_h2$ model is not able to precisely explain this important aspect of the immunology of leprosy because it is dichotomous. There has also been a practice of grouping patients into "tuberculoid" and "lepromatous" categories for the purpose of experiments and this may lead to oversimplification of conclusions and less data about the borderline states.

Macrophages under the influence of cytokines, particularly TNFα together with lymphocytes form granulomas. TNFα is essential for the formation of granulomas (Algood *et al.*, 2005).The granuloma envelops infected macrophages and APCs (Russell, 2007). CD4+ cells are found mainly within the granuloma and CD8 cytotoxic T cells in the mantle area surrounding it (Modlin *et al.*, 1988). T lymphocytes in tuberculoid granulomas produce the anti-microbial protein granulysin (Ochoa *et al.*, 2001). Lepromatous disease is characterised by poor granuloma formation. mRNA production is predominantly for cytokines IL4, IL5 and IL10 (Yamamura *et al.*, 1991). IL4 has been shown to down regulate TLR2 on monocytes (Brightbill *et al.*, 1999) and IL10 will suppress production of IL12 (Libraty *et al.*, 1997). There is a preponderance of CD8 cells in LL skin lesions.

Lepromatous patients (LL and BL) were shown to produce greater amounts of IgA, IgG and IgM antibodies to *M. leprae* than BT and TT patients (Melsom *et al.*, 1982). The role of specific antibodies directed against *M. leprae* in the pathogenesis of leprosy is unclear. Anti-PGL-1 antibodies of the IgA, IgG and IgM subtypes are found in the serum of leprosy patients. The detection of the IgM antibody raised against the terminal trisaccharide of PGL-1 forms the basis of the lateral flow test which is an additional tool for classifying but not diagnosing leprosy (Oskam *et al.*, 2003). The test is not sensitive in individuals with PB disease as only 15–40 % of these patients have detectable antibodies. Patients with LL also have increased production of immunoglobulins and antibodies that are not specific for *M. leprae* such as rheumatoid factor, anti-cardiolipin antibodies and cryoglobulins (Bullock *et al.*, 1970).

The balance and complex interaction of cytokines, chemokines, adhesion molecules, their receptors and the cells of the innate and adaptive immune system all play a role in

ultimately determining the particular immune response of the individual to the organism and the resultant immunopathology.

## 1.2.7 Clinical Features

Patients commonly present with skin lesions, numbness or weakness caused by peripheral nerve involvement or more rarely a painless burn or ulcer in an anaesthetic hand or foot. A leprosy reaction may be a presenting feature of the disease (Pfalzgraff and Ramu, 1994). In non-endemic areas the diagnosis is frequently delayed because leprosy is not considered and patients may present to a wide range of specialists (Lockwood and Reid, 2001).

### 1.2.7.1 Cutaneous

Early skin lesions may be rather poorly defined hypopigmented or erythematous macules. Sensation in these early stages may be unaltered.

TT leprosy is characterised by a single or very few lesions. These are macules or plaques with well defined edges. In dark skin hypopigmentation predominates over the erythema or copper colour more usually seen in lighter skin. The lesions are frequently anaesthetic. The anaesthesia is due to destruction of dermal nerve fibres. Anaesthesia may not be present in facial lesions. Involvement of autonomic fibres is often marked and results in dry lesions with a tendency to scale due to loss of sweating. Hairs are reduced in number or may be completely absent. The TT form carries a good prognosis and lesions will often self-heal.

Individuals with BT leprosy have similar lesions to those with TT leprosy but the margins of lesion are less pronounced and less infiltrated. BT lesions tend to be more numerous and larger (fig.1.03a). TT lesions tend to heal before enlarging to greater than 10cm whereas BT lesions may involve a large part of a limb or the trunk. The BT lesions of an affected individual may vary in size and shape.

BB leprosy is very unstable immunologically. Patients may have macular or papular or plaque-like skin lesions or even a combination. Larger lesions may have a geographic appearance and some lesions have an ill-defined outer margin with a well-defined ("punched-out") inner margin.

BL leprosy usually starts with a few macular lesions which become more widespread and symmetrically distributed. The macules become progressively more infiltrated. Papular and nodular lesions may develop and are more defined than those seen in LL. Skin lesions at the lepromatous (BL/LL) end of the spectrum may not have demonstrable sensory loss.

Lepromatous disease may be present for many years before diagnosis. The early skin changes are widely and symmetrically distributed macules. They are poorly defined with mild hypopigmentation and erythema. Flesh coloured or occasionally erythematous papules and nodules may be present. The skin if left untreated thickens due to dermal infiltration giving rise to the "leonine facies" (fig.1.03b).



**Figures 1.03a. BT leprosy and 1.03b. LL**

Hair is lost from affected skin notably from eyelashes and eyebrows (madarosis). *M. leprae* have been demonstrated in hair follicles located in the dermal papilla and the outer root sheaf during anagen and telogen in untreated lepromatous patients. The formative process of the hair shafts, root sheaths and pigmentation was not affected but the authors postulated that changes in the biochemical environment of the dermal papilla may be responsible for hair loss (Gummer *et al.*, 1983).

The nail changes observed in leprosy result from the peripheral neuropathy and are not specific to the disease. Trauma, vascular impairment and infection all contribute in varying degrees (Patki and Baran, 1991).

### 1.2.7.2 Neural

Nerve involvement in leprosy affects sensory, motor and autonomic function of peripheral nerves. Sensory loss is the earliest and most frequently affected modality but a predominantly motor loss can also occur. Enlarged nerves can also be damaged due to entrapment within fibro-osseous tunnels. Reactions cause further nerve damage. The

presence of a skin lesion overlying a major nerve trunk is associated with a significantly increased risk of impairment in that nerve (van Brakel *et al.*, 2005b). Silent neuropathy is an insidious deterioration in sensory or motor function without signs or symptoms of inflammation (van Brakel and Khawas, 1994).

The effect of the disease on nerves leads to disability and deformity such as clawing of the hand. Deformity also occurs through impaired sensation leading to trauma and secondary infection (including osteomyelitis) which causes tissue damage. The increased dryness of the involved skin makes it more vulnerable to damage.

At enrolment in the Bangladesh Acute Nerve Damage Study (BANDS), a prospective cohort study of 2664 patients with leprosy, 6.46% of posterior tibial nerves were impaired (Croft *et al.*, 1999). In the same study 3.23% of the ulnar, 2.2% of the median, 1.18% of the lateral popliteal, 0.79% of the facial and 0.09% of the radial nerves were impaired. Other nerves affected by the disease include the greater auricular, radial and the radial cutaneous nerves. The majority of (83.33%) patients in this cohort had PB disease. The MB patients of the ILEP Nerve Function Impairment and Reaction (INFIR) recruited in India had much higher rates of NFI with 29.9% of posterior tibial nerves and 12.9% of ulnar nerves impaired (van Brakel *et al.*, 2005a).

In TT leprosy damage to peripheral nerves is limited. However in BT leprosy damage to peripheral nerves may be marked and enlargement and tenderness are features. Nerve pain misdiagnosed as joint pain may result in a person being labelled as having arthritis. Nerve involvement results in sensory and/or motor impairment. Nerve function may deteriorate rapidly. Tenderness is less of a feature in BL leprosy. In LL the destruction of dermal nerves leads to a glove and stocking neuropathy, peripheral nerve involvement tends to occur late and is usually symmetrical.

Pure neuritic leprosy (PNL) affects peripheral nerve trunks in the absence of cutaneous signs. PNL may be any disease type (Pannikar *et al.*, 1983; Uplekar and Antia, 1986). PNL accounts for approximately 5% of cases of leprosy in India and Bangladesh (Croft *et al.*, 1999; van Brakel *et al.*, 2005b). The prevalence of PNL in an Ethiopian cohort was 0.5% (Saunderson *et al.*, 2000b).

The presence or absence of anti-PGL-1 antibodies in the serum has been shown to predict which patients are at greatest risk of NFI when used in conjunction with the WHO classification in Bangladesh (Schuring *et al.*, 2008). Seronegative PB patients are at lowest risk of NFI with a cumulative incidence of 3.5%. Seropositive PB and seronegative MB

patients have a medium risk of NFI of 13% and seropositive MB patients have a high cumulative risk of 53%.

### 1.2.7.3 Ocular

A compilation of 47 surveys conducted between 1983 and 1994 in 24 countries with 4772 patients with leprosy showed that the disease had caused of blindness in 3.2% of those studied (Ffytche, 1998). Blindness can have devastating consequences for those who probably already have sensory loss of the hands and feet. The disease compromises the eye through nerve damage and by inflammation due to direct bacillary invasion of the skin or eye itself. These factors can occur in combination and result in the four main causes of visual loss: lagophthalmos (an inability to close the eyes normally), corneal ulceration, acute or chronic iridocyclitis and secondary cataract.

Lagophthalmos results from damage to the zygomatic and temporal branches of the facial (VIIth) nerve. It gives rise to exposure keratopathy. Reduced corneal and conjunctival sensation due to involvement of the ophthalmic branch of the trigeminal (Vth) nerve predisposes to corneal ulceration.

### 1.2.7.4 Nasal

Involvement of the nasal mucosa in LL gives rise to nasal stuffiness which is an early symptom and later epistaxis may occur (Barton, 1976). Infiltration of nasal structures may lead to a saddle deformity due to septal perforation and destruction of the anterior nasal spine (Pfalzgraff and Ramu, 1994). Nasal deformity contributes significantly to the stigma associated with leprosy (Schwarz and Macdonald, 2004).

Laryngeal involvement although extremely rare nowadays, was life threatening before effective chemotherapy was available.

### 1.2.7.5 Systemic

The involvement of other systems seen in LL and BL disease is due to bacillary infiltration of structures and organs. *M. leprae* is found in lymph nodes, bone marrow, the liver, spleen, kidneys and adrenal glands. The lungs do not appear to be affected (Chinen *et al.*, 1997).

Testicular atrophy results from bacillary infiltration in LL and also the acute orchitis of erythema nodosum leprosum (ENL, Type 2 reactions). In a small study of 30 Indian patients with BL leprosy and LL 30% had reduced testicular volume and 10% had gynaecomastia (Abraham *et al.*, 1990). In a study from Japan of 86 men (with a mean age

31

of 65.5 years), who had previously been treated for leprosy, 43% had lower than normal bone mineral density compatible with osteoporosis. These individuals had significantly lower free testosterone than those who did not have osteoporosis. Unfortunately the authors did not include any data concerning the type of leprosy these individuals had had (Ishikawa *et al.*, 2001).

## 1.2.8 Disability

The WHO classifies leprosy disability into three grades (WHO, 1988):

| WHO Disability Grade | Criteria |
| --- | --- |
| 0 | No disability |
| 1 | Loss of sensation in the hands or feet |
| 2 | Visible damage or disability |

**Table 1.01.  WHO Leprosy Disability classification**

A complete motor and sensory neurological assessment is carried out to ensure that nerve function is not deteriorating especially as this can be asymptomatic. 40.9% of the newly diagnosed Indian INFIR cohort had WHO disability grade one and 9.6% grade two at enrolment (van Brakel *et al.*, 2005b). The BANDS cohort had a prevalence of grade one and grade two disability of 9.61 and 5.97% overall (PB and MB patients) at enrolment. However the rate of grade one disability was 28.48% and grade two 18.24% in the MB patients (Croft *et al.*, 1999). In Brazil almost 6% of the new cases reported to WHO in 2008 had grade two disability at presentation (WHO, 2009).

## 1.2.9 Classification of leprosy

The classification of patients is important to determine the appropriate treatment. Classification also enables the clinician to predict those at risk of complications and to give as accurate a prognosis as possible. There are two systems used to classify leprosy patients.

 The Ridley-Jopling System (Ridley and Jopling, 1966) was developed to help improve the understanding of the disease and was intended for research purposes. The system uses clinical and histopathological features and the bacteriological index to classify patients. It categorises leprosy patients into a spectrum with polar TT and LL forms and middle types of BT, BB and BL leprosy. Patients with different disease types exhibit different immunological responses to *M. leprae* (Modlin *et al.*, 1988) (fig.1.02). It is useful as the borderline states are unstable immunologically and can be complicated by reactions. Following the introduction of MDT it was used to decide which patients received PB or MB MDT.

The definition of a PB case was originally any individual with indeterminate leprosy or TT or BT leprosy. MB cases were defined as individuals with a BI $\geq 2$ at any site (WHO, 1982). The PB and MB categories were changed slightly in 1988 when an MB case was defined as any individual with a positive slit skin smear (WHO, 1988). It was subsequently acknowledged that high quality slit skin smear facilities were not widely available (WHO, 1994). A simpler classification based on the number of skin lesions was introduced for use in the field when slit-skin smears are unavailable (WHO, 1998). It is a quick and useful tool which can be employed by a wide variety of health care providers.

| Leprosy type | Number of skin lesions |
|---|---|
| Paucibacillary (PB) | 1-5 |
| Multibacillary (MB) | 6 or more |

**Table 1.02.  WHO Operational Classification of leprosy**

The MB group as it is currently defined is very heterogeneous. It includes some individuals with BT leprosy and all those with BB, BL and LL. In the INFIR study approximately 60% of the cohort of MB patients had a negative bacterial index (BI) (van Brakel *et al.*, 2005b). A similar figure of 63.29% was reported for the BANDS cohort (Croft *et al.*, 1999). The Ridley-Jopling classification is the recommended classification system for use in studies examining immunological processes or genetic susceptibility to leprosy or its complications (Lockwood *et al.*, 2007).

## 1.2.10 Diagnosis and investigations

The diagnosis of leprosy remains a principally clinical one. It is important to take a history to determine risk factors for the disease and the type of symptoms being experienced. The patient should be examined in a quiet room with good light.

The presence of the cardinal signs of leprosy: skin lesions with definite sensory loss or thickened peripheral nerves or the demonstration of *M. leprae* on slit-skin smears or on histology of tissue (skin or nerve) is diagnostic. (Table 1.03).

| Cardinal signs of leprosy |
|---|
| Skin lesions with definite sensory loss |
| Thickened peripheral nerves |
| Acid-fast bacilli on skin smears or tissue biopsy |

**Table 1.03. Cardinal features of leprosy**

Sensory loss is not a feature of the skin lesions affecting patients with BL leprosy or LL. In the Ethiopian ALERT MDT Field Evaluation Study (AMFES) sensory loss in skin lesions was present in 70% of the 594 individuals with leprosy (Saunderson and Groenen, 2000). In a population survey in Karonga district in Malawi anaesthesia was found in only 48.5% of leprosy skin lesions confirmed by histopathology (Ponnighaus and Fine, 1988). The majority of the Malawians found to have leprosy had PB disease. In a study of 225 suspected and definite cases of leprosy in India three examiners identified skin patches with sensory loss in 70.5%, 85.5 % and 86.9% of the cases (Gupte *et al.*, 1990).

A cohort study of MB patients in Mumbai found that 85% (302 of 357) of patients had nerve enlargement on palpation. This was graded on a four point scale as: "no enlargement", "slightly enlarged", "moderately enlarged" and "very enlarged" (Khambati *et al.*, 2009). The ulnar nerve was most commonly enlarged as determined by clinical examination in both this study and the INFIR cohort (van Brakel *et al.*, 2005b). The investigators of the Mumbai study reported that 74% of ulnar nerves were thickened clinically compared to 61.3% of ulnar nerves being definitely thickened. The proportion of thickened posterior tibial nerves in both studies was 50% and 46.4% respectively. In Malawi enlarged nerves were more common in patients who self reported compared to those who were actively detected (Ponnighaus and Fine, 1988).

The cardinal signs elicited by clinical examination are variable in their sensitivity and specificity. The diagnosis may be supported by slit-skin smears (Pfalzgraff and Ramu, 1994). The BI is a logarithmic scale (1-6) quantifying the density of *M. leprae* on a slit-skin smear and is used to assess response to treatment. The proportion of patients enrolled into BANDS that were slit-skin smear negative was 92.83%. Bangladesh appears to have much more PB leprosy than other countries and the reason for this is not clear. In the Ethiopian AMFES cohort the proportion of slit-skin smear negative individuals was much lower at 55%.

The histological examination of a skin biopsy is the gold standard for diagnosis of leprosy and rarely a nerve biopsy may be needed to confirm the diagnosis. A nerve biopsy is performed on a purely sensory nerve (e.g. radial cutaneous or sural nerve).

## 1.2.11 Nerve function assessment

The assessment of nerve function is done by testing sensation and motor function in the face, hands and feet. Motor function is assessed by using the MRC grading system of muscle power (Brain, 2000). The muscles tested in the hand are the abductor digiti minimi

and the first dorsal interosseous innervated by the ulnar nerve. Abductor pollicis brevis and extensor carpi ulnaris innervated by the median nerve and radial nerve respectively. The lateral popliteal nerve in the lower limb which supplies the tibialis anterior and extensor hallucis longus and orbicularis oculi in the face are tested. It is important to ensure that the muscle being tested is isolated by careful positioning. The effect of other muscles is thus removed so that they are unable to provide the movement being tested and give an erroneous result for a muscle that may be paralysed. This may occur if the extrinsic extensor and flexor muscles are allowed to abduct the little finger when abductor digiti minimi is affected (van Brakel *et al.*, 1996).

The method of sensory testing used depends on the availability of equipment and personnel trained to use it. The use of a ball-point pen at four sites on each hand and foot is recommended in the Global Strategy for Further Reducing the Leprosy Burden and Sustaining Leprosy Control Activities (2006-2010) (WHO, 2006). The ball-point pen is used to gently depress the skin such that a dimple of approximately 1 cm across is created at each test site (Anderson and Croft, 1999). The ball-point pen has been shown to be reliable (Anderson and Croft, 1999) and was used in BANDS. Semmes-Weinstein monofilaments (SWM) are able to detect more subtle loss than the ball-point pen (Koelewijn *et al.*, 2003) but require more training of personnel and are less widely available. SWM are standardised graded nylon filaments attached to a handle. The stimulus is applied to the test site until the thread just bends and the patient is asked to indicate where they felt the stimulus (Brandsma, 1981). Three test points are used for each nerve (median and ulnar) in the hand and four for the posterior tibial on the foot (fig. 2.01) (Roberts *et al.*, 2007).The graded weights used in leprosy studies are 200 mg, 2 g, 4 g, 10 g and 300 g. SWM are very reliable when used by trained personnel (Anderson and Croft, 1999). The level of agreement was high but it is important to ensure that training is regularly repeated and inconsistencies associated with technique are corrected (Roberts *et al.*, 2007). SWM have been shown to have good concordance with sensory nerve conduction and quantitative sensory testing (QST) such as thermal thresholds but are less sensitive (van Brakel *et al.*, 2005a).

## 1.2.12 Differential Diagnosis

The manifestations of leprosy are protean and the differential diagnosis is therefore wide. The lesions of vitiligo are depigmented rather than hypopigmented. The hypopigmented lesions of pityriasis alba can be difficult to distinguish from early disease. Pityriasis versicolor and dermatophyte infection may both cause diagnostic difficulty; tinea corporis and faceii because lesions are erythematous plaques. Other granulomatous conditions such

as sarcoid, granuloma multiforme, cutaneous tuberculosis and granuloma annulare may resemble leprosy. In countries where *Leishmania donovani* is endemic post-kala-azar dermal leishmaniasis is a differential diagnosis in LL. The lesions of cutaneous T cell lymphoma may also mimic borderline types of leprosy.

Nerve thickening is a feature of the rare neurological conditions such as hereditary sensory motor neuropathy Type III and Refsum's disease. Amyloid which itself can complicate leprosy can cause nerve thickening.

## 1.2.13 Treatment of the infection

The WHO recommends that patients diagnosed as having leprosy should receive a multidrug combination. MDT was introduced in 1982 following the emergence of resistance to dapsone-only regimes (WHO, 1982). The first-line agents are rifampicin, clofazimine and dapsone. Between 1985 and 2005 14 million individuals received MDT (WHO, 2005).

PB patients are treated with rifampicin and dapsone for six months and the recommendation for individuals with MB disease is three drugs for 12 months (Table 1.04).

| Type of leprosy | Drug treatment | | Duration of treatment (months) |
|---|---|---|---|
| | **Monthly supervised** | **Daily, self administered** | |
| **Paucibacillary** | Rifampicin 600mg | Dapsone 100mg | 6 |
| **Multibacillary** | Rifampicin 600mg, clofazimine 300mg | Clofazimine 50mg, dapsone 100mg | 12 |

**Table 1.04 WHO-recommended MDT regimes**

Rifampicin is the only bactericidal agent in the regimen. *M. leprae* are rapidly killed by rifampicin. The infectivity of *M. leprae* in the mouse footpad is lost after three to four days of rifampicin (Shepard *et al.*, 1974). The infectivity of patients is markedly reduced within four days of a single dose of rifampicin (Hogerzeil and Rees, 1975). The public health risk posed by lepromatous patients is thought to cease to be significant within a "few" days of starting rifampicin (Waters *et al.*, 1978). However no studies have been performed to examine how quickly *M. leprae* loses its viability following the treatment of patients with WHO MDT.

The relapse rates following MDT are low. In PB disease reported rates of relapse are between 0.19 and 2.4% (Boerrigter *et al.*, 1991; Chopra *et al.*, 1990). In MB disease the published rates of relapse are between 0 and 7.7% (Fajardo *et al.*, 2009; Girdhar *et al.*, 2000). The highest reported relapse rate was in 20 of 260 (7.7%) Indian multibacillary patients who were treated with 24 months of MB MDT and all but two had a BI $\geq 4$ (Girdhar *et al.*, 2000).

WHO reduced the recommended treatment period for multibacillary disease from 24 to 12 months (WHO, 1994) but many clinicians advocate 24 months for patients with a BI > 4 at the time of diagnosis because Girdhar et al demonstrated that 90% of relapses occurred in patients with a BI greater than 4 (Girdhar *et al.*, 2000). Individuals in this study who were treated until they were smear negative had a lower relapse rate.

MDT appears to be generally well tolerated but there is little prospective data concerning the rate of adverse effects requiring omission of a component of the three drug regimen. An orange-red discolouration of body fluids occurs for 48 hours after ingestion of rifampicin. It may also cause hepatitis.

Clofazimine treatment causes red-brown skin and conjunctival discolouration and darkening of involved skin which can range from red through to purple or black (Jopling, 1976). This unpleasant effect may make the drug unacceptable to some patients particularly if cosmetically sensitive sites are affected. The discolouration fades slowly on withdrawal of the drug. Clofazimine also causes an ichthyosis on the shins and forearms (Jopling, 1976). Clofazimine crystals may be deposited in other tissues – and in the bowel can cause an enteropathy (Atkinson *et al.*, 1967).

In a retrospective study of 194 Brazilian patients 43.8% experienced adverse effects attributed to dapsone (Deps *et al.*, 2007). Dapsone causes haemolysis which may be severe especially in individuals with glucose-6-phosphate dehydrogenase deficiency (Degowin *et al.*, 1966) and is associated with a severe hypersensitivity syndrome (Lowe and Smith, 1949; Pandey *et al.*, 2007). Dapsone therapy may also cause hepatitis.

In individuals unable to take clofazimine or dapsone then other agents such as minocycline, clarithromycin, ofloxacin or pefloxacin are active against *M. leprae* (Britton and Lockwood, 2004) and can all be used as second line agents. Minocycline causes slate grey skin discolouration in some individuals (Simons and Morales, 1980).

## 1.2.14 Prevention of disability

The early detection of deterioration in nerve function and the rapid introduction of corticosteroid therapy are essential to minimise nerve damage and thus prevent disability.

Secondary damage to neuropathic areas must be prevented. It is important to make the patient aware of activities that put these areas at risk and to give advice about orthotics and protective footwear. Individuals should be taught self-examination and to recognise any areas of trauma. It has been demonstrated in Nepal that training people in self care can reduce the requirement for admission to hospital with plantar ulceration (Cross and Newcombe, 2001). 254 patients were taught self-examination and compared to the same number of randomly selected control patients who had not undergone the training. The OR of admission to hospital for a plantar ulcer for individuals who did not receive training was 1.8 (95% CI = 0.15-0.01).

Damaged neuropathic areas should be protected from further damage by resting the area and any secondary infection treated with appropriate antibiotics. Surgical intervention may be required to debride necrotic tissue and allow drainage of any collection. Reconstructive surgery may have a role in trying to improve function if contractures occur, there is foot drop or when there is eye involvement.

## 1.2.15 Leprosy and human immunodeficiency virus (HIV) infection

In 1991 approximately 10 years after the recognition of the HIV epidemic an editorial in the International Journal of Leprosy stated that there was:

> "Sparse but tantalizing evidence…HIV may increase the incidence of leprosy…..either through shortening the incubation period or by increasing disease penetrance" (Miller, 1991)

The hypothesis that advanced HIV infection would increase susceptibility to *M. leprae* and increase the proportion of patients developing LL was not substantiated nor does leprosy appear to develop more quickly. A case-control study from Uganda did not detect a significant difference in the proportion of HIV positive individuals diagnosed with leprosy and matched controls (Kawuma *et al.*, 1994).

The skin biopsies from individuals with both HIV and *M. leprae* infection (co-infected) have the typical histopathological changes of leprosy. The proportion of CD4+ lymphocytes in the granulomas of BT patients with HIV in Brazil was similar to that of HIV negative BT patients despite the fact that the HIV positive patients had low CD4 counts ranging from (0-379 cells/mm$^3$) (Sampaio *et al.*, 1995). The response to MDT is also similar to that of HIV negative individuals. *M. leprae* does not appear to accelerate the

decline in immune function in HIV disease which tuberculosis (TB) does (Aaron *et al.*, 2004).

The reported series of co-infected patients show that all Ridley-Jopling types are seen but there appears to be an overrepresentation of patients from the tuberculoid end of the spectrum. Fifteen of 22 co-infected individuals were classified as either TT or BT leprosy in a retrospective study from Brazil. Only one individual had LL (Pereira *et al.*, 2004). Three individuals had indeterminate leprosy. The Ridley-Jopling classification was not reported for three individuals. Ten individuals had HIV as their initial diagnosis. Five were diagnosed with leprosy first and seven were diagnosed with both simultaneously. Two individuals developed a T1R during the first six months of antiretroviral therapy (ART). Deps and Lockwood suggested that the occurrence of leprosy or a T1R during the first six months of ART be part of the definition of leprosy as an immune reconstitution inflammatory syndrome (IRIS) (Deps and Lockwood, 2008). Eight individuals developed leprosy in a cohort of 1002 HIV+ patients started on ART in Pune, India between 2003 and 2006 (Vinay *et al.*, 2009).  The incidence of leprosy after starting ART in this retrospective study was 5.22 per 1000 person years. Four of these Indian patients were diagnosed with leprosy in the first six months of ART but the authors state that the two individuals they regarded as having had IRIS presented 28 and 43 months after the initiation of ART.

A retrospective study of 1026 leprosy patients from Brazil found that a greater proportion of the 54 patients with HIV co-infection had BT leprosy compared with HIV negative leprosy patients. The HIV positive group had a significantly greater number of reactions (type not specified) at diagnosis than the HIV negative group but the cumulative rate of reactions in the two groups was similar overall (Sarno *et al.*, 2008). T1Rs have been increasingly reported in individuals with HIV co-infection as part of an immune reconstitution inflammatory syndrome following the commencement of anti-retroviral therapy (Deps and Lockwood, 2008).

A Ugandan study of  nine HIV positive patients with T1Rs reported a similar response to steroids to individuals in the HIV negative group (Bwire and Kawuma, 1994). This small study was not well designed in terms of outcome measures or comparability of severity of the two groups. The current treatment of T1Rs in HIV infected individuals is with corticosteroids just as in uninfected patients. The reported cases of T1Rs in co-infected individuals, whether ART related or not, have all used corticosteroids. One individual required the introduction of azathioprine to control repeated relapses of his steroid dependent T1R (Lawn *et al.*, 2003). The adverse effect of additional immunosuppression in HIV positive patients with T1Rs is unknown.

The influence of CD4 counts, viral load and ART on T1Rs and associated neuropathy requires investigation in prospective cohort studies. The contrast between the interaction of *M. leprae* and HIV and that of Mtb and HIV is striking and may provide important insights into all three diseases.

### 1.2.16 Pregnancy

The interaction between leprosy and pregnancy is well recognised. The development of T1Rs and neuritis is increased in the postpartum period when cell-mediated immunity returns to the pre-pregnant level (Duncan and Pearson, 1982; Lockwood and Sinha, 1999). ENL reactions occur throughout pregnancy and lactation and the onset of nerve damage is earlier than in those who are not pregnant (Duncan and Pearson, 1984). There is little evidence that pregnancy promotes infection or relapse of the disease.

## 1.3 Literature review of leprosy reactions

Leprosy reactions are immunologically mediated complications of the disease which can occur before, during or after successful completion of MDT. T1Rs, ENL and neuritis are immune mediated. Lucio's phenomenon which is regarded as a reaction is probably a result of vascular occlusion rather than immune activation. The main focus of this thesis is T1Rs and associated or isolated NFI. The other types of reaction are discussed briefly first.

### 1.3.1 Erythema nodosum leprosum (Type 2 reactions)

In a retrospective study of 481 BL and LL patients conducted in Hyderabad ENL occurred in approximately 50% of LL and 10% of BL leprosy cases (Pocaterra *et al.*, 2006). The OR for developing ENL was 8.4 for individuals with LL and 5.2 for individuals with a BI $\geq 4$. A retrospective study of 563 Nepali patients with BL leprosy and LL found that 19% experienced ENL. The greater the infiltration of the skin and BI > 4 significantly increase the risk of developing ENL (Manandhar *et al.*, 1999).

The histology of ENL lesions classically shows an intense perivascular infiltrate of neutrophils throughout the dermis and subcutis (Job, 1994). However in a study of ENL lesions from Pakistani patients neutrophils were not seen in 36% (Hussain *et al.*, 1995). Tissue oedema and vessels exhibiting fibrinoid necrosis may also be present. ENL is an immune complex mediated disease. Direct immunofluorescence studies have demonstrated granular deposits of immunoglobulin and complement in the dermis in ENL lesions but not in those of uncomplicated LL disease (Wemambu *et al.*, 1969). There is evidence of T

lymphocyte and macrophage activation and expression of mRNA for TNFα and IL12 in the skin (Moraes *et al.*, 1999). The ratio of CD4:CD8 cells is increased in ENL compared to uncomplicated LL (Kahawita and Lockwood, 2008). High levels of circulating TNFα have been demonstrated in the plasma of some individuals with ENL (Sarno *et al.*, 1991). *In vitro* peripheral blood mononuclear cells (PBMC) from individuals with ENL secrete increased amounts of TNFα following stimulation by *M. leprae* or *M. leprae* antigens compared to individuals with other forms of leprosy (Barnes *et al.*, 1992).

ENL is a systemic disorder affecting many organ systems. The onset is acute but it may pass into a chronic phase and it can be recurrent. ENL produces fever and in the skin painful and tender red papules or nodules (fig.1.04) occur in crops often affecting the face and extensor surfaces of the limbs. The lesions may be superficial or deep causing a panniculitis. Bullous ENL has been described (Rijal *et al.*, 2004) and lesions may ulcerate. Subcutaneous tissue involvement may lead to tethering and fixation to joints causing loss of function. ENL reactions may also produce uveitis, neuritis, arthritis, dactylitis, lymphadenitis and orchitis. The recurrent inflammation of eyes can lead to blindness and the testes to sterility.



**Figure 1.04 Cutaneous ENL**

The majority of ENL reactions require immunosuppression. The more severe ones require high doses of corticosteroids, usually starting with prednisolone 60mg daily. This controls the acute episode but the recurrent nature of the condition means that steroid-induced side effects may become a significant problem. Thalidomide 300-400mg daily has a dramatic effect in controlling ENL and preventing recurrences (Walker *et al.*, 2007). Its use is limited due to teratogenicity (phocomelia) and possible neurotoxicity (although neurotoxicity does not appear to be a problem in leprosy patients).

Clofazimine and pentoxifylline have both been used in ENL but they are less effective than prednisolone or thalidomide (Iyer and Ramu, 1976; Moreira *et al.*, 1998). Colchicine and chloroquine have also been used with limited effect. TNFα blockade with the biological drug infliximab has been used to treat a woman with ENL (Faber *et al.*, 2006). In leprosy endemic settings the risk of TB may be a contraindication to the use of these drugs. The current cost of these agents will also limit their use.

The use of thalidomide and pentoxifylline have been shown to reduce the levels of TNFα *in vivo* in subjects whose ENL has shown clinical improvement (Moreira *et al.*, 1998; Sampaio *et al.*, 1998) . However a study by Haslett et al has demonstrated low TNFα levels in individuals with milder ENL reactions and paradoxically these levels increased during therapy with thalidomide (Haslett *et al.*, 2005). This effect has been noted in toxic epidermal necrolysis as well as other diseases (Wolkenstein *et al.*, 1998). The authors postulate that ENL with systemic involvement may produce the high circulating TNFα levels previously seen and that this may not be the case in milder forms of the condition. Thalidomide has costimulatory effects on lymphocytes as well as inhibiting macrophage TNFα production which may explain the increase in TNFα during treatment in this setting.

### 1.3.2 Lucio's phenomenon

This is a very rare reactional state occurring in lepromatous disease which presents as painful irregular patches. They become purpuric and bullae form. The bullae breakdown leaving widespread areas of ulceration (Moschella, 1968; Rea and Jerskey, 2005). Healing is with scarring. Lucio's phenomenon is associated with severe systemic upset and may be fatal. The mechanism is a cutaneous vasculopathy which is thought to be due to infiltration of the skin causing an inflammatory microthromboembolic occlusion of the dermal vasculature (Azulay-Abulafia *et al.*, 2006; Sehgal *et al.*, 1987). Other authors have described a predominantly vasculitic process in the skin biopsies of patients (Rea and Ridley, 1979). Lucio's phenomenon was first described in Mexico and was thought to be confined to the western hemisphere but cases from India have been reported (Kaur *et al.*, 2005).

### 1.3.3 Neuritis and silent neuropathy

Neuritis is present if an individual has any of the following: spontaneous nerve pain, paraesthesia, tenderness, or new sensory or motor impairment (van Brakel *et al.*, 2005b). It indicates inflammation in the nerve. Nerve pain, paraesthesia or tenderness may precede nerve function impairment (NFI), which, if not treated rapidly and adequately becomes permanent.

van Brakel and Khawas proposed the term "Silent Neuropathy" (SN) to describe the phenomenon of nerve function impairment occurring in the absence of symptoms of inflammation. Other authors had used terms such as "quiet nerve paralysis" (Srinivasan *et al.*, 1982) and "silent neuritis" (Duncan and Pearson, 1982). It is therefore only detected if health workers perform a careful examination of the peripheral nervous system. In Nepal 13% of patients developed SN including 6.8% of new patients who presented with SN. The majority of SN was present at diagnosis or developed during the first year of MDT (van Brakel and Khawas, 1994). In the Mumbai cohort study 3% of participants had SN which had been present for less than six months at the time of enrolment (Khambati *et al.*, 2008). The BANDS investigators reported a cumulative incidence of SN of 28% in MB cases after five years follow-up (Richardus *et al.*, 2004).

SN can occur in isolation from other types of reaction but may precede or be preceded by T1R (van Brakel and Khawas, 1994). They postulated that SN may be due to Schwann cell degeneration or possibly increased CMI (as happens in T1R) however it remains unclear if this is the case why the skin is not affected at the same time. The treatment of SN is the same as for T1R. The duration of SN cannot always be ascertained from the history and so a trial of prednisolone is usually given.

## 1.3.4 Type 1 (reversal) reactions

T1Rs are a major cause of NFI in leprosy and affect up to 30% of susceptible individuals (Ranque *et al.*, 2007). T1Rs may be a presenting feature of leprosy or occur during MDT or even after it has been successfully completed.

### 1.3.4.1 Epidemiology

There have been relatively few epidemiological studies of T1Rs or neuritis in leprosy. Tables 1.05 and 1.06 summarise some of the reports of the frequency of T1Rs. The large variation in these rates is due to the different methodologies used and the changing definitions of PB and MB categories.

30.1% of individuals with borderline leprosy in Nepal develop a T1R (van Brakel *et al.*, 1994). Half of these individuals had demonstrable new NFI. These figures are from a retrospective study conducted at a leprosy referral centre and similar studies conducted in India have reported T1R rates of 8.9% in a cohort from Hyderabad presenting in one year (1985) and followed for almost 6 years, 10.7% in Orissa between 1992 and 2002 and 24.1% in Chandigarh over 15 years (Kumar *et al.*, 2004; Lockwood *et al.*, 1993; Santaram and Porichha, 2004). The cumulative rate in Hyderabad was 24% for PB (tuberculoid and

borderline tuberculoid) patients in the five year period 1982 to 1987 (Hogeweg *et al.*, 1991). 19.8% (60 of 303) of INFIR cohort had a T1R at recruitment (van Brakel *et al.*, 2005b). Thirty-nine per cent (74 of 188) experienced a reaction or NFI during the two year follow-up period. A T1R occurred in 10% (19 of 188) of individuals during the study period (van Brakel *et al.*, 2008). 35.7% of a cohort of MB patients in Malawi experienced a T1R or a deficit in nerve function (Ponnighaus and Boerrigter, 1995). 19.9% of individuals enrolled in a prospective study from a referral centre in Thailand developed a T1R, each patient was followed for a minimum of three years after being diagnosed with leprosy (Scollard *et al.*, 1994). A prospective hospital based study from Vietnam demonstrated a prevalence of T1Rs of 29.1% in 237 patients with mainly BB and BL leprosy (Ranque *et al.*, 2007). A retrospective study conducted in the field in Bangladesh identified T1Rs in 8.8% of individuals (Richardus *et al.*, 1996). A prospective study in Bangladesh with five years follow-up demonstrated a cumulative incidence of T1Rs of 17% in MB patients (Richardus *et al.*, 2004). A prospective field study of 594 individuals with up to 10 years follow-up from Ethiopia reported a rate of T1Rs of 16.5% (Saunderson *et al.*, 2000a).

| Location of study | Type of study | Number of patients | Type of leprosy | Duration of follow-up (years) | Frequency of Type 1 reactions and/or nerve function impairment (%) |
|---|---|---|---|---|---|
| PROSPECTIVE STUDIES | | | | | |
| **Ethiopia** (Saunderson *et al.*, 2000a) | Cohort study | 594 | New patients | 6-11 | 16.5 |
| **Bangladesh** (Richardus *et al.*, 2004) | Cohort study | 2664 | Paucibacillary (PB) and Multibacillary (MB) | PB 3 MB 5 | PB 0.9 MB 17 |
| **Naini and Faizabad, India** (van Brakel *et al.*, 2005b) | Cohort study | 303 | Multibacillary | 2 | 19.8 |
| **Thailand** (Scollard *et al.*, 1994) | Cohort study | 176 | All newly diagnosed types | 3 minimum | 19.9 |
| **Vietnam** (Ranque *et al.*, 2007) | Case-control study | 237 | All types except indeterminate | Not clear. | 29.1 |
| **Malawi*** (Ponnighaus and Boerrigter, 1995) | Randomized trial of MB MDT | 305 | Multibacillary BI ≥2 at any site | Mean follow-up 3 years | 35.7 |

**Table 1.05. The frequency of Type 1 reactions. *These prospective studies used definitions of PB and MB leprosy which differ from the current WHO definitions.**

| Location of study | Type of study | Number of patients | Type of leprosy | Duration of follow-up (years) | Frequency of Type 1 reactions and/or nerve function impairment (%) |
|---|---|---|---|---|---|
| **RETROSPECTIVE STUDIES** | | | | | |
| **Hyderabad, India** (Lockwood *et al.*, 1993) | Leprosy research centre clinic records review | 494 | All types | ≤6 | 8.9 |
| **Orissa, India** (Santaram and Porichha, 2004) | Regional leprosy centre records review | 942 | Patients registered between 1992-2002 | Not clear | 10.7 |
| **Hyderabad, India*** (Hogeweg *et al.*, 1991) | Leprosy research centre clinic records review | 1226 | Paucibacillary (Tuberculoid and borderline tuberculoid 1982-87) | Not clear | 24 |
| **Chandigarh, India** (Kumar *et al.*, 2004) | Tertiary referral clinic records review | 2867 | All types except pure neuritic leprosy | 3-13 | 24.1 at presentation. 33 overall. |
| **Brazil** (Nery *et al.*, 1998) | Leprosy clinic records review | 162 | Untreated slit skin smear positive patients | Not clear | 25.9 |
| **Nepal** (van Brakel *et al.*, 1994) | Leprosy hospital clinic records review | 386 | Untreated patients except those with pure neuritic leprosy | Mean 1.73 | 30.1 |

**Table 1.06. The frequency of Type 1 reactions. *These retrospective studies used definitions of PB and MB leprosy which differ from the current WHO definitions.**

The prospective study from Bangladesh suggests that nerve function impairment and T1Rs occur more than 1.7 times more frequently in men than women (Croft *et al.*, 2000a). This finding needs further confirmation in other studies.

Indian and Ethiopian cohort studies show that patients continue to experience reactions and neuropathy in the third year after diagnosis and beyond (Saunderson *et al.*, 2000a; van Brakel *et al.*, 2008).
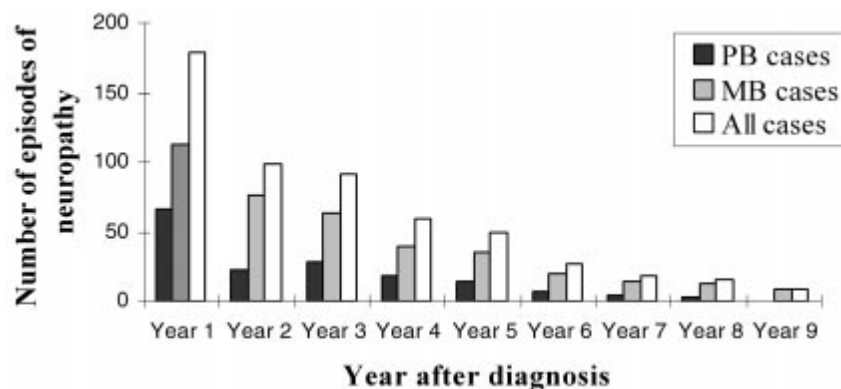


**Figure 1.05. Number of episodes of neuropathy by year after diagnosis in the AMFES cohort (n=594) (Saunderson *et al.*, 2000c)**

45

### 1.3.4.2 Risk factors

Borderline disease is a strong risk factor for the occurrence of T1Rs (Ranque *et al.*, 2007) but small numbers of patients with the polar forms of leprosy may also experience T1Rs (Kumar *et al.*, 2004). Older patients (≥ 15 years) may be at increased risk of T1R than children with leprosy (Ranque *et al.*, 2007). Individuals who have WHO disability grades 1 and 2 at diagnosis are significantly more likely to have severe T1Rs (Schreuder, 1998a). T1Rs can occur at any time but are frequently seen after starting MDT or during the puerperium (Lockwood and Sinha, 1999).

Borderline patients with positive slit-skin smears were more likely to experience a T1R (Roche *et al.*, 1991). A study of Brazilian patients with slit-skin smear negative single lesion paucibacillary leprosy showed that individuals with *M. leprae* DNA detectable by PCR in the skin were more likely to experience a T1R than those in whom *M. leprae* DNA was undetectable (Sousa *et al.*, 2007). Individuals with borderline forms of leprosy who are seropositive for anti-PGL-1 antibodies have an increased risk of T1R (Roche *et al.*, 1991).

Of the 188 participants of the INFIR cohort (n=303) who did not have a T1R or NFI at baseline 69 experienced a T1R and five ENL during the two year follow-up period (Smith *et al.*, 2009). Abnormality in sensory nerve conduction in the ulnar and radial cutaneous nerves at baseline was predictive of a future T1R or ENL. An abnormality in any nerve sensory conduction (except the median nerve) at the assessment immediately prior to the event was predictive.

### 1.3.4.3 Genetic susceptibility

Ethiopian patients with a microsatellite polymorphism in the *TLR2* gene had an increased frequency of T1R. However individuals with the SNP 597 C → T in the *TLR2* gene had a lower frequency of T1R (Bochud *et al.*, 2008).

The SNP 1805 T → G in the *TLR1* gene has been associated with a decreased risk of leprosy T1R in Nepali patients (Misch *et al.*, 2008). This polymorphism appears to lead to a loss of expression of the receptor on the surface of peripheral blood monocytes (Johnson *et al.*, 2007).

### 1.3.4.4 Pathology

The histological features of a T1R are oedema with disorganisation of the granuloma and widespread inflammatory cells largely lymphocytes but also including neutrophils occasionally. The number of AFB may be significantly reduced in BL lesions (Job, 1994).

The diagnosis is usually made clinically but a skin biopsy is sometimes used to help support the diagnosis. Interestingly, even experienced pathologists may under diagnose reaction in skin sections from patients with clinically apparent T1R (Lockwood *et al.*, 2008). Four pathologists were asked to examine the skin sections from 99 patients with a clinical diagnosis of T1R and 52 controls. The agreement between the histological and clinical diagnosis of T1R was low at approximately 50%. The proportion of T1R diagnoses made by the four pathologists ranged between 32-62% of the patients with a clinical diagnosis of T1R. Important diagnostic features appear to be epithelioid cell granuloma oedema, dermal oedema, the presence of plasma cells and granuloma fraction and epidermal expression of HLA-DR.

### 1.3.4.5 Immunology

T1Rs are delayed hypersensitivity reactions that occur predominantly in borderline forms of leprosy (Job, 1994). *M. leprae* antigens have been demonstrated in the nerves and skin of patients experiencing T1Rs. The antigens were localised to Schwann cells and macrophages (Lockwood *et al.*, 2002). Human Schwann cells express TLR2 (Oliveira *et al.*, 2003). *M. leprae* infection may lead to the expression of MHC II on the surface of the cells and this may give rise to antigen presentation which triggers CD4 lymphocyte killing of the cell mediated by cytokines such as TNFα (Ochoa *et al.*, 2001).

A small study from Brazil showed that three patients with BL leprosy who experienced a T1R had a greater number of CD80 positive staining cells in their skin biopsy than the one BL leprosy patient and the two LL patients who did not experience a reaction. The reactional patients also had a greater number of CD80 positive PBMCs identified by flow cytometry than non-reactional individuals (Santos *et al.*, 2007).

There was increased TNFα protein detectable in the skin and nerves of 14 patients during T1Rs. The assessment was made by counting the proportion of cells positively stained using a anti-TNFα mouse antibody (Khanolkar-Young *et al.*, 1995). The results of the immunhistochemistry experiment were supported by TNFα mRNA expression determined using in-situ hybridisation. BT patients had greater levels of staining and mRNA expression than BL leprosy patients.

T1Rs appear to be mediated via Th1 type cells and lesions in reaction express the pro-inflammatory IFNγ, IL12 and the oxygen free radical producer iNOS (Little *et al.*, 2001). This study was performed on serial skin biopsies taken from 15 Indian patients with T1R at baseline, day 7, day 28 and day 180 of treatment with prednisolone. Cytokine expression and cellular infiltration persisted at pretreatment levels until at least day 7. The levels

gradually fell in the majority but some individuals showed persistence of infiltration and cytokine expression even at six months. The expression of mRNA of various chemokines including IL-8, monocyte chemoattractant protein 1 and CCL5 (or RANTES) is higher in the skin during reaction (Kirkaldy *et al.*, 2003). Stefani and colleagues recently showed that 10 smear negative newly diagnosed BT patients with T1R had significantly elevated levels of plasma CXCL10 and IL-6 compared to BT non-reactional controls. None of these individuals had neuritis (Stefani *et al.*, 2009).

The levels of circulating cytokines do not reflect the local changes taking place in the skin during T1Rs. Treatment of the reaction causes clinical improvement but changes in the inflammatory cytokines lag behind by some considerable time and in some may remain unchanged (Andersson *et al.*, 2005). A similar seemingly paradoxical finding has also been demonstrated in tuberculous meningitis (TBM) (Simmons *et al.*, 2005). This variation in the inflammatory activity within different compartments may help to explain why treatment is not always effective. The inflammatory cytokines produced during a T1R may affect local conversion of endogenous corticosteroids (the cortisol-cortisone shuttle) in the lesional skin of leprosy patients with T1Rs (Andersson *et al.*, 2007). The gene expression of the enzyme 11β-hydroxysteroid dehydrogenase type 2 which converts the active cortisol back to inactive cortisone is decreased in the skin of patients with T1R compared to non-reactional controls. This supports the hypothesis that local endogenous active glucocorticoid levels are increased during T1R in response to the marked inflammation that has been triggered but are insufficient to suppress it. In patients with untreated pulmonary TB there is an increase in the urinary metabolites of cortisol compared to successfully treated and healthy controls (Baker *et al.*, 2000).

### 1.3.4.6 Clinical features

A T1R is characterised by acute inflammation in skin lesions or nerves or both. T1Rs predominantly affect the borderline states of leprosy. Skin lesions become acutely inflamed and oedematous and may ulcerate (fig. 1.06). Oedema of the hands, feet and face can also be a feature of a reaction but systemic symptoms are unusual.

T1Rs are frequently recurrent and this can lead to further nerve damage (van Brakel *et al.*, 1994). The detection of NFI is done clinically. Graded SWM (or a ball-point pen) are used to detect sensory loss. Voluntary muscle testing is used to assess motor nerve function. A recent study by van Brakel et al, using nerve conduction studies and quantitative sensory testing, has demonstrated that individuals experiencing neuritis, NFI or reactional episodes either alone or in combination have evidence of sub-clinical neuropathy up to 12 weeks prior to clinically detectable changes (van Brakel *et al.*, 2008).

**Figure 1.06. Inflamed skin and peripheral oedema in patients experiencing leprosy T1R**

In the INFIR Cohort study the 12 individuals who were diagnosed with a T1R limited to the skin had demonstrable sub-clinical nerve involvement using sensory nerve conduction and/or warm detection thresholds (P. Nicholls, personal communication). Sub-clinical changes in nerve function are predictive of further nerve impairment (Smith *et al.*, 2009).

### 1.3.4.7 Treatment of Type 1 reactions

The use of adrenocorticotrophic hormone in the management of leprosy reactions was first reported by Roche *et al* in 1951 (Roche *et al.*, 1951). The response of NFI to corticosteroids is highly variable with 33-73% of nerves recovering (Croft *et al.*, 2000b; Saunderson *et al.*, 2000c). There are few good data for making evidence-based treatment decisions about managing T1Rs or NFI. This was highlighted by the Cochrane systematic review "Corticosteroids for treating nerve damage in leprosy" by van Veen *et al* (van Veen *et al.*, 2007). Three randomized controlled trials were included in the review. The sole trial which examined the effect of corticosteroids in T1R did not fulfil the initial inclusion criteria of the review.

| Country, Year and Type of study | Criteria for review | Number analysed | Measures | Conclusion |
|---|---|---|---|---|
| **India** (Santaram and Porichha, 2004) | All reactions | 101 Type 1 reactions of 942 cases | "Satisfactory response" | 95.2% of all reactions had satisfactory response |
| **Indonesia** (Bernink and Voskens, 1997) Field study | Nerve function impairment in all types of reaction | 154 | Improvement, the same or worse | 75% of nerves improved in all types of reaction. |
| **Nepal** (van Brakel and Khawas, 1996) | Nerve function impairment | 168 | Comparison of nerve function at 3 and 6 months after steroids | Up to 47% showed no functional improvement |
| **India** (Lockwood *et al.*, 1993) All cases from 1985 | Type 1 reaction | 44 Type 1 reaction of 494 cases | Improvement in symptoms and signs | 93% 0f skin lesions and 50% of neuritic episodes responded |
| **Ethiopia** (Becx-Bleumink and Berhe, 1992) | All reactions | 365 Type 1 reactions | Recurrent reaction Nerve function loss | Approx a third of BL patients relapse as steroids cut. 25% of nerves do not improve |
| **India** (Kiran *et al.*, 1991) | ≤6 months of facial nerve damage with lagophthalmos | 27 (36 eyes) | Degree of eyelid lag in mm | 64% had a good response |
| **Ethiopia** (Naafs *et al.*, 1979) | Neuritis of selected patients | 48 | VMT deficit | A longer course is better than a short one. |

**Table 1.07. Retrospective reports of steroids in Type 1 reactions and/or nerve function impairment**

Table 1.07 summarises reports of retrospective studies of the effect of corticosteroids on T1Rs and/or nerve function impairment in patient series from Ethiopia, India, Nepal and Indonesia. Only limited conclusions can be drawn from these series. These studies suggest more favourable responses to corticosteroids than the prospective data from the more rigorous studies in Tables 1.08 and 1.09. Despite this they clearly indicate a less than satisfactory response of T1Rs or isolated nerve function impairment to corticosteroids. Tables 1.08 and 1.09 summarise the published studies of prospective cohorts in which systemic corticosteroids or other immunosuppressants were used to treat T1Rs and/or nerve involvement due to leprosy. Studies that were not formal clinical trials were included if there was a clearly stated clinical outcome. There are only five randomized studies four of which were conducted in south Asia.

The trial from Brazil (Garbino *et al.*, 2008) is difficult to interpret in a meaningful way because the authors used a scoring system which had not been validated and grouped

individuals with T1R and ENL together. The amount of prednisone used by each individual varied but was not stated.

| Country, Year and Type of study | Entry criteria | No. | Intervention | Outcome measures | Conclusion |
|---|---|---|---|---|---|
| **Brazil** (Garbino *et al.*, 2008) Randomised, controlled | Type 1 reactions or ENL associated with ulnar neuropathy | 21 | Prednisone 120mg daily initially compared with 60mg daily initially for controls. Tapered variably. | Clinical Score and motor nerve conduction | Difficult to compare groups. Clinical Score was not validated. Both types of reaction analysed together. "Significant improvement over time" |
| **India** (Rao *et al.*, 2006) Double-blind randomised controlled, parallel group | "Severe" Type 1 reactions | 334 | 3 prednisolone regimes: 3.5g over 5 months 2.31g over 5 months 2.94g over 3 months | Amount of extra prednisolone required | The 5 month regimes were equally effective and less additional prednisolone was required by these two groups than by the 3 month group |
| **Nepal** (Marlowe *et al.*, 2004) Randomised, controlled | Type 1 reactions skin or skin and nerve | 40 | 12 weeks azathioprine and 8 weeks prednisolone compared to 12 weeks prednisolone alone | Skin signs, nerve tenderness, sensory and motor testing and amount of extra prednisolone required | Equally effective |
| **Nepal, Bangladesh** (Richardus *et al.*, 2003b) Randomised placebo controlled, double blind | NFI of 6-24 months duration. | 92 | 16 week standard prednisolone regime | Sensory and motor test scores | No difference |
| **Nepal, Bangladesh** (van Brakel *et al.*, 2003) Randomised placebo controlled, double blind | Isolated mild sensory impairment | 75 | 16 week standard prednisolone regime | Improvement in monofilament scores. | No difference between treated and untreated groups. |

**Table 1.08. Prospective randomised studies using steroids in Type 1 reactions and/or nerve function impairment.**

| Country, Year and Type of study | Entry criteria | No. | Intervention | Outcome measures | Conclusion |
|---|---|---|---|---|---|
| **Ethiopia, Nepal** (Marlowe *et al.*, 2007) Open, uncontrolled | Severe acute Type 1 reactions | 43 | 12 weeks ciclosporin 5mg/kg and prednisolone 40mg for first 5 days Ciclosporin increased to 7.5mg/kg if deterioration | Skin and nerve score Improvement in clinical outcomes and relapse | Variable improvement in skin and nerve signs. High levels of recurrence of reaction particularly in Ethiopian patients. |
| **Ethiopia** (Saunderson *et al.*, 2000c) Prospective observation field study | Neuropathy including nerve tenderness | 594 | Steroid regimes for PB (12 weeks) and MB (24 weeks) patients | Motor and sensory testing and symptom improvement | 73% of all neuropathy given steroids responded fully in 73 patients with no impairment at diagnosis |
| **Bangladesh** (Croft *et al.*, 2000b) Prospective, open, uncontrolled | NFI | 132 | 16 week standard prednisolone regime | Improvement | 33% of motor nerves and 37% of sensory nerves fully recovered at 12 months. 67% of nerves improved |
| **Thailand** (Schreuder, 1998b) Observation study | Newly diagnosed leprosy patients | 640 | Not clear | Nerve function | Nerve damage at presentation improves in only 44% compared to 82% improvement in damage developing whilst on treatment |
| **Nepal** (Wilder-Smith and Wilder-Smith, 1997) | Skin signs - obligatory Nerve signs - optional Oedema or fever - optional | 18 | Prednisolone starting at 40mg and tapered according to individual response | Nerve function | 21.2% improved sensory function and 1.3% improved motor function |
| **India** (Kiran *et al.*, 1985) ?Prospective Open. | Impaired VMT or ST | 33 | Semi-standardized prednisolone regime | Nerve score | Good result in 74% of nerves (No controls) |
| **Ethiopia** (Touw-Langendijk *et al.*, 1984) Open, uncontrolled | Recent nerve function loss | 36 | 6 month course of prednisolone | Sensory and motor function | 63% of affected nerves (59/93) "improved" |

**Table 1.09. Non-randomised prospective studies using steroids in Type 1 reactions and/or nerve function impairment.**

Different methodologies employing different entry criteria and outcome measures have made it difficult to compare studies. The grouping together of all individuals with T1R regardless of whether new NFI is a feature of the reaction makes it difficult to assess the impact on nerve function of the treatments being studied. Studies have also used different features of nerve involvement such as nerve function impairment and neuritis as entry criteria and outcome measures.

It is difficult to compare studies that use improvement as an outcome with those that use the more stringent criterion of recovery. Some published studies have even looked at T1Rs

and ENL together despite their different aetiology, clinical presentation and response to treatment.

Several studies have indicated that some nerve function impairment will improve without steroid therapy. This improvement may be spontaneous or attributable to MDT (Croft *et al.*, 2000b; Saunderson *et al.*, 2000c; Schreuder, 1998b). The BANDS cohort included 69 individuals with NFI who should have received prednisolone but did not. In these patients 33% of involved motor nerves and 62% of sensory nerves had some degree of improvement at 12 months follow-up (Croft *et al.*, 2000b). The AMFES cohort included 141 individuals with NFI at the time of enrolment which had been present for longer than six months and so were not treated with steroids. Between a quarter and a third of nerves with this longstanding impairment fully improved during the long period of follow-up (Saunderson *et al.*, 2000c).

The effective killing of *M. leprae* by MDT may improve neuropathy which is due to direct bacillary invasion of nerves and allow some axonal regeneration. The phenomenon of spontaneous improvement in nerve function is another confounder in determining the size of the effect of any intervention being studied. It would now be unethical to conduct a trial of the effect of steroids compared to inactive placebo.

The treatment of T1Rs is aimed at controlling the acute inflammation, easing pain and reversing nerve damage. MDT is initiated in those presenting with a T1R or continued in those who develop a reaction whilst on it. Individuals with inflamed skin plaques, neuritis or nerve function impairment are treated with oral corticosteroids. Different regimes have been employed in the management of T1Rs.

A randomized study of three different prednisolone regimes suggested that duration of treatment, rather than the starting dose of prednisolone, may be more important in controlling T1Rs (Rao *et al.*, 2006). This was an Indian multicentre study of 334 patients treated with prednisolone. Prednisolone 30 mg tapered slowly to zero over 20 weeks (total dose = 2.31 g) was superior to prednisolone 60 mg tapered over 12 weeks (total dose 2.94 g). There was no significant difference between prednisolone 30 mg or 60 mg (total dose 3.5 g) tapered over 20 weeks. Individuals both with and without nerve involvement were enrolled into the study. The primary outcome measures were failure to respond to treatment and physician determined requirement for additional prednisolone rather than improvement in nerve function or skin signs.

The Trials In Prevention of Disability (TRIPOD) were three randomized controlled trials of MB patients conducted in Nepal and Bangladesh. The first and largest of these TRIPOD 1

examined the role of a four month course of prophylactic prednisolone in the prevention of reactional episodes, neuritis and nerve function impairment (Smith *et al.*, 2004). 636 individuals were enrolled and received either prednisolone 20mg for 12 weeks and tapered to zero over a further four weeks (total dose 1.96 g) or placebo. The prednisolone had a protective effect whilst patients were taking it but at 12 month follow-up this effect had been lost

The current WHO document: The Global Strategy for Further Reducing the Leprosy Burden and Sustaining Leprosy Control Activities (2006-2010) states that "Severe reversal reactions should be treated with a course of steroids, usually lasting 3-6 months" (WHO, 2006). Only 60% of individuals will show improvement in nerve function with 12 weeks of oral prednisolone (van Brakel and Khawas, 1996). Skin lesions will readily respond.

The TRIPOD 3 study randomized 92 MB patients with NFI that had been present for between 6 and 24 months to either a 16 week course of prednisolone (total dose 2.52 g) or placebo (Richardus *et al.*, 2003b). There was no significant improvement in this longstanding NFI.

A trial in which individuals with ulnar neuritis were randomized to either six weeks prednisolone or medial epicondylectomy and six weeks prednisolone demonstrated improvement in nerve function in both groups but did not show any added benefit of surgery (Pannikar *et al.*, 1984). A study from Senegal in 31 patients with neuritis who were treated with prednisone for six months did not demonstrate any additional benefit of early surgery in those nerves randomized to receive a decompression procedure and epineurotomy (Boucher *et al.*, 1999).

Azathioprine in combination with an eight week course of prednisolone was as effective as a 12 week course of prednisolone in the management of T1Rs in a pilot study in Nepal (Marlowe *et al.*, 2004). Ciclosporin has been used in pilot studies in Nepal and Ethiopia with some success (Marlowe *et al.*, 2007).

**1.3.4.8 Aim and hypothesis 1**

I have shown that although the clinical entity of T1R and NFI is well recognised, the assessment of the clinical severity of these complications of leprosy is not currently possible due to a lack of a validated tool. This has made the interpretation of the small number of controlled trials and cohort studies difficult. These and future studies are diminished in their clinical relevance because of this.

- **AIM 1:** To develop and validate a severity scale for T1Rs.

- **HYPOTHESIS 1:** The development of a reliable and valid severity scale for T1Rs and leprosy associated NFI can be done using symptoms and signs assessed on clinical examination

## 1.4 Literature review of the actions of corticosteroids

Corticosteroids bind to specific glucocorticoid receptors (GR) in the cytoplasm of the cell. The receptors then may dissociate from their chaperone proteins (such as heat shock protein (hsp)-90) and this exposes sites on the receptor necessary for transport of the GR-steroid complex across the nuclear membrane. In human embryonic cells made to express mouse-GR reduced acetylation of hsp90 is associated with less stables steroid binding capacity (Murphy *et al.*, 2005). The relocation is under the control of nuclear import proteins such as importin α and importin β (fig.1.07) (Pratt *et al.*, 2004). Alternatively active transport of the chaperone-GR steroid complex may occur along cytoplasmic microtubules but it is not clear whether hsp90 is still bound to the GR at the point of entry into the nucleus (Grad and Picard, 2007).

Once in the nucleus the GR-steroid complexes form dimers and bind to the promoter region of steroid responsive genes known as glucocorticoid response elements (GRE). Activation of GRE leads to the transcription of genes encoding anti-inflammatory mediators such as annexin-1, MAP kinase phosphatase-1, IκBα, secretory leukocyte protease inhibitor and glucocorticoid-induced leucine zipper (GILZ) (Barnes, 2006; Perretti and D'Acquisto, 2006).

Activated GR-steroid complexes may also interact with the coactivator molecules and transcription factor complexes in the nucleus. This inhibits the activity of histone acetyltransferases (HATs), preventing chromatin remodelling and thus reducing the production of proinflammatory cytokines. It has been shown in vitro by immunoprecipitation that the corticosteroid dexamethasone when bound to GR recruits histone deacetylases to reverse chromatin remodelling in a dose-dependent fashion (Ito *et al.*, 2000). The activity of HATs requires the steroid receptor coactivator 3 and has been shown to increase the expression of cathelicidin mRNA in normal human epidermal keratinocytes in response to Vitamin D (Schauber *et al.*, 2008).

In higher concentrations (such as is the case with pulsed methylprednisolone (MP) therapy) corticosteroids may also have nongenomic effects such as inhibiting transcription factors and destabilising mRNA by binding to adenine/uridine rich elements (Barnes, 2006). There is some indirect evidence to support this post-transcriptional effect of corticosteroids. In an alveolar carcinoma cell line (A549) IL1β induced cyclo-oxygenase 2 mRNA expression

increases until three hours and then gradually declines over the following three hours whereas the addition of dexamethasone results in a rapid decline of mRNA to almost undetectable levels (Newton *et al.*, 1998). The addition of an RNA polymerase II blocker to the system instead of dexamethasone had no appreciable effect.
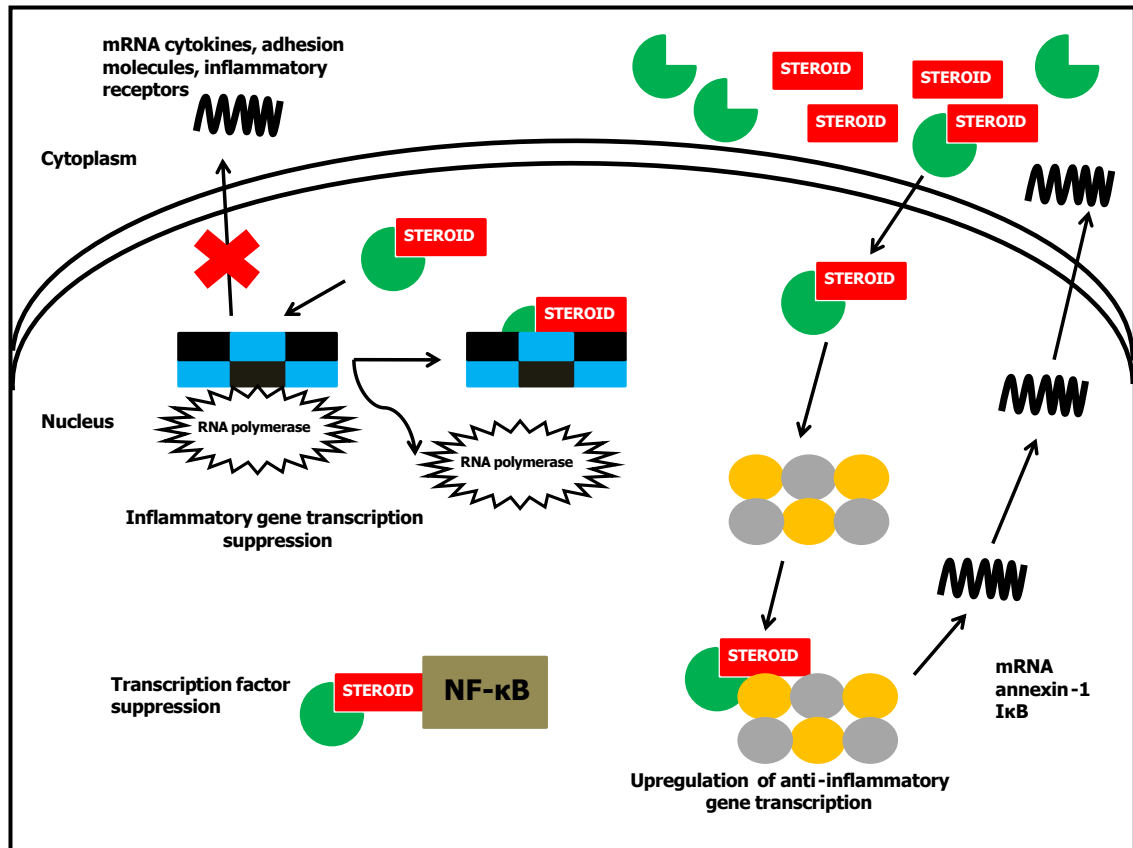


**Figure 1.07. Cellular mechanism of action of corticosteroids**

## 1.4.1 Corticosteroid resistance

A proportion of individuals with inflammatory conditions such as asthma, rheumatoid arthritis (RA) and inflammatory bowel disease who are non responsive to corticosteroid therapy are described as "resistant" (Barnes and Adcock, 2009). The molecular mechanisms that have been postulated to underlie this include reduced corticosteroid-corticosteroid receptor binding, defective nuclear translocation and reduced histone acetylation. It is not known how common the phenomenon of corticosteroid resistance due to such physiological factors is in patients with leprosy reactions.

## 1.4.2 Adverse effects of corticosteroids

The risks associated with the administration of any drug are a concern. The use of potent immunosuppressants is potentially problematic in areas endemic for severe infectious

diseases such as TB. Immunosuppression may also cause potentially fatal hyperinfection with *Strongyloides stercoralis* (Leang *et al.*, 2004).

The First European Workshop on Glucocorticoid Therapy designated doses of prednisone between > 30mg and ≤ 100mg as "high doses" which are associated with severe side effects if used long term. This group also considers that side effects are considerable and dose dependent at "medium doses" of between > 7.5mg and ≤ 30mg (Buttgereit *et al.*, 2002). There are few data concerning the long term sequelae of corticosteroids used to treat patients with T1Rs.

Taking corticosteroids may cause bone demineralization leading to osteoporosis. This is a dose dependent phenomenon and the rate of loss of bone mineral density is considerable in the first six months of corticosteroid therapy. Men with leprosy are at increased risk of osteoporosis and this is associated with hypogonadism (Ishikawa *et al.*, 1999). The role of previous corticosteroid therapy in exacerbating the osteoporosis affecting people who have had leprosy has not been assessed. Osteoporosis may become increasingly important if longer courses or higher doses of corticosteroids are conclusively proven to be superior in the management of T1Rs. There are no studies of the extent of bone demineralization in leprosy patients treated with steroids or interventions that might improve or prevent it.

Diabetes and hyperglycaemia may occur during treatment with low doses of corticosteroids. A case-controlled study of patients in a Medicaid programme in the USA showed that at low steroid doses hypoglycaemic agents may be required (Gurwitz *et al.*, 1994). In a large, retrospective series of 581 Indian patients with T1R, 2.2% developed diabetes requiring an oral hypoglycaemic agent during the initial phase of treatment with corticosteroids (Sugumaran, 1998).

The formation of cataracts is a recognised complication of corticosteroid therapy but may also complicate leprosy (particularly smear positive disease) per se (Daniel and Sundar Rao, 2007). Cataract was identified in 4% of individuals treated for T1R by Sugumaran but all of these patients had been on steroids for more than 12 months (Sugumaran 1998). Age-related cataract is now the commonest cause of blindness in leprosy affected people (Hogeweg and Keunen, 2005).

Analysis of the adverse events attributable to prednisolone in the three TRIPOD trials suggests that the drug is safe when used under field conditions in standardised regimens (Richardus *et al.*, 2003a). The trials used a total prednisolone dose of 1.96 g and 2.52 g. The steroid treated group were significantly more likely to experience minor adverse events but there was no difference in the likelihood of major adverse events between the

prednisolone and placebo groups. Three hundred of the 815 patients enrolled in the three studies were followed for 24 months and none developed TB or hypertension during that time. It should be noted that these 815 patients represent a very small proportion of leprosy patients treated with corticosteroids and lack of data concerning the rate of adverse events in people treated under field conditions where monitoring may be absent due to a lack of suitably trained staff.

### 1.4.3 Pulse methylprednisolone therapy

High dose intravenous (IV) methylprednisolone is beneficial when used in the early phase of an exacerbation of Th1 cytokine mediated relapsing chronic diseases. These conditions include RA (Weusten *et al.*, 1993) and multiple sclerosis (MS) (Filippini *et al.*, 2000).

In 18 patients with MS treated with IV methylprednisolone 1 g for three days there was a significant suppression of mitogen stimulated IFNγ, TNFα and IL2 production by blood leucocytes ex vivo after treatment (Wandinger *et al.*, 1998). Methylprednisolone has also been shown to reduce serum levels of TNFα in RA (Youssef *et al.*, 1997). Eleven patients given 1 g intravenously showed significantly reduced serum levels of TNFα at 4 and 24 hours. In a comparative study of lymphocyte-suppressive potency between prednisolone and methylprednisolone in 44 individuals with RA the latter was more effective in those with greater disease activity as defined by rheumatoid factor titres (Hirano *et al.*, 2000).

IL10 is an anti-inflammatory cytokine produced by blood monocytes and its levels rise during T1R or relapse of reactions. It has been postulated that this is a response to the release of TNFα (Lockwood *et al.*, 2002). Methylprednisolone in vitro up-regulates monocyte IL10 in whole blood in addition to suppression of the pro-inflammatory cytokines involved in T1Rs namely TNFα, IFNγ and IL2 (Hodge *et al.*, 1999).

The *ex vivo* effect of dexamethasone on PBMCs from an unspecified number of healthy volunteers has been studied using DNA microarray analysis. Dexamethasone down regulated the expression of pro-inflammatory cytokine genes such as IL1β, IL1α, IL8, IFNγ and pro-inflammatory chemokine genes MCP2 and MCP3 whilst up-regulating the expression of anti-inflammatory cytokines TGFβ3 and IL10 (Galon *et al.*, 2002). One of the genes most strongly down-regulated was IL1Ra, a soluble receptor antagonist released during inflammation.

A Phase III study to investigate the difference in effect of oral versus IV methylprednisolone in MS (Oral Megadose Corticosteroid Therapy of Acute Exacerbations of Multiple Sclerosis (OMEGA)) is currently underway (Burton *et al.*, 2009).

### 1.4.4 Aim and hypothesis 2

Systemic corticosteroids are the mainstay of treatment of T1Rs and NFI although conclusive evidence of their efficacy is lacking. The optimal dose and duration of treatment with corticosteroids is unclear. Physicians working in leprosy endemic areas are experienced in using systemic corticosteroids to manage leprosy reactional states. Methylprednisolone is an affordable and readily available corticosteroid in many leprosy endemic areas. The drug is available as an IV preparation facilitating the administration of larger doses of steroid in a relatively short time - pulse therapy. Methylprednisolone has been used with success in disorders with similar immunological features to T1Rs. These factors make it an appropriate drug to study in the management of T1Rs.

- **AIM 2:** To assess the safety and effect of high dose IV methylprednisolone in leprosy T1Rs and NFI in a leprosy endemic setting.

- **HYPOTHESIS 2:** High dose IV methylprednisolone and oral prednisolone is not associated with a significantly greater rate of adverse events compared to oral prednisolone alone.

## 1.5 Literature review of innate immunity and toll-like receptors

The innate immune system protects the host organism and responds to pathogens by triggering inflammation via complement activation, the recruitment of inflammatory cells and initiation of adaptive immunity.

TLRs and other PRRs play a vital role in the activation of the innate immune system.

### 1.5.1 Toll-like receptors

TLRs are membrane bound PRRs which are highly conserved throughout the plant and animal kingdoms. They were originally discovered in *Drosophila melanogaster* (Anderson *et al.*, 1985) and shown to be important in the fly's resistance to fungal infection by *Aspergillus fumigatus* (Lemaitre *et al.*, 1996). Homologous mammalian TLRs were subsequently discovered.

Ten human TLRs have been identified to date and these are located either on the cell surface or intracellular endosomal membranes.

## 1.5.2 Structure of toll-like receptors

TLRs are type I integral membrane glycoproteins. The extracellular component is comprised of an N-terminal leucine-rich repeat (LRR) domain, there are between 19 and 25 repeats of 24 to 29 leucine residues (Matsushima *et al.*, 2007). There is a short transmembrane portion and a C-terminal intracellular portion which has a high degree of homology with the IL-1R domain and is referred to as the Toll-IL-1R (TIR) (Gay and Keith, 1991). TLRs are therefore classed as members of the IL1R superfamily.

## 1.5.3 Pathogen-associated molecular patterns (PAMPs)

TLRs bind distinct groups of ligands which are highly conserved on and by pathogenic organisms. These are known as PAMPs. These bind to the LRR domain of the TLRs. The LRR domain creates a large concave binding surface which has a high affinity for its ligand.

TLR2 forms heterodimers with TLR1 and TLR6 in order to recognise and bind with certain PAMPs (Weber *et al.*, 2003).

The human TLRs and their ligands are shown in Table 1.10.

| Receptor | Cellular location | Immune cell type | Microbial ligands | Micro-organisms recognised |
|---|---|---|---|---|
| TLR1/TLR2 | Cell membrane | Macrophages Dendritic cells B lymphocytes | Triacylated lipopeptides | Bacteria and mycobacteria |
| TLR2 | Cell membrane | Macrophages Dendritic cells Mast cells | Lipoteichoic acid Porins Peptidoglycan Lipoaribinomannan | Gram-positive bacteria Gram-negative bacteria Gram-positive and negative Mycobacteria |
| TLR3 | Endosomal | Dendritic cells B lymphocytes | Double-stranded RNA | Viruses |
| TLR4 | Cell membrane | Macrophages Dendritic cells B lymphocytes | Lipopolysaccharides Heat shock proteins | Gram-negative bacteria Bacterial and host |
| TLR5 | Cell membrane | Macrophages Dendritic cells | Flagellin | Flagellated bacteria |
| TLR6/TLR2 | Cell membrane | Macrophages Dendritic cells | Diacylated lipopeptides Lipoteichoic acid | Mycoplasma Group B Streptococci |
| TLR7 | Endosomal | Macrophages Dendritic cells B lymphocytes | Single-stranded RNA (Imidazoquinoline) | Viruses |
| TLR8 | Endosomal | Monocytes Neutrophils | ?Single stranded RNA | Unknown |
| TLR9 | Endosomal | Macrophages Dendritic cells B lymphocytes | Hypomethylated CpG DNA | Gram-positive and negative bacteria and mycobacteria |
| TLR10 | Cell membrane | Macrophages B lymphocytes | Unknown | Unknown |

**Table 1.10. Human toll-like receptors, cellular location and ligands**

## 1.5.4 Damage-associated molecular patterns (DAMPs)

The recognition of PAMPs by TLRs does not explain why commensal organisms do not trigger inflammation via TLRs and other PRRs of the innate immune system. Matzinger postulated that the innate immune system also recognises danger signals or DAMPs (Matzinger, 1998).

DAMPs such as heat shock proteins, S100 proteins, the products of cellular damage by reactive oxygen species and the chromatin associated protein high-mobility group box 1 (HMGB1) commonly occur in infections because of tissue damage. DAMPs have also been implicated in autoimmunity and inflammation as mammalian host DNA and uric acid can trigger inflammation via PRRs. Genomic double-stranded DNA from lysed fibroblasts induced maturation of murine APCs indicated by an increase in CD11c cells expressing CD40 (Ishii *et al.*, 2001).Treating the cell lysates with proteinase K and DNase I prevented this maturation. Uric acid crystals activated the NALP3 inflammasome to release IL1β in THP1 cells ( a human leukaemia cell line) (Martinon *et al.*, 2006). The innate immune system binds ligands expressed on invading pathogens and also recognises the products of inflammation and cellular damage.

## 1.5.5 Toll-like receptor signal transduction

Once a ligand has bound to its TLR, the receptor dimerises and activates an orchestrated proinflammatory response via a signalling cascade (Gay and Gangloff, 2007). The signalling may proceed via one of two pathways. These pathways are Myeloid differentiation primary response gene (88) (MyD88)-dependent (Tauszig-Delamasure *et al.*, 2002) or MyD88-independent (Fitzgerald *et al.*, 2003) and both lead to the production of proinflammatory cytokines and type I interferons. All TLRs except TLR3 signal via MyD88. TLR3 utilises TIR-domain-containing adaptor protein inducing IFNβ (TRIF, also known as TICAM1) (Yamamoto *et al.*, 2003).

The MyD88 dependent pathway is activated by homophilic association of the TLR cytoplasmic IL1R domain with the IL1R like portion of MyD88 (fig. 1.07). A further adaptor molecule TIR-domain-containing-adaptor (TIRAP) is also recruited to this complex (Gay and Gangloff, 2007). This is followed by the recruitment of IL1R-associated kinase 4 (IRAK-4) and IRAK1. IRAK4 is then activated via phosphorylation and subsequently phosphorylates and activates IRAK1. Phosphorylated IRAK1 associates with TNF receptor-associated factor 6 (TRAF6). TRAF6 activates transforming growth factor (TGF)-β-activating kinase 1 (TAK1) which phosphorylates the inhibitor of IκB kinase-2 (IKK) and mitogen-activated protein (MAP) kinase kinase 6 (MKK 6).

The activated inhibitor of IκB, IKK, then phosphorylates IκB. IκB is bound to NFκB in the cytoplasm, once phosphorylated it dissociates from NFκB. The free NFκB is then able to enter the nucleus and exert its effect on proinflammatory gene transcription (Barnes, 2006).

The signalling cascade initiated by TLR bound ligand can also result in the activation of the interferon regulatory factors (IRF), c-Jun N-terminal kinases (JNKs) and other kinases such as p38. The signalling cascade for TLR3 is MyD88-independent and is mediated by TRIF and results in the activation of IRF3. TLR4 can also use a MyD88-independent pathway by recruiting TRIF-related adaptor molecule (TRAM) which then activates TRIF.
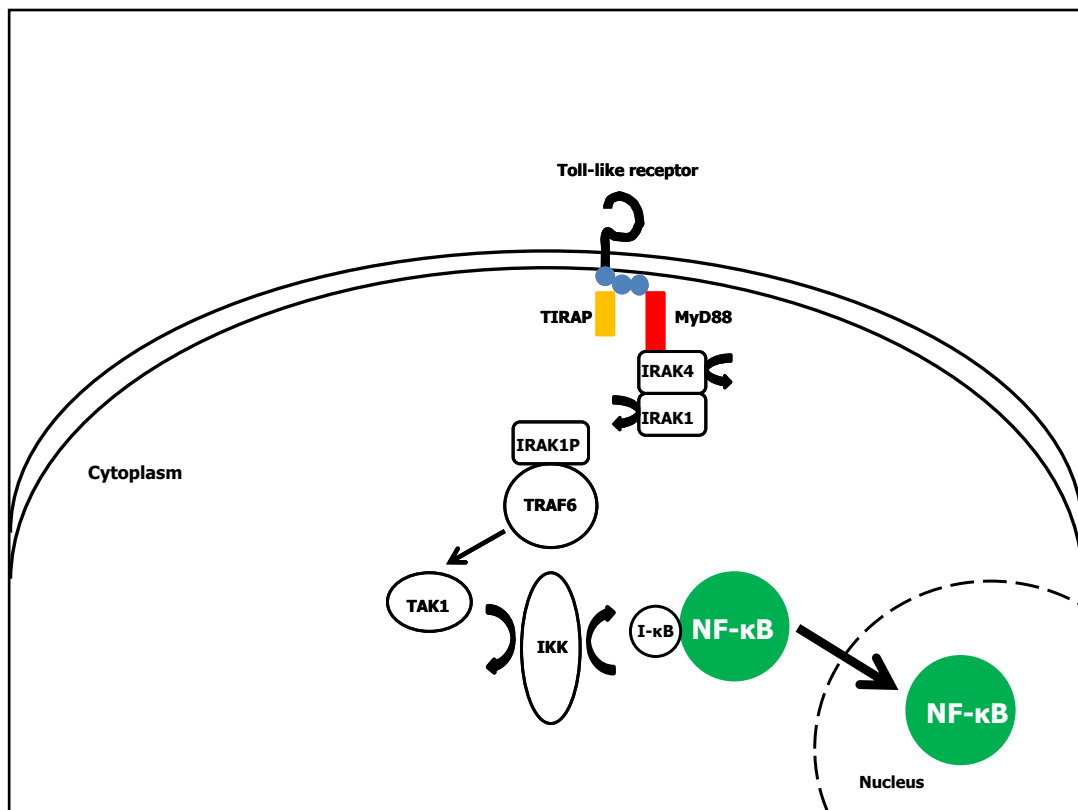


**Figure 1.08. MyD88-dependent toll-like receptor signal transduction**

In the nucleus NFκB associates with AP-1 and large coactivator molecules such as cyclic AMP response element binding (CREB) binding protein (CBP). The coactivator molecules have histone acetyltransferase activity. Reversible acetylation of histones causes chromatin remodelling. The remodelling of chromatin results in the normally closed structure of the chromatin opening up and allowing the binding of RNA polymerase II to the DNA and thus activating transcription of genes coding for proinflammatory agents (Barnes, 2006).

Other molecules such as CD14, a glycosylphosphatidylinositol protein expressed on the cell surface amplifies TLR2 specific responses and the recognition of lipopolysaccharide by TLR4. It can also act as a transporter of microbial ligands to TLRs (Akashi-Takamura and Miyake, 2008).

### 1.5.6 Toll-like receptors and disease

TLR are expressed in tissues during mycobacterial diseases, in the skin during infectious and primary inflammatory dermatoses and also in neural tissue in central and peripheral nervous system disorders.

### 1.5.7 Toll-like receptors and mycobacterial diseases

Nine HIV negative individuals with pulmonary TB expressed a wide variety of TLRs in lung tissue granulomas whereas two control patients with pulmonary neoplasia did not. The TLR receptor expression was identified using rabbit polyclonal anti-human TLR antibodies. TLR1, TLR2, TLR4 and TLR9 were expressed most frequently (Fenhalls *et al.*, 2003). TLR2 and TLR4 were expressed on alveolar macrophages and epithelioid macrophages and giant cells associated with the tuberculous granulomas.

TLR2 deficient mice are markedly susceptible to infection with Mtb and this susceptibility to infection is much less marked in TLR9 deficient mice. However mice deficient in both TLR2 and TLR9 are much more susceptible to Mtb than mice which are deficient in only one of these TLRs. This suggests that in mice, at least, TLR9 may play a role in combating infection with Mtb (Bafica *et al.*, 2005). TLR9 deficient mice form larger granulomas following pulmonary infection with Mtb but these are associated with decreased production of cytokines such as IFNγ and IL12 (Ito *et al.*, 2007). The immunopathology associated with infection may be reduced although granuloma formation is preserved. Human myeloid precursors *in vitro* are converted to competent CD1 expressing APCs following infection with live Mtb or exposure to lipid components of the mycobacterial cell wall. This conversion is TLR2 dependent (Roura-Mir *et al.*, 2005).Human monocytes infected with Mtb and then activated via TLR2 up-regulate the VDR and produce cathelicidin. Cathelicidin is an antimicrobial which is active against Mtb (Liu *et al.*, 2006).Macrophages derived from healthy donors and stimulated with lipid fractions from different strains of Mtb show up and down regulation of TLR2 and TLR4. TLR2 is upgraded by the Canetti strain and down regulated by H37Rv and Beijing strains. The same holds for TLR4 although the results for the H37Rv strain conflicted depending on the polarity of the lipids (Rocha-Ramirez *et al.*, 2008).

The apoptosis of peripheral morphonuclear neutrophils (PMNs) in patients with pulmonary TB occurs following the interaction of Mtb with TLR2 and is mediated via the kinase p38. In pleural effusions such PMNs take on a dendritic cell phenotype and express CD83, so-called transdifferentiation. Monocytes in tuberculous pleural fluid show increased expression of TLR2 and TLR4 compared to their counterparts in the peripheral blood

(Prabha *et al.*, 2008). Peripheral CD4+ T lymphocytes show increased expression of TLR2 in patients with pulmonary TB compared to healthy donors. In individuals with pleural disease the expression of TLR2 was significantly greater on CD4+ cells from pleural fluid than from the peripheral blood in patients with uncomplicated TB.

Individuals with latent TB (tuberculin skin test positive but no clinical symptoms, signs or radiological signs of TB) and co-existent filarial infection show reduced mRNA expression of TLR2 and TLR9 (Babu *et al.*, 2009). The expression of mRNA of these receptors was also reduced in TB infected individuals with filarial infection following stimulation with either PPD or Mtb-culture filtrate protein compared to similar individuals who did not have filarial infection.

In 151 Turkish patients with TB (pulmonary, pleural, lymph node, bone and renal) the SNP 753 G→A in the *TLR2* gene which results in a substitution of arginine to glutamine was associated with an increased risk of TB compared to 116 controls (Ogus *et al.*, 2004). The microsatellite polymorphisms with shorter GT repeats within intron II of the *TLR2* gene were associated with an increased risk of developing pulmonary or extra-pulmonary TB in Korean subjects (n=176) and confirmed in a further 82 patients used as a validation cohort (Yim *et al.*, 2006). This polymorphism was also associated with an increased risk of non-tuberculous mycobacteria pulmonary disease with *Mycobacterium avium-intracellulare* complex or *Mycobacterium abscessus* in another cohort of 193 HIV negative Korean patients (Yim *et al.*, 2008). In a study of Tanzanian patients with HIV and pulmonary TB, the 24 individuals with TB were nearly three times more likely to have the SNP 896 A → G of the *TLR4* gene than 80 HIV positive controls without active TB (Ferwerda *et al.*, 2007). This SNP encodes a glycine instead of an aspartic acid residue.

Two studies have shown that individuals with the SNP 558 C → T in the gene encoding TIRAP are more likely to have TBM (Dissanayeke *et al.*, 2009; Hawn *et al.*, 2006). In the study by Hawn *et al* of 175 Vietnamese individuals with TBM the odds ratio of having the SNP was 3.02 (95% CI 1.79 – 5.09) compared to that of the controls. The blood of individuals homozygous for the thymidine allele produced significantly less IL6 when stimulated with bacterial lipopeptides (Hawn *et al.*, 2006). Khor and colleagues reported that individuals from West Africa and Algeria who were heterozygous for the SNP 539 C →T in the *TIRAP* gene were protected against TB. The same *TIRAP* gene polymorphism was found significantly less frequently in controls than in a UK cohort with invasive pneumococcal disease (the isolation of *Streptococcus pneumoniae* from a normally sterile site). This polymorphism results in a leucine substitution of serine at position 180 in the TIRAP protein. The leucine variant TIRAP results in less activation of NFκB and it is

hypothesised that the reduced host inflammatory response may result in less severe disease (Khor *et al.*, 2007). However this association was not reproduced in similar studies of individuals from Russia, Ghana and Indonesia (Nejentsev *et al.*, 2008).

The SNP 336 A → G in the gene *CD209* which encodes DC-SIGN was present more frequently in 914 control subjects than 1262 individuals with pulmonary TB from West Africa and Malawi (OR 0.86, 95%CI 0.77–0.96) (Vannberg *et al.*, 2008) this polymorphism results in reduced expression of DC-SIGN *in vitro* (Sakuntabhai *et al.*, 2005).

In a Taiwanese study of eight children who had *Mycobacterium bovis* bacilli Calmette-Guerin (BCG) infection following BCG vaccination none had any abnormality of TLR2 signalling (Lee *et al.*, 2009b).In Mycobacterium ulcerans disease (MUD) TLR9 is expressed on the surface of plasmacytoid dendritic cells. *M. ulcerans* is a largely extracellular pathogen and it is not clear what might cause this expression of TLR9 which is expressed intracellularly (Peduzzi *et al.*, 2007). *In vitro M. ulcerans* induces TLR2 and TLR4 expression on HaCaT cells and Dectin-1 in both HaCat cells and primary keratinocytes (Lee *et al.*, 2009a).

In mycobacterial infections there is evidence from human and mouse studies that TLRs are important in recognising pathogen and instituting an immune response. However there is also evidence that TLR expression may be associated with more severe disease. The evidence supporting their role in such immunopathology is that they are associated with increase cytokine activity and more severe disease in mouse models (Ito *et al.*, 2007). In human mycobacterial disease such as TBM an immunopathological component has been implicated in the high rates of mortality and neurological complications (Green *et al.*, 2009). This is supported by the fact that dexamethasone reduces mortality in these patients (Thwaites *et al.*, 2004). Polymorphisms in genes encoding TLRs or molecules involved in TLR signal transduction may reduce TLR induced inflammation (Khor *et al.*, 2007).

## 1.5.8 Toll-like receptors and cutaneous diseases

TLRs are expressed by human skin cells *in vitro* and *in vivo*. The expression of TLRs occurs in skin diseases associated with infection or colonisation by micro-organisms and immunologically mediated diseases such as psoriasis.

Human keratinocytes express TLR2 and TLR4. Activation of these TLRs was associated with the production of inflammatory cytokines which led to microbial killing (Pivarcsi *et al.*, 2003). The mRNA of all TLRs except TLR7, TLR8 and TLR9 was shown to be

expressed in human epidermis, cultured primary foreskin keratinocytes and HaCaT cells (Kollisch *et al.*, 2005). Exposure of primary human keratinocytes *in vitro* to *Staphylococcus aureus* is associated with TLR2-dependent increased expression of the antibacterial agent human β-defensin 3 (Menzies and Kenoyer, 2006; Sumikawa *et al.*, 2006). Human Langerhans cells stimulated via TLR2 acquire migratory capacity and stimulate the proliferation of allogeneic CD4+ T lymphocytes (Peiser *et al.*, 2008). Human melanocytes express functional TLR4 (Ahn *et al.*, 2008).

In acne lesions TLR2 is expressed on macrophages surrounding the pilosebaceous unit. In acne inversa lesions, the expression of both TLR2 mRNA and protein is increased in the dermis compared to normal skin (Hunger *et al.*, 2008). The TLR2 was expressed on cells that also expressed DC-SIGN. *Propionibacterium acnes* triggers the release of inflammatory cytokines via TLR2 *in vitro* (Kim *et al.*, 2002). The expression of both TLR2 and TLR4 was increased in the epidermis of acne lesions compared with normal skin (Jugeau *et al.*, 2005). Zinc is used topically in mild to moderate acne and has mild antimicrobial effects against *P. acnes* and also anti-inflammatory properties (Bojar *et al.*, 1994). Incubation of extracts of *P. acnes* stimulated cultured primary foreskin keratinocytes with zinc has been shown to reduce the expression of TLR2 (Jarrousse *et al.*, 2007). This suggests that zinc may improve acne by reducing TLR2 induced inflammation.

Seborrhoeic dermatitis is associated with the presence of *Malassezia furfur* in affected skin. Human keratinocytes when infected experimentally with *Malassezia furfur* increase their expression of TLR2 mRNA (Baroni *et al.*, 2006). Lithium gluconate which is used topically in the treatment of seborrhoeic dermatitis reduces TLR2 and TLR4 expression in lipopolysaccharide stimulated cultured normal human epidermal keratinocytes (Ballanger *et al.*, 2008). These findings support an analogous role in reducing TLR mediated inflammation for lithium in seborrhoeic dermatitis as for zinc in acne.

In mice infected with the protozoan *Leishmania braziliensis* MyD88 deficient animals experienced more severe and prolonged illness than wild type animals. Interestingly TLR2 deficient animals demonstrated enhanced resistance to infection despite a similar parasite burden to wild type animals (Vargas-Inchaustegui *et al.*, 2009). This finding suggests that TLR2 may contribute to the clinical phenotype of cutaneous ulceration in this murine model.

TLR3 and TLR9 are expressed in the lesions of viral warts and molluscum contagiosum and the expression of mRNA of these TLRs is greater than in normal skin (Ku *et al.*, 2008).

Epidermal keratinocytes in normal human skin constitutively express TLR1, TLR2 and TLR5. In the epidermis of lesions of psoriasis TLR2 staining is greatest at the top of the epidermis, the site of least proliferation of keratinocytes (Baker *et al.*, 2003). Curry also reported increased staining of TLR2 as well as TLR1 and TLR4 in psoriasis lesions (Curry *et al.*, 2003). TLR2 staining was markedly increased in the affected skin of individuals with plaque psoriasis (Begon *et al.*, 2007). TLR9 protein and mRNA expression was greater in 22 individuals with lichen planus compared to normal controls (Li *et al.*, 2007).

In mycosis fungoides epidermal keratinocytes show increased expression of TLR2, TLR4 and TLR9 protein. It was postulated that they may facilitate the persistence of clonal lymphocytes within the epidermis (Jarrousse *et al.*, 2006).

In a small study of 24 individuals with pulmonary sarcoidosis there was a greater degree of TLR2 and TLR4 expression on peripheral blood mononuclear cells than healthy controls (Wiken *et al.*, 2009).

## 1.5.9 Toll-like receptors and peripheral nerve disorders

TLRs are expressed by the specialised cells of the peripheral and central nervous systems including microglial cells, astrocytes, oligodendrocytes and Schwann cells and neurons (Okun *et al.*, 2009).

Wallerian (traumatic) degeneration of peripheral nerves may cause the release of proteins which activate TLR4 and lead to the release of monocyte chemoattractant protein-1 by rat Schwann cells *in vitro* (Karanth *et al.*, 2006). TLR2 and TLR4 activation is associated with recovery of locomotor function following experimental sciatic nerve injury in mice (Boivin *et al.*, 2007). Transection of L5 spinal nerves in rats leads to increased mRNA expression of TLR4 in the spinal cord (Tanga *et al.*, 2004).

Rat and mouse Schwann cells are activated following incubation with necrotic neuronal cells. The inflammatory response produced is attenuated in TLR2 and TLR3 knockout mice (Lee *et al.*, 2006).

Anti-ganglioside-like lipo-oligosaccharides antibody which is associated with Guillain-Barré syndrome enhances the expression of TLR4 on rat Schwann cells and the production of IL-1β and TNFα (Hao *et al.*, 2009). In experimental autoimmune neuritis (a rat model of Guillain-Barré syndrome) there is increased expression of TLR2 and CD14 on inflammatory cells in sciatic nerves (Zhang *et al.*, 2009).

TLR4 knockout mice experience reduced pain hypersensitivity compared to wild type animals when subjected to an experimental sciatic nerve injury which induces neuropathic pain (Bettoni *et al.*, 2008). TLR2 knockout mice experience less nerve injury induced allodynia than C57BL/6 mice (Kim *et al.*, 2007). It has been postulated that HMGB-1 may be the ligand responsible for activating TLRs following insults which result in neuropathic pain (Kim *et al.*, 2009). There is increasing recognition of the complication of neuropathic pain in individuals with leprosy (Hietaharju *et al.*, 2000; Saunderson *et al.*, 2008; Stump *et al.*, 2004).

There is an association between SNPs in the *TLR4* gene and peripheral neuropathy complicating Type 2 diabetes mellitus (Rudofsky *et al.*, 2004). SNP 896 A → G and SNP 1196 C → T which result in a glycine substituted for an aspartic acid and a threonine for an isoleucine respectively.

## 1.5.10 The effect of corticosteroids on toll-like receptor expression

Corticosteroids have marked effects on gene expression. In healthy subjects treated with dexamethasone DNA Microarray experiments showed that of the 9182 genes studied 9% were considered down-regulated and 12% up-regulated in PBMCs (Galon *et al.*, 2002). PBMCs from these dexamethasone treated healthy volunteers showed increased gene expression of TLR2 and TLR4 whilst TLR3 gene expression was downregulated (Galon *et al.*, 2002). Dendritic cells increase their expression of TLR2 and TLR4 following incubation with corticosteroids (dexamethasone, methylprednisolone and prednisone) although the cells showed reduced functional capabilities (Rozkova *et al.*, 2006). Human epidermal keratinocytes show increased TLR2 mRNA expression following incubation with budesonide (Kis *et al.*, 2006) or dexamethasone (Shibata *et al.*, 2009). Primary human corneal epithelial cells express less *TLR3* following incubation with dexamethasone (Hara *et al.*, 2009). Dexamethasone acts synergistically in the presence of TNFα to increase the expression of TLR2 on respiratory epithelial cells (Homma *et al.*, 2004) and IL1β in HeLa cells (Sakai *et al.*, 2004).

Corticosteroids increase GILZ mRNA expression by human monocytes and in mice. GILZ gene expression in a monocytic cell line was associated with a reduced expression of TLR2 following ligand stimulation (Berrebi *et al.*, 2003).

The monocytes of patients with systemic lupus erythematosus treated with pulsed corticosteroids showed reduced expression of CD14 compared with patients who did not receive corticosteroid therapy (Sumegi *et al.*, 2005).

TLR2 and TLR4 are expressed on human adrenal cortex cells but not medulla (Bornstein *et al.*, 2004). TLR2 deficient mice produce less corticosterone than wild type mice even in the unstressed state. It is postulated that there is an interaction which is bidirectional between the innate immune system via TLRs and the hypothalamic-pituitary axis (Bornstein *et al.*, 2006).

Corticosteroids up-regulate TLR expression in *in vitro* experiments and in healthy subjects but their effect in individuals during pathological processes may be different. Galon and colleagues showed that although TLR4 gene expression was up-regulated by dexamethasone in healthy subjects when they activated isolated immune cells by incubating with anti-human CD3 and anti-CD28 antibodies TLR4 gene expression was down regulated (Galon *et al.*, 2002).

### 1.5.11 Aim and hypothesis 3

- **AIM 3:** To quantitatively measure TLR gene and protein expression during corticosteroid treatment and validate a housekeeping gene for PCR assays in individuals with T1R who receive prednisolone and MDT.
- **HYPOTHESIS 3:** The expression of TLR1, TLR2, TLR4 and TLR9 in skin lesions is associated with T1Rs and declines during corticosteroid therapy.

## 1.6 Summary

Leprosy T1Rs are a complication of infection by *M. leprae*. The delayed hypersensitivity exhibited affects the skin and nerves and may lead to permanent loss of nerve function. The treatment of T1R is with corticosteroids but not all patients' nerve function will fully recover. The design of clinical trials has been limited by difficulties in defining outcome measures for T1Rs.

TLRs are expressed in normal and diseased skin and nerves. The expression of certain TLRs is increased during infections and inflammatory processes of the skin and nerves in both the end organ and immune cells.

## 1.7 Aims

1. To develop and validate a severity scale for T1Rs.
2. To assess the safety and effect of high dose IV methylprednisolone in leprosy T1Rs and NFI in a leprosy endemic setting.

3. To quantitatively measure TLR gene and protein expression during corticosteroid treatment and validate a housekeeping gene for PCR assays in individuals with T1R who receive prednisolone and MDT.

## 1.8 Hypotheses

1. The development of a reliable and valid severity scale for T1Rs and leprosy associated NFI is possible using symptoms and signs determined by clinical examination
2. High dose IV methylprednisolone and oral prednisolone is not associated with a significantly greater rate of adverse events compared to oral prednisolone alone.
3. TLR1, TLR2, TLR4 and TLR9 expression in skin lesions is associated with T1Rs and declines during corticosteroid therapy.

# CHAPTER TWO

## The development and validation of a severity scale for leprosy Type 1 reactions and nerve function impairment

## 2.1 Introduction

Clinical trials with appropriate outcome measures are needed to determine the most effective treatment regimens for T1Rs (van Brakel *et al.*, 2007b). It has proved difficult to compare the small number of studies because of the different outcome measures used. There are also difficulties in comparing the severity of T1Rs between different cohorts and even between different arms of clinical trials.

A tool which enables clinicians to accurately assess the severity of leprosy T1Rs would be useful in defining outcomes for clinical trials. It would facilitate the even distribution of patients with similar disease severity between the arms of clinical trials. A measure of reaction severity could also be used in treatment guidelines to indicate the need for therapy. A quantitative measure of reaction severity may be a useful prognostic tool.

A scale devised as part of the INFIR Cohort study examined 21 items for the basis of a severity scale of both types of leprosy reactions and retrospectively assessed the performance of this scale (van Brakel *et al.*, 2007a). There was good agreement between items in the scale.

A different scale (with 24 items) was used by Marlowe *et al* in a different INFIR study of azathioprine and prednisolone in T1Rs but it was not validated (Marlowe *et al.*, 2004).

An "indice névritique" – a composite scale using various assessments of nerves including electrophysiological studies – was developed by Naafs and colleagues but has not been validated (Naafs and Dagne, 1977; Naafs *et al.*, 1979).

Garbino compiled a Clinical Score for ulnar neuropathy complicating Type 1 and ENL reactions (Garbino *et al.*, 2008). This was a composite of an assessment of spontaneous nerve pain with a visual analogue score, graded clinical assessment of nerve enlargement, monofilament sensory testing and voluntary muscle testing. The score was not validated.

Using the INFIR scales as a starting point we decided to develop and validate a scale to measure the severity of T1Rs and NFI in leprosy. This was initially based on 24 items with a final version based on 21 items.

## 2.2 Participants and Methods

### 2.2.1 Expert opinion

To establish content validity a questionnaire was sent to eight leprologists who were not involved in the development of the current scale. The questionnaire used open questions to

ascertain the signs they believed to be important in T1R, which signs indicated a more severe reaction and how they categorised T1R severity.

## 2.2.2 Scale development

The severity scale for leprosy T1Rs was developed by modifying the two previous scales used in the INFIR studies and informed by the responses of the leprologists to the questionnaire.

The scale that was developed and tested had 24 items grouped into three parts (see Appendix A):

Section A contained six items each of which scored between 0 and 3 depending on the assessment of their severity by the examiner using the scale.

Section B was an assessment of sensory function of each of the trigeminal, ulnar, median and posterior tibial nerves. Cotton wool was used to assess the trigeminal nerve. Graded SWM were used for the ulnar, median and posterior tibial nerves.

The ulnar and median nerves were examined using a 2 g and 10 g monofilament at three sites on the palmar aspect of the hand for each nerve (ulnar and median) and the posterior tibial nerves were assessed using 10 g and 300 g at four sites on the sole of the foot (fig. 2.01).

**Figure 2.01. Sites of sensory testing on the palms and soles**
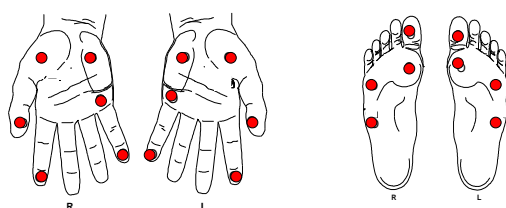


A score from 0 to 6 was assigned depending on the ability of the patient to successfully recognise the weighted monofilaments and the number of sites in which they were felt. For

example, on the hand if a person could feel the 2 g monofilament at the three sites innervated by the ulnar nerve then a score of zero was recorded. If the 2 g was felt at two sites and the 10 g at the third site a score of one was recorded. If however the 10 g monofilament was not felt at one site then a score of 4 was recorded even if the patient was able to feel the 2 g monofilament at the other two sites.

Section C measured motor function of ten nerves (facial, ulnar, median, radial, posterior tibial) by voluntary muscle testing (VMT) using the Medical Research Council (MRC) grading system (Brain, 2000). Normal muscle power (MRC Grade 5) scored zero on the scale. Active movement against gravity and resistance (Grade 4) scored one and active movement against gravity (Grade 3) scored two. An MRC grade of less than three scored three on the severity scale.

The sum of the total for each section gives the overall severity scale score which ranged from 0-96, the lower the score the less severe the reaction.

### 2.2.3 Scale testing

The assessment of the severity scale was performed at the specialist leprosy referral centres of DBLM Hospital, Nilphamari, Bangladesh and Oswaldo Cruz Institute, Rio de Janeiro, Brazil between June 2006 and November 2007.

Ethical approval was granted for the external validation of the scale and the assessment of inter-observer agreement by the Ethics Committee of the London School of Hygiene and Tropical Medicine (4021), the Bangladesh Medical Research Council and the Institutional Review Board of the Oswaldo Cruz Institute.

Patients attending the centres with evidence of a T1R or nerve function impairment of less than 6 months duration were eligible. Eligible individuals were invited to participate by the attending physician.

Written informed consent was obtained from individuals who participated in the external validation of the scale and also from those enrolled in the study of inter-observer agreement.

### 2.2.4 Validation of the scale

Individuals were examined independently by a worker who was trained to use the scale and experienced leprologists (> 20 years experience) who categorized the reaction as mild or moderate or severe. Neither assessor (nor the patient) was aware of the result of the others examination. All of the demographic and clinical data were recorded on a standard form.

The Ridley-Jopling classification was used to classify the type of leprosy each patient had (Ridley and Jopling, 1966).

## 2.2.5 Inter-observer agreement

Inter-observer agreement was tested at the two centres in a subsequent stage of the study using the same eligibility criteria. Two assessors independently used the scale to assess individuals diagnosed as having T1Rs. The scale was applied in the same way as in the validation part of the study. The time interval between the two assessments was kept as short as was practicable. Four pairs of assessors were used.

## 2.2.6 Data management

The data were entered into an Access database. The data were analysed using the Statistical Package for the Social Sciences (SPSS version 14. SPSS Inc, Illinois, Chicago).

## 2.2.7 Statistical Methods

The item to total score correlation was examined using Spearman rank correlation.

The internal consistency or reliability was assessed using Cronbach's alpha. An alpha between 0.7 and 0.9 was considered acceptable (Streiner and Norman, 2003). The contribution of each item in the scale was assessed by calculating Cronbach's alpha for the scale if that item were removed.

The ability of the scale to discriminate between different clinical severity categories was determined using analysis of variance. The threshold for accepting statistical significance was $p < 0.05$.

Inter-observer reliability was evaluated using Intra-Class Correlation of the total score of each examiner using a two-way analysis of variation (5% level of significance) and the strength of agreement criteria of Landis and Koch (Landis and Koch, 1977). A Bland Altman plot of the difference between pairs of observations and the mean of those pairs was used to highlight any potential systematic differences between raters.

Receiver operating characteristic curves were used to determine cut off points for mild, moderate and severe reactions by calculating the sensitivity and specificity of the scale scores for mild and moderate groups and moderate and severe groups respectively.

## 2.3 Results

### 2.3.1 Expert opinion

The questionnaire sent to eight leprologists was returned by seven. The features of T1R that were considered important indicators of severity were extent and degree of inflammation of skin lesions, the presence of peripheral oedema, nerve tenderness and nerve function impairment. These items are all part of the clinical severity scale we have developed and thus gives our scale face validity.

**Question 1**: What clinical signs would be important to include in an attempt to objectively measure a Type 1 reaction?

The responders listed between three and 10 clinical signs they regarded as important when measuring a T1R. These are shown in Table 2.01 with the number of leprologists who included them in their response to question 1.

| Clinical sign | Number (n=7) |
|---|---|
| Skin lesion oedema | 5 |
| Skin lesion erythema | 4 |
| Skin lesion ulceration | 4 |
| Number or percentage of skin lesions involved | 4 |
| Body surface area involved | 1 |
| Peripheral oedema | 4 |
| Fever | 2 |
| Neuritis | 7 |
| Nerve function impairment | 6 |
| Nerve enlargement | 5 |
| Patient discomfort | 2 |
| Ridley-Jopling Classification | 1 |

**Table 2.01. Important signs of T1R –expert opinion**

**Question 2**: How would you measure these signs?

Six of the respondents stated that they would use VMT and monofilament sensory testing. There was less agreement concerning the measurement of other signs but categories such as; mild or moderate or severe and absent or present were given by three respondents.

**Question 3**: Which signs, if any, are more likely to indicate a more severe Type 1 reaction?

Nerve function impairment, nerve tenderness, peripheral oedema and ulceration of skin lesions were reported as indicating a severe reaction by the majority of leprologists who responded to the questionnaire (Table 2.02).

| Signs associated with more severe reaction | Number (n=7) |
|---|---|
| Skin ulceration | 4 |
| Peripheral oedema | 5 |
| Nerve function impairment | 5 |
| Nerve pain or tenderness | 5 |
| Speed of onset | 1 |
| Fever | 2 |
| Ridley-Jopling classification | 1 |
| Duration | 1 |
| Large number of patches | 1 |
| New patches | 1 |

**Table 2.02.  Signs indicative of a severe T1R –expert opinion**

**Question 4**: How do you categorise the severity of a Type 1 reaction?

Four respondents categorise T1Rs as mild or moderate or severe. Two classify reaction severity on the basis of whether an individual requires corticosteroids or does not require corticosteroids. One leprologist used mild or severe to categorise T1R severity.

### 2.3.2 Scale testing

### 2.3.2.1 Validity

81 individuals were recruited (56 from Bangladesh and 25 from Brazil). 64 (79%) were male and 17 (21%) female. The clinical features are summarised in Table 2.03.

| | Study of Validity Number (%) |
|---|---|
| **Number enrolled** | 81 |
| **Male** | 64 (79) |
| **Female** | 17 (21) |
| **Mean Age in years (range)** | 39.5 (11-86) |
| **Type of leprosy** | |
| **BT** | 56(69.1) |
| **BB** | 6 (7.4) |
| **BL** | 18 (22.2) |
| **LL** | 4 (4.9) |
| **PNL** | 1 (1.2) |
| **First episode of Type 1 reaction** | 52 (64.2) |
| **Type of reaction** | |
| **Skin and nerves** | 56 (69.1) |
| **Skin only** | 18 (22.2) |
| **Nerves only** | 7 (8.6) |

**Table 2.03. Description of individuals enrolled in the validation study**

77

The median severity score for the 81 individuals was 10. The range was 59, with a minimum of two and a maximum of 61. The distribution of scores was right skewed (fig. 2.02).
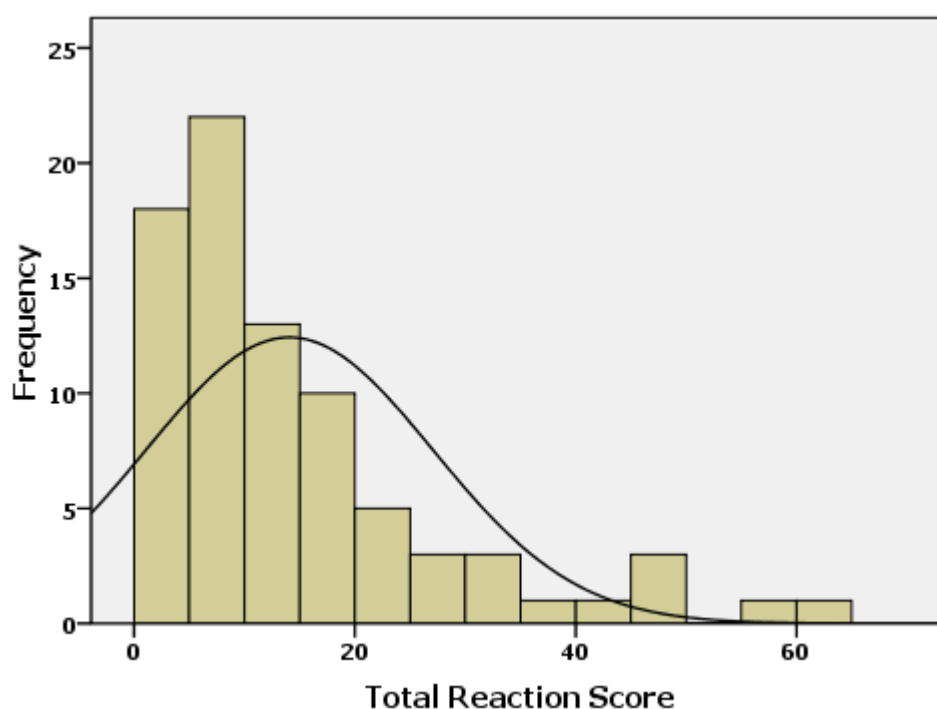


**Figure 2.02. Distribution of scores for the validation study (n=81)**

The range of the item to total score correlation was -0.09 to +0.73. Nerve pain and nerve tenderness appeared to show no correlation with the total score.

The internal consistency of the scale was assessed using Cronbach's alpha. The Cronbach's alpha was 0.819. Removal of the following individual items resulted in an increase in the alpha: the degree of inflammation of skin lesions, the number of raised inflamed lesions, nerve pain, nerve tenderness, fever, function of right trigeminal nerve, function of the left trigeminal nerve, motor function of the right and left radial nerves (Table 2.04). This indicates that removal of one or more of these items might improve the remaining items ability to measure the severity of T1Rs.

| Type of item | Item | Cronbach's Alpha if Item Deleted |
|---|---|---|
| **Skin and oedema signs** | *Degree of inflammation of skin* | *.822* |
| | *Number of raised and/or inflamed lesions* | *.824* |
| | Peripheral oedema due to reaction | .814 |
| **Nerve symptom** | *Nerve pain and/or paraesthesia* | *.826* |
| **Nerve sign** | *Nerve tenderness (worst affected nerve only)* | *.825* |
| **Systemic sign** | *Fever (•C)* | *.820* |
| **Sensory function of nerve** | *Right trigeminal* | *.821* |
| | *Left trigeminal* | *.821* |
| | Right ulnar | .799 |
| | Left ulnar | .789 |
| | Right median | .795 |
| | Left median | .803 |
| | Right posterior tibial | .797 |
| | Left posterior tibial | .800 |
| **Motor function of nerve** | Right facial | .817 |
| | Left facial | .816 |
| | Right ulnar | .810 |
| | Left ulnar | .807 |
| | Right median | .809 |
| | Left median | .808 |
| | *Right radial* | *.821* |
| | *Left radial* | *.821* |
| | Right lateral popliteal | .809 |
| | Left lateral popliteal | .816 |

**Table 2.04. Cronbach α for the scale when individual item indicated is removed.**
**An increase in α indicates that removal of the item is improving agreement of the remaining scale items. (The overall α for the original 24 item scale was 0.819)**

Principal component analysis (PCA) identified a general factor or component to which all but nerve pain, nerve tenderness and the number of inflamed lesions contributed accounting for 23.5% of total variance.

| | Component | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| **SKIN** | | | | | | | |
| Degree of inflammation of skin lesions | .036 | .151 | .593 | .229 | -.290 | -.282 | -.122 |
| Number of raised or inflamed skin lesions | -.006 | .074 | .658 | .014 | -.473 | .006 | .012 |
| Peripheral oedema due to reaction | .300 | -.043 | .671 | -.131 | -.225 | .283 | .087 |
| **NERVES** | | | | | | | |
| Nerve pain | -.190 | -.050 | .401 | -.335 | .190 | .482 | .349 |
| Nerve tenderness | -.201 | .182 | .550 | -.204 | .305 | .491 | .104 |
| **SYSTEMIC SIGN** | | | | | | | |
| Fever | .148 | -.207 | .437 | .309 | -.080 | .202 | -.123 |
| **SENSORY** | | | | | | | |
| Right trigeminal | .022 | .789 | .060 | .252 | .446 | .125 | -.241 |
| Left trigeminal | .022 | .789 | .060 | .252 | .446 | .125 | -.241 |
| Right ulnar | .690 | -.099 | .083 | -.381 | .280 | -.302 | -.031 |
| Left ulnar | .851 | -.191 | .030 | .204 | .035 | .044 | -.070 |
| Right median | .725 | -.103 | .201 | -.097 | .218 | -.254 | -.207 |
| Left median | .683 | -.343 | .133 | .376 | .203 | -.014 | -.239 |
| Right posterior tibial | .683 | .286 | -.173 | -.285 | -.235 | .204 | -.240 |
| Left posterior tibial | .631 | .381 | -.056 | -.274 | -.160 | .167 | -.311 |
| **MOTOR** | | | | | | | |
| Right facial | .304 | .644 | -.025 | .149 | -.230 | -.191 | .509 |
| Left facial | .331 | .647 | .006 | .166 | -.123 | -.260 | .467 |
| Right ulnar | .632 | -.092 | -.356 | .188 | -.015 | .277 | .393 |
| Left ulnar | .587 | -.078 | .068 | -.424 | .193 | -.160 | .157 |
| Right median | .698 | -.259 | .119 | .475 | .076 | .171 | .178 |
| Left median | .685 | -.061 | .277 | .002 | .102 | -.279 | .167 |
| Right radial | .147 | -.266 | -.249 | .552 | .198 | .313 | .156 |
| Left radial | .041 | -.117 | .039 | -.407 | .563 | -.091 | .275 |
| Right lateral popliteal | .563 | .094 | -.214 | -.268 | -.269 | .272 | -.170 |
| Left lateral popliteal | .396 | .106 | -.482 | -.191 | -.281 | .351 | .055 |

Extraction Method: Principal Component Analysis. 7 components extracted.

**Table 2.05.  Loadings for each item by component (factor)**

The important variables in the second factor accounting for 11.6% of the total variance were those related to the eye, namely, trigeminal nerve sensation and facial nerve motor function. The third factor which accounted for 10.7% contrasted individuals with skin signs and no NFI with those who only had NFI.

The second factor explains the amount of variance that remains unaccounted for after the first component has been extracted. The second component is not correlated with the first

component, they are orthogonal. This is true of the relationship of the third factor to the second and so on.

The contribution of the different scale variables to each component are shown in Table 2.05.

The Scree plot shows the eigenvalue for each component. The eigenvalue is an index of the variance accounted for by each component. The PCA identified seven components with an eigenvalue greater than one accounting for 73.8% of the total variance.
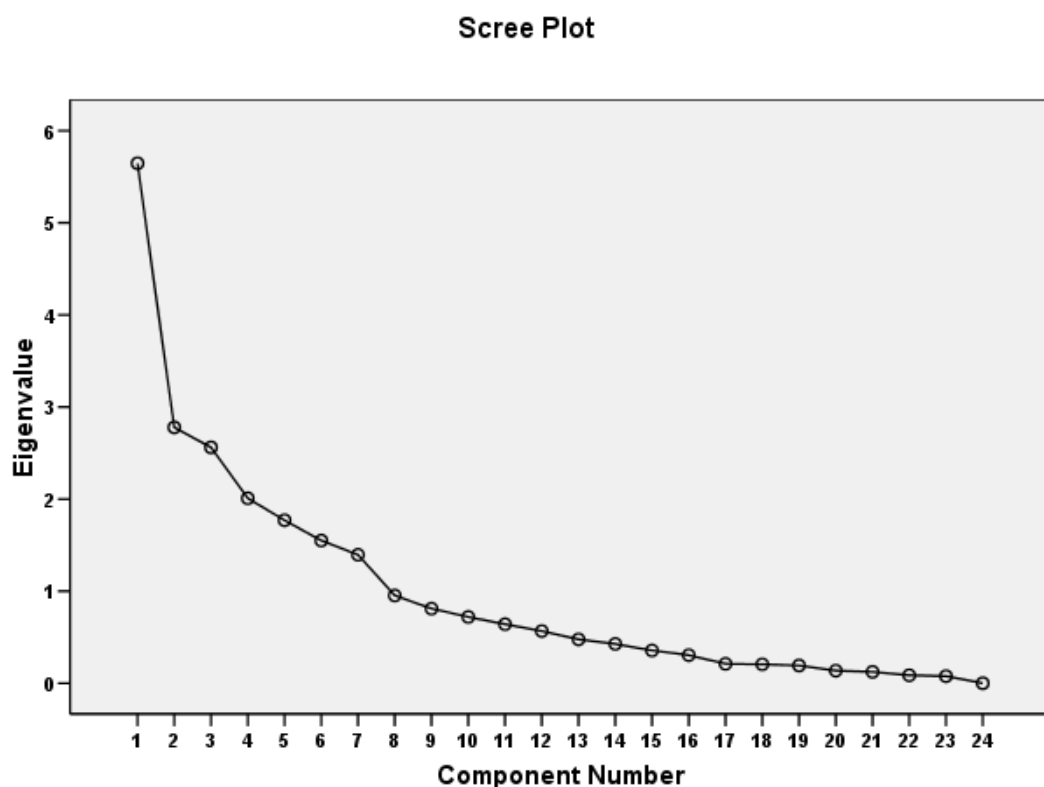


**Figure 2.03. Scree plot of eigenvalues for each component (factor)**

The expert assessment of the severity of the T1Rs was categorized as mild in 19 individuals (23.5%), moderate in 40 (49.4%) and severe in 12 (14.8%). The severity was not recorded in 10 cases.

The median scores for each category of reaction severity are shown in the box plot in fig. 2.04 with the inter-quartile range (IQR).

Outliers are indicated by either a circle or an asterisk which is labelled with the individuals unique study identifier. A circle indicates a result is 1.5 to 3 times the IQR. An asterisk is a more extreme outlier at > 3 times the IQR.
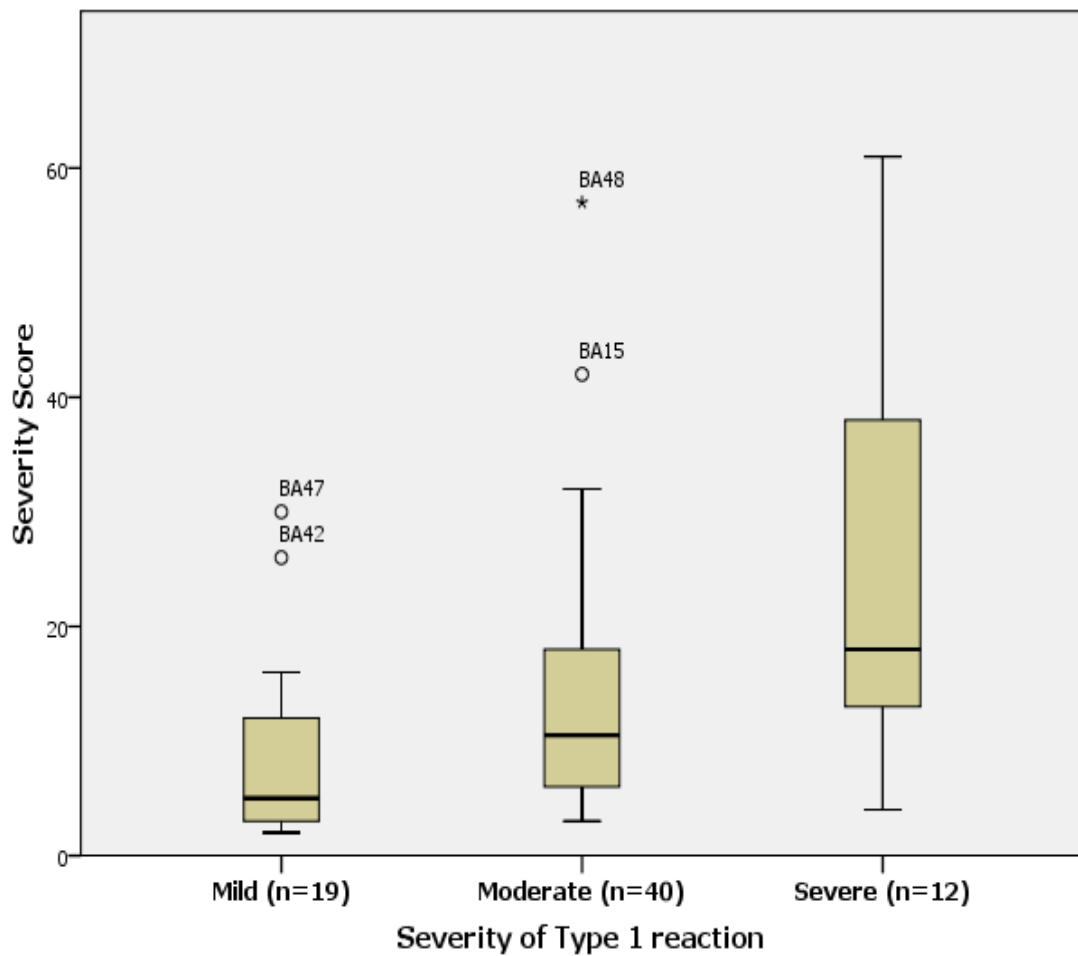
**Figure 2.04. Severity scores and expert assessment of severity of T1R**

The median scores for each category were: mild = 5.0 (IQR=11), moderate = 10.5 (IQR=13) and severe = 18.0 (IQR=29).

The differences between the mild and moderate group and the moderate and severe groups did not reach statistical significance (p=0.053 and 0.052 respectively). The performance of the scale was not materially affected by excluding the seven individuals who did not have skin involvement.

### 2.3.2.2 Inter-observer agreement

Thirty nine individuals (27 from Bangladesh and 12 from Brazil) were recruited to the second stage of the study to assess inter-observer agreement. The details of these participants are presented in Table 2.06.

| Study of Interobserver Agreement | |
| --- | --- |
| | Number (%) |
| Number enrolled | 39 |
| Male | 29 (74.4) |
| Female | 10 (25.6) |
| Mean Age in years (range) | 40.9 (11-95) |
| Type of leprosy | |
| BT | 17 (43.6) |
| BB | 3 (7.7) |
| BL | 15 (38.5) |
| LL | 4 (10.3) |
| PNL | 0 (0) |
| First episode of Type 1 reaction | 19 (48.7) |
| Type of reaction | |
| Skin and nerves | 28 (71.8) |
| Skin only | 9 (23.1) |
| Nerves only | 7 (5.1) |

**Table 2.06. Description of participants in the reliability study**

The Intra-Class Correlation coefficient based on a two-way analysis of variance with random effects is 0.994. The strength of agreement is very good (Landis and Koch, 1977).

A Bland and Altman plot (Bland and Altman, 1995) (fig.2.05) of the difference between the scores for pairs of observers plotted against the mean of the scores shows good agreement between observers with 95% of differences less than two standard deviations from the mean.
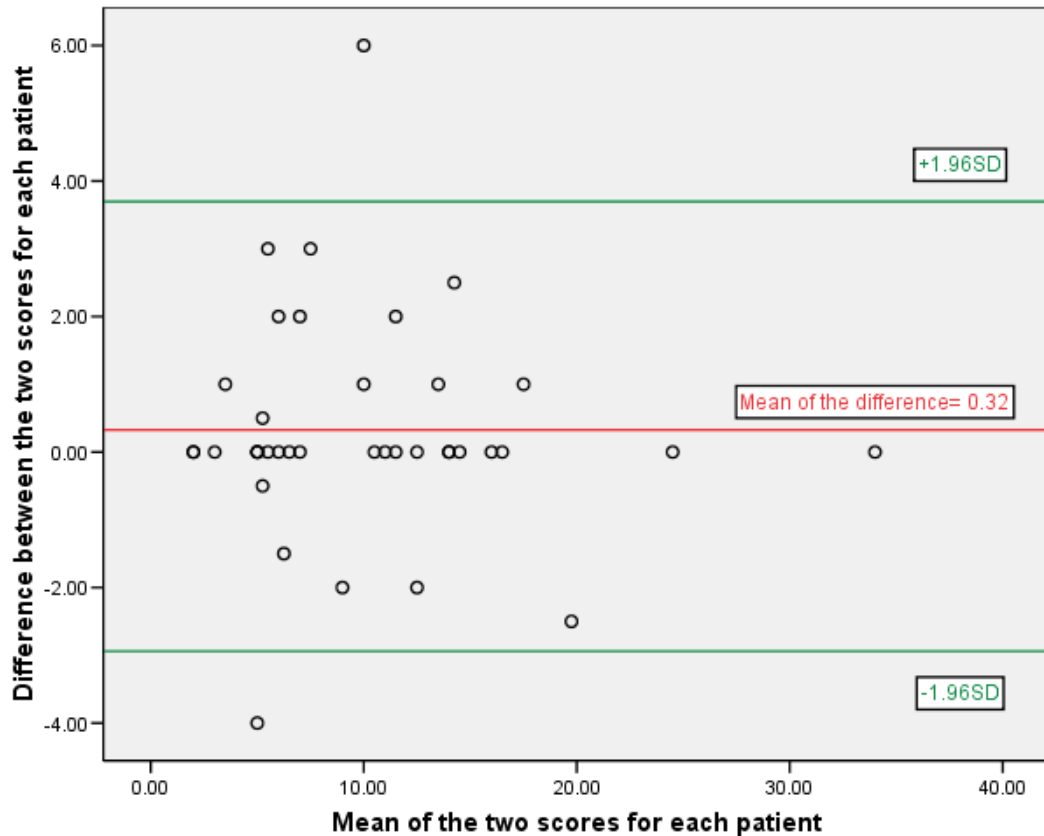
**Figure 2.05. Plot using scores for each individual from both examiners.**

### 2.3.2.1 The Final scale

The scale was adjusted and the analysis repeated in the light of the data obtained.

The items nerve pain, nerve tenderness and fever were removed (see Appendix 4). The rationale for removing these items was that nerve pain and nerve tenderness performed least well of all the items in the scale (in terms of Cronbach's alpha). Fever was removed because it occurred in only four of the 120 participants in the study as a whole.

We felt it was important to retain the cutaneous signs and trigeminal and radial nerve function items as these are important clinical features of T1Rs.

The scores for the sensory testing (using SWM and cotton wool) were reduced by 50% to make the maximum score possible for each sensory nerve three. This is the maximum score possible for each of the motor and cutaneous items.

These adjustments result in the final scale which consists of 21 items and has a range of 0-63. The maximum score possible for sections A, B and C are 9, 24 and 30 respectively.

The severity score for each of the 71 participants who had been categorised as mild or moderate or severe was recalculated using the final scale.

For this adjusted version of the scale Cronbach's alpha remained satisfactory at 0.833.

The median scores for each severity group were: mild = 5.0, moderate = 7.5 and severe = 15.25. The differences between the mild and moderate groups (p=0.038) and the moderate and severe groups (p=0.048) reached statistical significance.
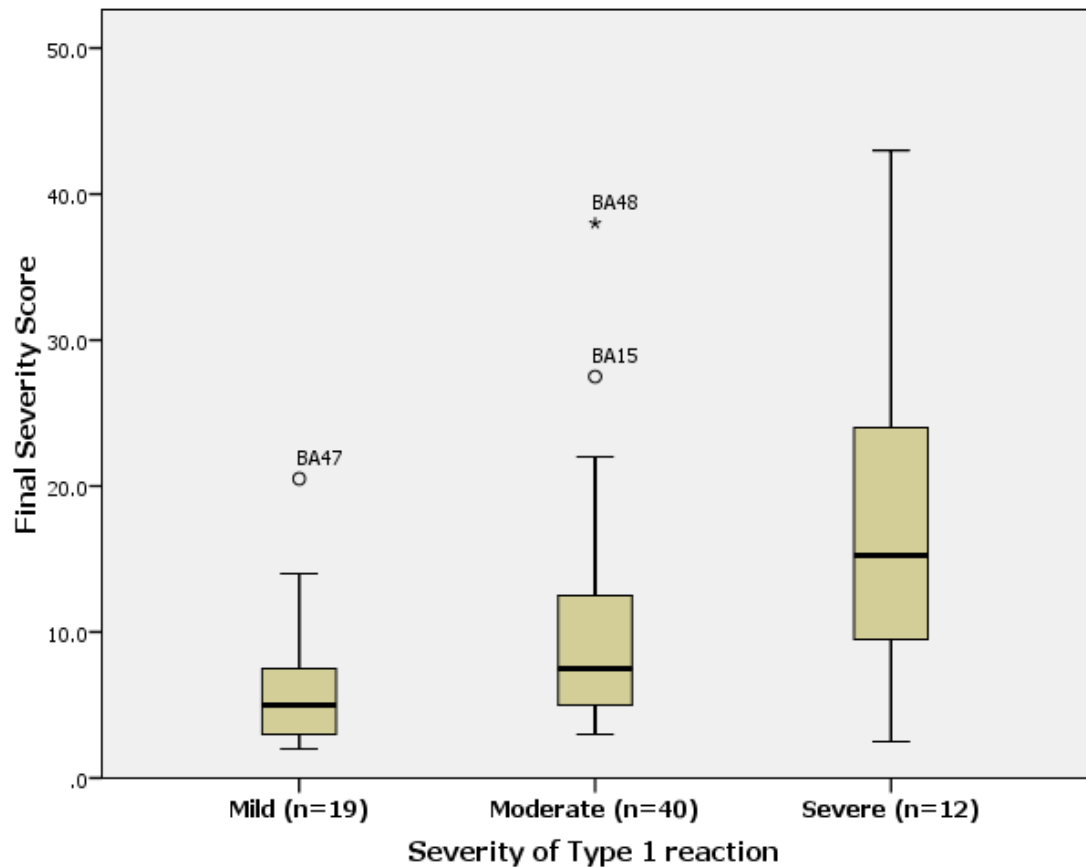


**Figure 2.06. Final severity scores and expert assessment of severity of T1R**

Receiver operating characteristic (ROC) curves can be used to determine cut off points between two groups (Streiner and Norman, 2003).

**Figure 2.07. ROC Curves (A) mild and moderate, (B) moderate and severe**

ROC curves for the final scale scores was plotted for individuals identified as mild or moderate by the expert raters and for those categorized as moderate or severe (fig.2.07). This facilitates the determination of cut off scores for each category.

Using the ROC curves in conjunction with a consideration of the clinical meaning of a given score we determined the following cut off points. This was done by choosing scores with a high sensitivity and reasonable specificity.

| Final Scale (Mild or Moderate) | Scores | Sensitivity | 1 - Specificity |
|---|---|---|---|
| **MILD SCORES** | 1.0000 | 1.000 | 1.000 |
| | 2.5000 | 1.000 | .895 |
| | 3.5000 | .875 | .579 |
| **MODERATE SCORES** | 4.5000 | .800 | .526 |
| | 5.2500 | .725 | .368 |
| | 5.7500 | .725 | .316 |
| | 6.5000 | .600 | .263 |
| | 7.2500 | .525 | .263 |
| | 8.0000 | .475 | .263 |
| | 8.7500 | .450 | .263 |
| | 9.2500 | .400 | .211 |
| | 10.0000 | .375 | .211 |
| | 11.0000 | .325 | .211 |
| | 11.7500 | .275 | .158 |
| | 12.5000 | .250 | .158 |
| | 13.5000 | .175 | .105 |
| | 14.5000 | .175 | .053 |
| | 15.2500 | .150 | .053 |
| | 15.7500 | .125 | .053 |
| | 17.2500 | .100 | .053 |
| | 19.5000 | .075 | .053 |
| | 21.2500 | .075 | .000 |
| | 24.7500 | .050 | .000 |
| | 32.7500 | .025 | .000 |
| | 39.0000 | .000 | .000 |

**Table 2.07. Scores and cut offs for mild and moderate**

A mild T1R is characterized using the final scale by a score of 4 or less (see Table 2.07). A moderate reaction is a score of between 4.5 and 8.5.

A severe reaction is a score of 9 or more (see Table 2.08).

| Final Scale (Moderate or Severe) | Scores | Sensitivity | 1 - Specificity |
|---|---|---|---|
| | 1.5000 | 1.000 | 1.000 |
| | 2.7500 | .917 | 1.000 |
| | 3.5000 | .917 | .878 |
| MODERATE SCORES | 4.5000 | .917 | .805 |
| | 5.5000 | .917 | .732 |
| | 6.5000 | .917 | .610 |
| | 7.2500 | .917 | .537 |
| | 7.7500 | .833 | .488 |
| | 8.2500 | .750 | .488 |
| | 8.7500 | .750 | .463 |
| SEVERE SCORES | 9.2500 | .750 | .415 |
| | 10.0000 | .750 | .390 |
| | 10.7500 | .750 | .341 |
| | 11.2500 | .667 | .341 |
| | 11.7500 | .583 | .293 |
| | 12.5000 | .583 | .268 |
| | 14.0000 | .583 | .171 |
| | 15.2500 | .500 | .146 |
| | 15.7500 | .417 | .122 |
| | 16.5000 | .417 | .098 |
| | 17.2500 | .333 | .098 |
| | 18.0000 | .250 | .098 |
| | 20.2500 | .250 | .073 |
| | 24.7500 | .250 | .049 |
| | 29.0000 | .250 | .024 |
| | 32.2500 | .167 | .024 |
| | 36.0000 | .083 | .024 |
| | 40.5000 | .083 | .000 |
| | 44.0000 | .000 | .000 |

**Table 2.08. Scores and cut offs for moderate and severe**

The area under the curve for mild and moderate categories is 0.701 for the final scale (0.688 for the original scale). The area under the curve for the moderate and severe categories is 0.734 for the final scale (0.731 for the original scale). These values indicate that the final scale is a fair discriminator between the severity categories traditionally used by clinicians.

## 2.4 Discussion

In many branches of medicine a single test or diagnostic criterion is either not available or insufficient to adequately measure or describe a clinical syndrome. This has led to difficulties in measuring the severity and prognosis of conditions. The response by researchers has been to develop composite measurement scales.

Psychologists have for many years been concerned with accurately measuring and predicting behaviour and there is a large literature on how to develop and test such measures (Cronbach and Meehl, 1955; Streiner and Norman, 2003).

The use of unpublished scales to measure outcome has been shown to be a significant source of bias in psychiatry (Marshall *et al.*, 2000). The lack of clear descriptions of scales and familiarity with them make clinical research difficult to interpret.

A reliable 21 item severity scale to measure leprosy T1Rs has been developed and prospectively validated.

The scale requires the examiner to be proficient in recognising the cutaneous signs of T1R, the assessment of VMT and the use of SWM. These skills are not widely practised even in many leprosy endemic countries and it is anticipated that the main use of this tool, at least initially, will be in research and referral settings.

The scale is easy to use and requires little additional training or equipment for workers based in referral centres. Using a standard assessment form the additional time required to use the scale is minimal.

T1Rs are a significant cause of nerve function impairment and this is the major concern of the physician managing a patient with this condition. The scale we have developed reflects the importance of NFI in the severity of T1Rs.

VMT and SWM in the assessment of NFI have been shown to be reliable (Anderson and Croft, 1999). Monofilaments have been shown to be concordant with other sensory function tests (van Brakel *et al.*, 2005a). These factors undoubtedly contribute to the robustness of the current scale but careful training and assessment of examiners is required (Roberts *et al.*, 2007).

In Nepal, Kets and colleagues reported monofilament touch sensibility thresholds in 136 healthy volunteers to be 200 mg for the hand and 2 g for the sole of the foot (Kets *et al.*, 1996). These findings were supported by those of Anderson and van Brakel who reported similar findings in 600 healthy Nepalis. In addition they reported that the normal threshold

for the heel was 10 g (Anderson and van Brakel, 1998). These thresholds were used in the scoring system developed for the INFIR studies in which five monofilaments were used.

The use of two monofilaments on the hands (2 g and 10 g) and feet (10 g and 300 g) simplifies the system used in the INFIR Cohort Study. However this also results in a higher sensory threshold before an individual's NFI impacts on their T1R severity scale score.

The rationale for using 2 g and 10 g on the hand is that thresholds of more than 2 g were associated with a loss of functional capability in leprosy patients in Nepal. Individuals unable to recognise a 2 g monofilament were not able to detect a 0.5 mm Braille-like dot in the corner of a smooth aluminium sheet. The majority of these individuals were also unable to differentiate between five different textures (smooth vinyl, sandpaper and textiles) (van Brakel *et al.*, 1997).

An inability to detect a 10 g monofilament on the foot is associated with an increase in plantar ulceration in leprosy patients in the United States (Birke and Sims, 1986). In a study conducted in Ethiopia a 10 g monofilament was a sensitive screening tool for detecting individuals with leprosy who were at risk of developing a plantar ulcer (Feenstra *et al.*, 2001).

In a study conducted in the United Kingdom an inability to feel a 10 g monofilament is an independent risk factor for ulceration in individuals with diabetes mellitus. The 10 g monofilament is recommended as a screening tool by the American Diabetes Association (American Diabetes Association, 2008).

The INFIR Cohort study also used a single monofilament test site for the purely sensory radial cutaneous and sural nerves (van Brakel *et al.*, 2005b). These two nerves are not commonly tested in routine clinical practice and are not included in the severity scale.

The radial cutaneous and sural nerves may be assessed using various forms of quantitative sensory testing before new impairment identified by monofilaments is demonstrable. Recently published data analysing 188 individuals from the INFIR Cohort who did not present with reaction or nerve involvement  has shown that impairment identified using monofilaments occurred in the radial cutaneous nerve in 7% of individuals and in the sural nerve in 6.1% (van Brakel *et al.*, 2008). However the definition of impairment in the radial cutaneous nerve was the inability to feel monofilaments less than 10 g or in the sural nerve less than 300 g (van Brakel *et al.*, 2005b).

The lack of a gold standard measure of T1Rs has resulted in us having to compare the scale with the variable and somewhat vague clinical categories of severity as mild, moderate or

severe. This has undoubtedly led to a degree of heterogeneity of T1R severity within these categories but despite this the scale has performed well.

The final scale has a high degree of inter-observer reliability. We were unable to test intra-observer reliability because of the effect of treatment on the signs of reaction. It would be unethical to withhold treatment. The assessment of intra-observer variation is desirable but not absolutely necessary in scales with a high level of inter-observer reliability (Streiner and Norman, 2003). This is because the sources of error that contribute to intra-observer variation will also contribute to inter-observer variation. There will also be additional sources of difference in inter-observer variation. The assessment of intra-observer variation has not been possible in the development of valid scales in other fields such as neurology (Wijdicks *et al.*, 2005).

Severity scales are widely used to quantify the severity of inflammatory dermatoses and neurological diseases. The Psoriasis Area Severity Index (PASI) was introduced (Fredriksson and Pettersson, 1978) without formal validation (Berth-Jones *et al.*, 2006) as an outcome measure in the first trial of the retinoid etretinate in psoriasis. It has become the most widely used severity measure of psoriasis and clinicians are required to use it to assess the severity of psoriasis in individuals being considered for biological therapies in the UK (Smith *et al.*, 2005).

The PASI does show good reliability in terms of inter- and intra-observer agreement (the intra-class correlation coefficient for each being $> 0.81$) (Berth-Jones *et al.*, 2006). The PASI has been criticised for not being a linear scale and for being difficult to use. A self administered version of the scale, the SAPASI, has been tested and declared valid although in order to test its validity it was compared to the PASI (Feldman *et al.*, 1996).

In atopic dermatitis it has been recognised that there are too many outcome measures used in clinical trials (Schmitt *et al.*, 2007). Twenty measures of severity were identified but only three had undergone sufficient validation. The authors recommended that future studies use the Eczema Area Severity Index, the Patient-oriented Eczema Measure and the Severity Scoring of Atopic Dermatitis index (SCORAD).

The most common neurological complication of HIV infection is a painful sensory neuropathy. The Subjective Peripheral Neuropathy Screen (SPNS) was developed to try and detect HIV-induced peripheral neuropathy or neuropathy secondary to antiretroviral therapy. This used self reported symptoms of neuropathy and was shown to be reliable (with a Cronbach alpha of 0.86) and valid. The SPNS differentiated between those who had

symptoms of neuropathy and controls. It also showed good agreement with objective quantitative sensory testing (McArthur, 1998).

The use of valid severity scales to measure inflammatory skin disease and peripheral neuropathy demonstrate that the development of quantitative approaches to such disorders is possible. Leprosy T1Rs combine features of inflammatory skin disease and peripheral neuropathy.

In its final form the adjusted severity scale for leprosy T1Rs and NFI is valid and sensitive. Neurological items are well represented and reflect the importance of nerve function impairment. The addition of weighting of the different components of the scale would add to its complexity. An important issue that requires further work is that of determining the Minimally Important Difference (MID) from a patient perspective in scores derived from the scale before and after treatment. This is important because it provides a meaningful patient centred outcome measure of change. This is discussed further in the final chapter. The ability of the scale to reflect change following corticosteroid treatment of T1R and NFI is examined in Chapter 3 using a Nepali cohort.

A consideration that has not been addressed is the performance of the scale in individuals who have nerve damage of greater than 6 months duration. The treatment of nerve damage present for this length of time with corticosteroids is not associated with significant clinical benefit compared to placebo (Richardus *et al.*, 2003b). Nerve damage greater than six months duration should not be included in the severity score. The issue of longstanding NFI can be problematic as patients who are presenting for the first time may be unsure as to the duration of the NFI and may have some acute NFI in a nerve which already has some pre-existing permanent impairment.

Longstanding nerve damage in an individual who experiences a T1R would lead to a higher score than an individual with an identical reaction but who has no pre-existing nerve damage. The severity of the T1R in the two individuals is presumably the same. However it could be argued that individuals who already have some degree of permanent nerve damage have less neurological reserve and are thus more at risk from even a mild reaction. This however needs to be formally tested.

This is the first prospective validation of a severity scale for leprosy T1Rs. It is hoped that this scale will prove a useful tool in more accurately assessing T1Rs particularly in clinical trials where the ability to accurately compare the severity of T1Rs in different patients is vital.

# CHAPTER THREE

## A phase two randomised controlled double blind trial of high dose intravenous methylprednisolone and oral prednisolone versus intravenous placebo and oral prednisolone in individuals with leprosy Type 1 reactions and /or nerve function impairment

## 3.1 Overview of trial

Oral corticosteroids are the mainstay of treatment of leprosy T1R. However treatment with a standardised 12 week course of oral prednisolone (total dose 1.68 g) which had been used in a previous pilot study in Nepal resulted in 37% of individuals requiring additional prednisolone (Marlowe *et al.*, 2004). The randomized controlled treatment trials TRIPOD 2 and TRIPOD 3 that were reported during the design of this study had both used a 16 week course of oral prednisolone (total dose 2.52 g) (Richardus *et al.*, 2003b; van Brakel *et al.*, 2003). It was decided to compare pulsed methylprednisolone and oral prednisolone with a 16 week course of oral prednisolone (total dose 2.52 g) alone. High dose IV MP had not been used previously in a trial of treatment of leprosy T1R so a Phase 2 trial was needed to confirm safety before considering whether to proceed to a larger Phase 3 trial of clinical efficacy.

A Phase 2 trial made it possible to assess the adverse effect profile of methylprednisolone when used in leprosy patients and patient tolerability for methylprednisolone in the Nepali setting.

We wished to recruit 60 individuals in total. This number was felt to be feasible in the time available. The recruitment started on 7th December 2005 and ended on 31st December 2007. The final assessment was completed and the data entered into the Access database on 5th November 2008.

## 3.2 Aims of trial

1.  To assess the safety and tolerability of high dose methylprednisolone in patients with leprosy T1Rs and patients with leprosy associated acute neuritis with nerve function impairment in Nepal.
2.  To assess the effect of high dose methylprednisolone on the clinical outcome of leprosy T1Rs and leprosy associated acute neuritis with nerve function impairment.

## 3.3 Methods

A double blind randomised placebo controlled trial was designed to compare early high dose IV methylprednisolone, followed by oral prednisolone (P) with oral prednisolone alone as the control. It is not ethical to use an inactive agent as placebo on its own. Individuals received IV normal saline placebo and oral prednisolone. The participants were treated with corticosteroids for 16 weeks. The total period of follow-up was 48 weeks from entry into the trial.

### 3.3.1 Participants

Participants in the trial were recruited from the leprosy service of Anandaban Hospital, Kathmandu, Nepal.

### 3.3.2 Study location

Nepal is a landlocked country situated between India and China with a population of approximately 23 million. It is one of the poorest countries in the South Asia region (only Afghanistan and Bhutan have lower purchasing power parity gross national incomes).

Skin disease is common in Nepal particularly in rural areas (Walker *et al.*, 2008). 4708 new cases of leprosy were reported in 2008. The overall leprosy prevalence rate of 2.0 per 10 000 population but this is higher in rural areas such as the Terai (Jain, 2008; WHO, 2009).

Anandaban Hospital is the leprosy referral centre for the central region of Nepal. The hospital provides a weekly outpatient clinic and a full range of inpatient services to leprosy affected people free of charge. Anadaban Hospital is funded by The Leprosy Mission Nepal.

### 3.3.3 Ethical Approval

The study was approved by the Nepal Health Research Council and the Ethics Committee of the London School of Hygiene and Tropical Medicine (Number 4022).

### 3.3.4 Consent

Informed consent was obtained by a native Nepali speaker after s/he had fully explained the trial and answered any questions. The trial consent forms and information leaflets were available in Devanagari script. The consent forms were signed by all participants (if they were unable to sign, a mark or thumb print was used instead and witnessed by the person obtaining the consent).

### 3.3.5 Good Clinical Practice

The study adhered to the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Good Clinical Practice. The trial monitors were Dr K V Krishna Moorthy, Blue Peter Research Centre, Hyderabad, India and Dr P S S Sundar Rao, The Leprosy Mission, Delhi, India.

### 3.3.6 International Standard Randomised Controlled Trial Number

The trial was registered with Current Controlled Trials Ltd (*www.controlled-trials.com)* in accordance with the policy of the International Committee of Medical Journal Editors (Moher *et al.*, 2001) and was assigned the unique identifier ISRCTN31894035.

### 3.3.7 Diagnosis of leprosy

A person was diagnosed as having leprosy if they had hypopigmented, anaesthetic skin patches and/or thickened nerves and/or acid-fast bacilli on slit skin smears. Individuals were also diagnosed as having leprosy if the histological features of the disease were present in a skin or nerve biopsy. All participants in the study had a confirmatory skin biopsy.

### 3.3.8 Definitions of terms used in the study

A **T1R** was present if an individual with leprosy experienced the acute development of erythema and oedema of skin lesions and/or inflammation of nerves and/or oedema of the hands, feet and face.

**New NFI** is defined as less than 6 months duration of reduction in sensory, motor or autonomic function on history or examination.

**Neuritis** is the presence of spontaneous nerve pain, tenderness or paraesthesia.

**Deterioration in symptoms or re-reaction** was defined as a sustained deterioration for a period of at least two weeks of nerve function, the development of nerve pain unresponsive to analgesics, palpable swelling of skin patches or new erythematous and raised skin patches. Any decline in nerve function which the study doctors believed required immediate additional prednisolone was also regarded as deterioration.

### 3.3.9 Eligibility

Two groups of individuals were eligible for entry into the trial:

1. Individuals diagnosed as having leprosy with clinical evidence of Type 1 reaction of less than six months duration.
2. Individuals diagnosed with leprosy with new (less than six months duration) nerve function impairment without inflammation of skin lesions (if skin lesions were present).

Participants with any type of leprosy of the Ridley-Jopling Classification (Ridley and Jopling, 1966) were eligible.

Participants had to be adults aged between 16-65 years and weigh more than 30 kg.

One criterion for an individual's eligibility for the study was modified in September 2006 following a review of the study by the trial committee (Dr Susmita Dhakal, Dr Rachel Hawksworth, Professor Diana Lockwood, Dr Peter Nicholls and Dr Stephen Walker).

At the time the committee met the trial had been recruiting for nine months. In that time only 14 participants had been enrolled. It was determined that recruitment had been optimal by reviewing a random sample of clinical records from the leprosy clinic.

At first, enrolment into the study required individuals with clinical evidence of a T1R to have associated nerve function impairment. This was changed so that individuals with T1Rs involving the skin only would also be eligible for enrolment.

The change to this eligibility criterion was approved by the two Ethics committees.

### 3.3.10 Exclusion criteria

Individuals unwilling to give consent or return for follow-up were excluded.

Individuals who had taken systemic steroids within three months of enrolment were excluded.

Individuals who had received other immunosuppressant therapy including thalidomide within three months of enrolment were excluded.

Individuals with severe active infection such as tuberculosis or severe intercurrent disease were not enrolled into the trial.

Individuals with a contraindication to high dose methylprednisolone such as peptic ulcer disease, diabetes mellitus, glaucoma and uncontrolled hypertension or known allergy to methylprednisolone were also excluded.

Pregnant women were excluded and females of child bearing capacity were not recruited unless they had at least one month of adequate contraception.

### 3.3.11 Randomisation

Block randomisation in groups of four using a table of random numbers generated by Dr Peter Nicholls was used. A standard envelope system was used for allocation concealment. The envelopes were pre-packed in London by Dr Claire Watson.

The allocation procedure was decentralized and operated solely by the chief pharmacist at Anandaban Hospital who kept a separate record of the allocation. The pharmacist had no contact with the study participants during their inpatient stay.

The participants were randomly allocated to the methylprednisolone/prednisolone or the prednisolone alone arm and so had an equal chance of being in either arm of the study.

All study participants, physicians, ward staff and other assessors (physio-technicians) were blinded to the allocation. Only Dr Peter Nicholls had access to the study data and the randomisation code. The allocation code was revealed to the researchers once recruitment, follow-up, data collection and laboratory analyses had been completed (March 2009).

### 3.3.12 Treatment regimen

Individuals were randomly allocated to receive 1 gram of IV methylprednisolone in normal saline infused (over one hour) daily for 3 days plus eight placebo tablets (Comprehensive Medical Services India, Chennai India) daily or an identical normal saline infusion plus eight prednisolone 5 mg tablets daily for three days.

Methylprednisolone (as the sodium succinate) was supplied as a powder in vials containing 1 g. It was reconstituted with water for injection, mixed thoroughly and added to 500 ml of Normal saline by the chief pharmacist.

The infusions were administered in hospital by the enrolling physician or the nursing staff. The participants were given the tablets by the nursing staff who observed them being taken.

 The study participants stayed in hospital until at least the second assessment. This was conducted on day 4 of the study; the day after the third infusion had been completed.

Individuals in the two groups from day 4 continued treatment with identical tablets in a standard reducing schedule of oral prednisolone until they had received a total of 16 weeks of treatment with corticosteroids (Table 3.01.).

An individual allocated to the methylprednisolone group received a total dose of corticosteroid equivalent to 6.15 g of prednisolone. Individuals in the prednisolone alone group received 2.52 g of prednisolone in total.

| Assessment Number | Time point | Prednisolone alone arm (Total corticosteroid dose = 2.52 g of prednisolone) | Methylprednisolone/Prednisolone arm (Total corticosteroid dose equivalent to 6.15 g of prednisolone) |
|---|---|---|---|
| 1 | Day 1 | Placebo infusion + Prednisolone 40 mg | Methylprednisolone 1 g IV + 8 placebo tablets |
| | Day 2 | Placebo infusion + Prednisolone 40 mg | Methylprednisolone 1 g IV + 8 placebo tablets |
| | Day 3 | Placebo infusion + Prednisolone 40 mg | Methylprednisolone 1 g IV + 8 placebo tablets |
| 2 | Days 4-7 | Prednisolone 40 mg | Prednisolone 40 mg |
| 3 | Days 8-14 | Prednisolone 40 mg | Prednisolone 40 mg |
| 4 | Days 15- 21 | Prednisolone 35 mg | Prednisolone 35 mg |
| | Days 22- 28 | Prednisolone 35 mg | Prednisolone 35 mg |
| 5 | Day 29-35 | Prednisolone 30 mg | Prednisolone 30 mg |
| | Days 36-42 | Prednisolone 30 mg | Prednisolone 30 mg |
| | Days 43-49 | Prednisolone 25 mg | Prednisolone 25 mg |
| | Days 50-56 | Prednisolone 25 mg | Prednisolone 25 mg |
| 6 | Days 57-63 | Prednisolone 20 mg | Prednisolone 20 mg |
| | Days 64-70 | Prednisolone 20mg | Prednisolone 20mg |
| | Days 71-77 | Prednisolone 15 mg | Prednisolone 15 mg |
| | Days 78-84 | Prednisolone 15 mg | Prednisolone 15 mg |
| 7 | Days 85-91 | Prednisolone 10 mg | Prednisolone 10 mg |
| | Days 92-98 | Prednisolone 10 mg | Prednisolone 10 mg |
| | Days 99-105 | Prednisolone 5 mg | Prednisolone 5 mg |
| | Days 106-112 | Prednisolone 5 mg | Prednisolone 5 mg |
| 8 | Day 113 | No corticosteroids | No corticosteroids |

**Table 3.01. Treatment regimen for the methylprednisolone study**

Deterioration in nerve function or skin signs was treated with further prednisolone. Individuals deteriorating on a dose of prednisolone less than 20 mg daily had the dose increased back to 20 mg and reduced as per the above regimen. The exception to this was if

they had a T1R involving a facial patch. Individuals taking a dose of prednisolone greater than 20 mg had their dose increased to 40 mg and tapered as per the above protocol.

All participants in the study were given the anti-helminthic agent albendazole 400 mg daily for the first three days of the study. This was done to minimise the risk of helminth infections and in particular hyperinfection by *Strongyloides stercoralis* (Leang *et al.*, 2005). The histamine $H_2$-receptor antagonist famotidine 40mg daily was prescribed to all individuals whilst they were taking corticosteroids.

Standard, appropriate WHO MDT was commenced in individuals who were newly diagnosed with leprosy and continued in those who had not received a full course of either six or 12 months for PB or MB disease respectively.

### 3.3.13 Baseline demographic and clinical data

The patients name, age, occupation and level of literacy were recorded. Details about the time since leprosy symptoms first developed, the Ridley-Jopling classification of their disease and treatment with MDT were all recorded. Each participant was assigned a unique trial number.

Nerve function impairment present for more than six months was recorded. The nerve involved and the functional modality affected (sensory or motor) was also documented.

A detailed history of their skin and nerve symptoms was taken. The number and morphology of skin lesions, the presence of peripheral oedema, nerve tenderness, paraesthesia or nerve pain were recorded.

The individual's weight, height, temperature and blood pressure were recorded.

The skin was examined and the features of the skin signs including number and morphology of lesions and the presence of erythema or ulceration were recorded.

Sensory testing (ST) was performed using two SWM (Sorri-Bauru, Bauru, São Paulo, Brazil) at designated test sites on the hands and feet (see fig. 2.01, Chapter 2). The ulnar and median nerves were tested with 2 g and 10 g monofilaments. The posterior tibial nerve was tested with the 10 g and 300 g monofilaments. Trigeminal nerve sensation was tested using cotton wool.

VMT was assessed using the modified Medical Research Council grading of power (Brain, 2000). The facial nerve was tested by assessing forced eye closure. The median nerve was tested using resisted thumb abduction, the ulnar nerve by resisted little finger abduction and

the radial nerve by resisted wrist extension. The lateral popliteal nerve was tested by resisted foot dorsiflexion.

ST and VMT assessments were carried out by trained physio-technicians and if necessary repeated by the study physicians.

The results of the examination findings were recorded and a Clinical Severity Score calculated using the severity scale that was undergoing validation testing in Bangladesh and Brazil (see Chapter 2).

### 3.3.14 Baseline laboratory investigations

All participants in the study had the following investigations performed on entry into the trial:

A full blood count, serum creatinine and random blood glucose were taken. A stool sample was examined for ova, cysts and parasites. A chest radiograph was performed. On the first three days of the study a daily sputum specimen was examined for the presence of AFB. A pregnancy test was performed on all females of childbearing capacity.

The investigations were performed at Anandaban Hospital and had to be normal or negative prior to commencement of the trial drugs. Any parasites identified on stool microscopy that were not sensitive to albendazole were treated appropriately. Chest radiograph abnormalities were reviewed by two physicians and a decision made as to whether the participant could continue in the study.

Slit skin smears were taken from four sites to determine the mean BI if the participant had not had one done in the three months prior to enrolment in the trial. This investigation is also performed at Anandaban Hospital.

A skin biopsy (6mm punch, Stiefel Laboratories Ltd, High Wycombe, UK) was performed to determine the Ridley-Jopling classification in those individuals who had not already had a biopsy. The skin was fixed in formalin and examined by an experienced leprosy histopathologist at one of two Leprosy Mission Hospitals in India, Dr Lakshmi Rajan (Delhi) or Dr Joyce Ponnaiya (Karigiri).

### 3.3.15 Clinical assessments during the study

Participants were assessed by a study physician prior to treatment and then at day 4 (after the three IV infusions) and then days 8, 15, 29, 57, 85, 113, 141, 169, 197, 225, 253, 281, 309 and 337. A total of 15 assessments during the 48 week follow-up period.

The assessments were standardised. Participants were questioned about skin and nerve symptoms and potential adverse effects related or attributable to corticosteroids.

The same skin and nerve function assessments were performed as at baseline. The individual's weight, temperature and blood pressure were recorded. The participant's urine was tested with urine test strips at each visit to screen for glycosuria.

The assessor recorded the total amount of prednisolone that was prescribed at that visit and whether there was any need for additional prednisolone.

### 3.3.16 Study specimens

All participants provided a skin biopsy (6mm punch) at baseline, day 4 or day 29 and week 17. These specimens were bisected. One half was snap frozen in liquid nitrogen. The other half was immersed in RNA*later*™ (Ambion Inc., Austin, Texas, USA) and kept at 4°C overnight. The following day the RNA*later*™ was discarded and the skin sample stored at -80°C.

These samples were transported to the UK in a liquid nitrogen Dewar flask (CP100 Jencons (Scientific) Ltd.) and stored at the London School of Hygiene and Tropical Medicine in liquid nitrogen.

### 3.3.17 Data recording and management

All data were recorded at each assessment on standardised forms. The study forms were kept in a separate set of case notes from the ordinary hospital record. All study records were kept in a locked area to which access was limited. The data were entered into a secure anonymised Microsoft Office Access database by Dr Walker.

### 3.3.18 Statistical analysis

The data were analysed using the Statistical Package for the Social Sciences (SPSS version 16. SPSS Inc., Chicago, Illinois).

An intention to treat analysis was used for calculating the effects of treatment on individuals in each group.

### 3.3.19 Outcome measures

The primary outcome measure was the frequency of adverse events in the two treatment arms. Adverse events were enquired about at each assessment. Study form 6 (see Appendix 3.11) contained a list of adverse events attributable to corticosteroids which participants

were asked if they had experienced. There was also a free text space available where other symptoms mentioned by the study participants or identified by the physician could be recorded.

Adverse events were defined as major or minor in accordance with the classification used in the TRIPOD studies (Richardus *et al.*, 2003a).

Minor adverse events were defined as moon face, dermatophyte fungal or yeast infections, acne and gastric pain requiring an antacid (in addition to the famotidine each individual was prescribed whilst on corticosteroids). Individuals were questioned about the symptoms of nocturia, polyuria and polydipsia as a method of screening for diabetes mellitus in addition to urinalysis.

Major adverse events were defined as psychosis, peptic ulcer, glaucoma, cataract, diabetes mellitus, severe infections (including tuberculosis), infected neuropathic ulcers and hypertension.

Secondary outcomes measures were:

- change in clinical nerve function impairment and Clinical Severity Score at days 4, 29, 113 and 337.
- time to the next steroid requiring reactional episode or acute nerve function impairment
- the amount of supplementary prednisolone required in addition to the reducing 16 week regimen.

A post-hoc physician assessment of neurological outcome was determined in those individuals who had nerve function impairment and had completed the course of treatment. The designated outcomes were one of: recovered, improved, unchanged or worse.

Recovery was defined as the ability to feel the 2 g monofilament at all test sites on the hands, the 10 g at all sites on the feet and have grade 5 power in all tested muscles.

The assessment was done by comparing participants' baseline sensory and motor examinations with that of their last recorded assessment. The clinical severity score was not used to determine this outcome.

### 3.3.20 Power calculation

The proportion of Nepali individuals experiencing a major adverse effect in the prednisolone treated groups in the three TRIPOD studies was 2.4% (4 of 167) and the rate

of minor adverse effects in the prednisolone groups in the three TRIPOD studies was 8.4% (14 of 167) (Richardus *et al.*, 2003a). The figure for the major adverse effects reported in the paper cited was a combined figure for Bangladesh and Nepal. A figure for the Nepali participants was calculated from the original TRIPOD data (supplied by Dr P. Nicholls). This was done because there was an appreciable difference in the adverse effects rates for the two countries. The rate of minor adverse effects for oral prednisolone in the three studies was 28% in Bangladesh compared to 8.4% in Nepal.

The prednisolone treated groups received a total dose of prednisolone of either 1.96 g (TRIPOD 1 (n=636)) or 2.52 g (TRIPOD 2 and TRIPOD 3 (n = 179)). In this study we planned to compare methylprednisolone 1 g IV and oral prednisolone with a 2.52 g total dose 16 week course of oral prednisolone. The combined major adverse effect rate for the Nepali participants who received prednisolone in TRIPOD 2 and TRIPOD 3 was calculated. Two individuals out of a total of 47 who received prednisolone experienced a major adverse effect (4.3%).

In order to have 80% power to show that methylprednisolone was not associated with a significantly greater ($\alpha < 0.05$) rate of major adverse effects it was calculated that the study would need 201 participants in each group based on a higher rate of 7%. Using this same assumption but with the TRIPOD data for all the Nepali participants (major adverse effect rate of 2.4%) then 64 individuals would be needed to be enrolled in each arm.

## 3.4 Results

### 3.4.1 Participants

42 patients were enrolled into the trial between 7th December 2005 and 31st December 2007. 33 males and nine females were recruited.

22 individuals were randomized to the prednisolone only group. There were no statistically significant differences between the groups with respect to gender, age, Ridley-Jopling classification, or treatment with MDT.

| Study Number | Gender | Age | Occupation | Classification Ridley Jopling | WHO | Reaction Type |
|---|---|---|---|---|---|---|
| AN01 | Male | 36 | Farmer | BL | MB | Nerves Only |
| AN02 | Male | 28 | Carpet weaver | BT | MB | Skin and Nerves |
| AN03 | Male | 23 | Labourer | BT | MB | Skin and Nerves |
| AN04 | Male | 49 | Farmer | BL | MB | Skin and Nerves |
| AN05 | Male | 64 | Farmer | LL | MB | Skin and Nerves |
| AN06 | Male | 24 | Student | BL | MB | Skin and Nerves |
| AN07 | Male | 16 | Student | TT | MB | Skin and Nerves |
| AN08 | Male | 24 | Shop worker | BT | MB | Skin and Nerves |
| AN09 | Male | 42 | Technician | BT | MB | Nerves Only |
| AN10 | Male | 65 | Farmer | BL | MB | Nerves Only |
| AN11 | Female | 17 | Student | BL | MB | Skin and Nerves |
| AN12 | Male | 55 | Farmer | BL | MB | Nerves Only |
| AN13 | Male | 63 | Farmer | BL | MB | Skin and Nerves |
| AN14 | Female | 18 | Unemployed | BT | PB | Skin and Nerves |
| AN15 | Female | 35 | Housewife | BT | MB | Nerves Only |
| AN16 | Female | 39 | Farmer | BT | PB | Nerves Only |
| AN17 | Female | 42 | Farmer | BB | MB | Skin and Nerves |
| AN18 | Male | 41 | Farmer | BL | MB | Skin and Nerves |
| AN19 | Male | 54 | Farmer | BL | MB | Skin and Nerves |
| AN20 | Female | 54 | Farmer | BT | MB | Skin and Nerves |
| AN21 | Male | 53 | Driver | LL | MB | Nerves Only |
| AN22 | Male | 16 | None | BT | MB | Nerves Only |
| AN23 | Male | 29 | Barber | BT | PB | Skin and Nerves |
| AN24 | Male | 33 | Labourer | BT | PB | Nerves Only |
| AN25 | Male | 44 | Labourer | BT | MB | Skin and Nerves |
| AN26 | Male | 28 | Carpenter | BT | MB | Skin and Nerves |
| AN27 | Male | 40 | Silver industry | BB | MB | Skin Only |
| AN28 | Male | 59 | Farmer | BL | MB | Skin Only |
| AN29 | Male | 28 | Driver | BT | MB | Skin Only |
| AN30 | Male | 35 | Farmer | BT | MB | Nerves Only |
| AN31 | Male | 18 | House servant | BB | MB | Skin Only |
| AN32 | Male | 36 | Weaver | BT | MB | Nerves Only |
| AN33 | Female | 35 | Housewife | BT | MB | Skin Only |
| AN34 | Male | 40 | Farmer | BL | MB | Nerves Only |
| AN35 | Male | 27 | | BT | MB | Skin Only |
| AN36 | Male | 62 | Farmer | BT | MB | Skin and Nerves |
| AN37 | Female | 17 | Housewife | BT | MB | Skin and Nerves |
| AN38 | Male | 55 | Driver | BT | MB | Nerves Only |
| AN39 | Female | 40 | Housewife | BT | MB | Skin Only |
| AN40 | Male | 41 | Farmer | BL | MB | Skin and Nerves |
| AN41 | Male | 23 | Waiter | BL | MB | Skin Only |
| AN42 | Male | 22 | Farmer | BT | MB | Nerves Only |

**Table 3.02. Description of MP study participants**

**Figure 3.01. CONSORT flow diagram for the pilot study of individuals randomized to either intravenous methylprednisolone and oral prednisolone or oral prednisolone alone.**

**42 individuals randomly allocated to treatment**

**Allocation**

**Prednisolone alone (n=22)**
**Received prednisolone (n=22)**
**Did not receive prednisolone (n= 0)**

**Methylprednisolone (n=20)**
**Received MP (n=20)**
**Did not receive MP (n=0)**

**Follow-Up**

**Lost to follow-up (n=5)**
**Discontinued intervention (n=1)**

**Lost to follow-up (n=3)**
**Discontinued intervention (n=1)**

**Analysis**

**Analyzed (n=22)**
**Excluded from analysis (n=0)**

**Analyzed (n= 20)**
**Excluded from analysis (n=0)**

|  |  | Prednisolone (n=22) | Methylprednisolone (n=20) |
| --- | --- | --- | --- |
| Sex | Female | 5 | 4 |
|  | Male | 17 | 16 |
| Median Age [years (Range;Min-Max)] | Female | 39 (19;35-54) | 17.5 (25;17-42) |
|  | Male | 40 (43;22-65) | 28.5 (48;16-64) |
| Ridley-Jopling Classification | TT | 0 | 1 |
|  | BT | 11 | 12 |
|  | BB | 0 | 3 |
|  | BL | 10 | 3 |
|  | LL | 1 | 1 |
| Multi-drug Therapy status | Started at enrolment | 3 | 5 |
|  | Current | 14 | 10 |
|  | Completed | 5 | 5 |

**Table 3.03. Description of study participants in each arm**

The two groups did not differ significantly in terms of the nature of the reaction, the type of nerve function impairment at baseline or the pattern of old (> 6 months) nerve function impairment.

|  |  | Prednisolone (n=22) | Methylprednisolone (n=20) | p value (Significance p<0.05) |
| --- | --- | --- | --- | --- |
| Reaction Type | Skin Only | 4 | 4 |  |
|  | Skin and Nerves | 8 | 13 |  |
|  | Nerves Only | 10 | 3 | 0.87 |
| Baseline Nerve Status | No loss | 3 | 4 |  |
|  | New | 10 | 14 |  |
|  | Old | 1 | 0 |  |
|  | Old and new | 8 | 2 | 0.149 |
| Type of nerve involvement | None | 3 | 4 |  |
|  | Sensory | 3 | 3 |  |
|  | Motor | 3 | 4 |  |
|  | Mixed | 13 | 9 | 0.821 |
| Old nerve impairment pattern | None | 13 | 18 |  |
|  | Sensory | 3 | 1 |  |
|  | Motor | 1 | 0 |  |
|  | Mixed | 5 | 1 | 0.145 |

**Table 3.04. Reaction type and nerve involvement of study participants**

A majority (69%) of participants had negative slit-skin smears. The preponderance of smear negative cases is similar to that in the INFIR Cohort study where 63.7% of the MB patients were smear negative (van Brakel *et al.*, 2005b). The number of participants in each arm of the study and their mean bacterial indices is shown in Table 3.05.

| Mean bacterial index | Prednisolone (n=22) (%) | Methylprednisolone (n=20) (%) |
|---|---|---|
| **0** | 13 (59.1) | 16 (80) |
| **Up to and including 1** | 1 (4.5) | 2 (10) |
| **Up to and including 2** | 3 (13.6) | 1 (5) |
| **Up to and including 3** | 4 (18.2) | 0 |
| **Up to and including 4** | 1 (4.5) | 1 (5) |
| **Up to and including 5** | 0 | 0 |
| **Up to and including 6** | 0 | 0 |

**Table 3.05. Mean bacterial index of participants in study**

## 3.4.2 Incomplete follow-up

| Study Number | Arm | Number of assessments | Number of days in the trial |
|---|---|---|---|
| AN12 | P | 4 | 19 |
| AN15 | P | 9 | 147 |
| AN18 | P | 13 | 281 |
| AN19 | MP | 12 | 202 |
| AN20 | P | 13 | 272 |
| AN26 | MP | 8 | 119 |
| AN37 | MP | 4 | 14 |
| AN42 | P | 13 | 299 |

**Table 3.06. Participants who did not complete follow-up**

Eight participants (19%) did not complete the full schedule of follow-up. Five were randomised to the prednisolone arm and three received methylprednisolone. Efforts were made to get these individuals to attend by telephoning or writing to them but without success. Two of these individuals stopped attending whilst on corticosteroids.

### 3.4.3. Nerve impairment greater than six months at baseline

Eleven (26.2%) participants had nerve function impairment which they reported as being present for more than six months. In the INFIR cohort 26.1% of individuals had longstanding NFI (van Brakel *et al.*, 2005b) and 24.6% in a Bangladeshi cohort studied retrospectively (Richardus *et al.*, 1996).

A total of 26 out of 504 (5.2%) nerves were affected. Twenty-one out of 336 (6.3%) sensory nerves and 15 out 420 (3.6%) motor nerves had detectable nerve function impairment reported to be of greater than six months duration. The ulnar and median nerves have both sensory and motor functions.

One individual (AN18) had six different nerves involved. Figure 3.02 shows the number and pattern of impairment for each individual. Table 3.07 shows each individuals impaired nerves and how their monofilament or motor scores differed between enrolment and the last assessment.
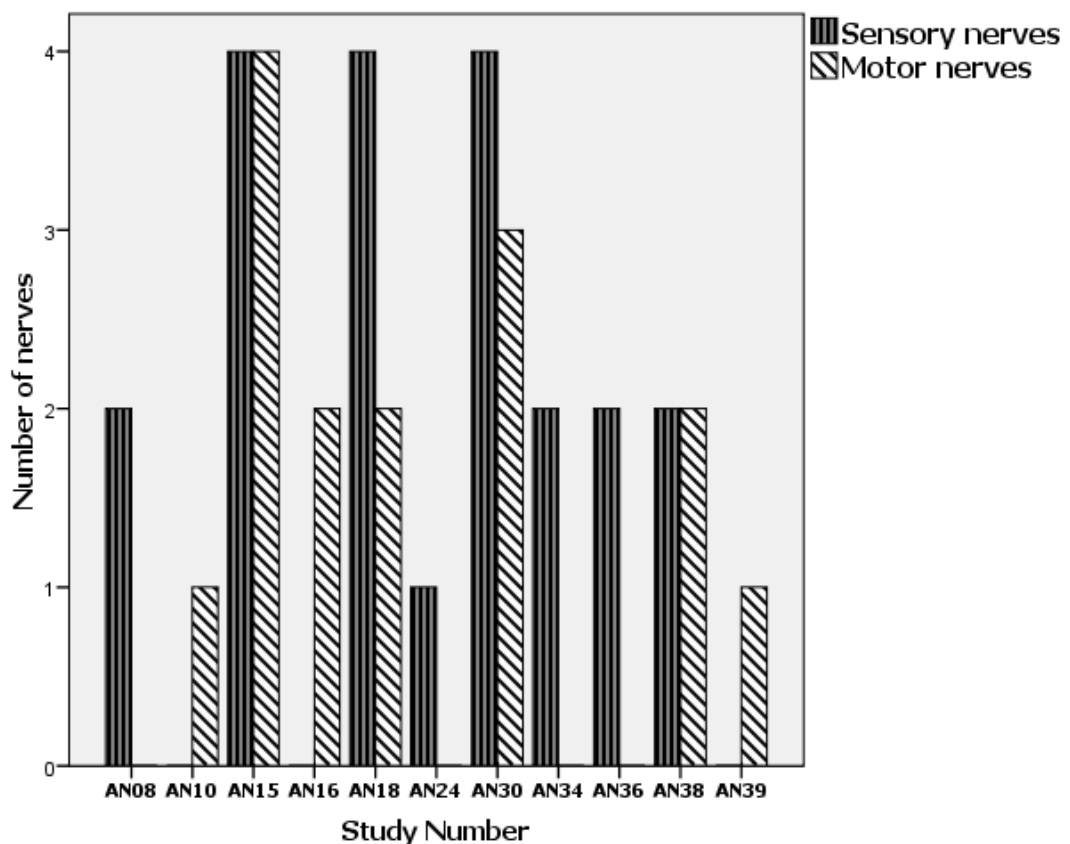


**Figure 3.02 Individuals with NFI greater than 6 months duration at baseline**

Figure 3.03 shows the frequency of involvement of each peripheral nerve. The posterior tibial and ulnar nerves were the most commonly affected. Longstanding nerve impairment was more frequently sensory which is in keeping with previous studies of nerve involvement in leprosy (Croft *et al.*, 1999; van Brakel *et al.*, 2005a).



**Fig. 3.03 Number of nerves impaired for more than six months**
**(Total number of nerves in study)**
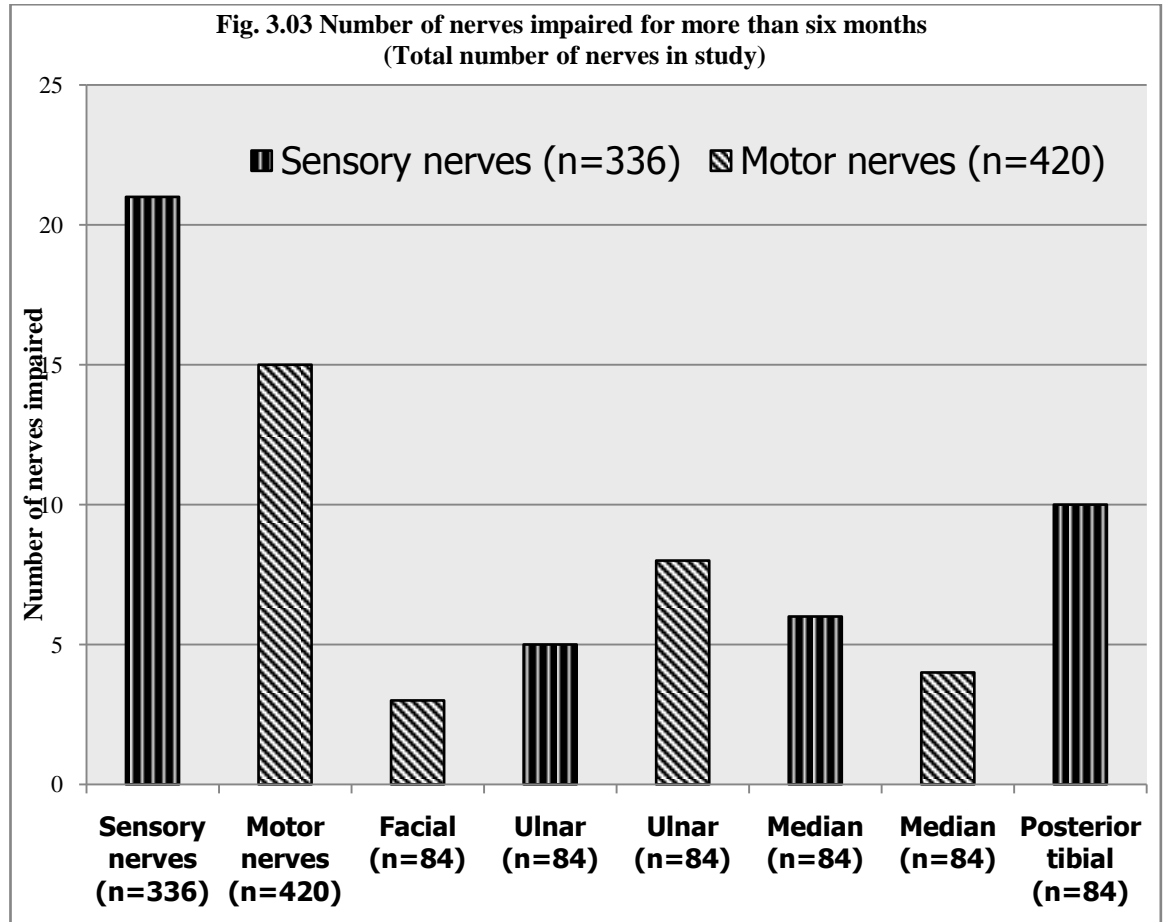
Sixteen (76.2%) sensory nerves and 6 (40%) motor nerves showed some improvement in monofilament score or VMT score respectively during corticosteroid therapy.

| Study number | Arm | Nerve | Baseline score | Last recorded score |
|---|---|---|---|---|
| AN8 | P | Left posterior tibial | 3 | 2 |
| | | Right posterior tibial* | 3 | 3 |
| AN10 | P | Left facial nerve | 1 | 0 |
| AN15 | P | Left ulnar* | 3 | 3 |
| | | Left ulnar (motor)* | 3 | 3 |
| | | Right ulnar* | 3 | 3 |
| | | Right ulnar (motor)* | 3 | 3 |
| | | Left median* | 3 | 3 |
| | | Left median (motor)* | 3 | 3 |
| | | Right median* | 3 | 3 |
| | | Right median (motor) | 2 | 3 |
| AN16 | P | Left ulnar (motor)* | 3 | 3 |
| | | Right ulnar (motor) | 1 | 0 |
| AN18 | P | Left facial (motor) | 2 | 2 |
| | | Right facial (motor) | 2 | 2 |
| | | Left ulnar | 1.5 | 2.5 |
| | | Left median | 2 | 2.5 |
| | | Left posterior tibial* | 3 | 3 |
| | | Right posterior tibial | 1.5 | 3 |
| AN24 | P | Right median | 2 | 2 |
| AN30 | P | Left ulnar* | 3 | 3 |
| | | Left ulnar (motor)* | 3 | 3 |
| | | Right ulnar (motor) | 1 | 0 |
| | | Left median* | 3 | 3 |
| | | Left median (motor)* | 3 | 3 |
| | | Left posterior tibial* | 3 | 3 |
| | | Right posterior tibial | 3 | 2.5 |
| AN34 | P | Left posterior tibial | 2.5 | 2 |
| | | Right posterior tibial | 1.5 | 0 |
| AN36 | MP | Left posterior tibial | 2.5 | 2.5 |
| | | Right posterior tibial | 0.5 | 1 |
| AN38 | MP | Left ulnar* | 3 | 3 |
| | | Left ulnar (motor)* | 3 | 3 |
| | | Left median* | 3 | 3 |
| | | Left median (motor)* | 3 | 3 |
| AN39 | P | Right ulnar (motor) | 1 | 0 |

**(\*indicates complete loss with no change)**

**Table 3.07. Nerves reported to have been affected for more than six months and their scores at the beginning and end of treatment**

In contrast to what was seen during corticosteroid therapy at the last recorded assessment only one (4.8%) sensory nerve (posterior tibial AN34) had recovered, three (14.3%) posterior tibial nerves had improved by a median monofilament score of 0.5. Thirteen

(61.9%) were unchanged (including 11 nerves with a maximum monofilament score of three) and four sensory nerves had deteriorated by a median score of 0.75.

Four (26.7%) motor nerves recovered but all had the mildest possible deficit at baseline; a VMT score of one (equivalent to MRC grade 4 power). Ten (66.7%) motor nerves were unchanged, including eight that had a maximal VMT score of three. One motor nerve (median AN15) deteriorated from a VMT score of two to three.

### 3.4.4 Adverse Events

Table 3.08 shows the number of individuals who experienced a particular adverse event. Twenty-three participants experienced at least one adverse event, twelve (54.5%) in the prednisolone arm and 11 (55%) in the methylprednisolone arm. Seven individuals experienced more than one adverse event. There were no statistically significant differences in the number of individuals experiencing a given adverse event between the two groups of the study.

Two individuals (one from each arm of the study) experienced a major adverse event. One was diagnosed with glaucoma and the other with infected neuropathic ulcers. None of the participants developed hypertension, tuberculosis or diabetes mellitus.

| | Adverse Event | Prednisolone | Methylprednisolone | chi square (Fisher's exact) |
|---|---|---|---|---|
| **Minor** | | | | |
| | Moon Face | 2 | 6 | 0.123 |
| | Acne | 5 | 5 | 1 |
| | Fungal infection | 0 | 1 | 0.476 |
| | Gastric pain | 5 | 2 | 0.414 |
| | NPP | 2 | 2 | 1 |
| | Weight gain | 1 | 0 | 1 |
| | | | | |
| **Major** | Glaucoma | 1 | 0 | 1 |
| | Infected ulcers | 0 | 1 | 0.476 |

**Table 3.08. Minor and major adverse events in both arms of the study**

The risk ratio of having an adverse event (of any type; major or minor) given that the participant received methylprednisolone was 1.0083 (95% CI: 0.5817 to 1.7480; $p=0.9764$) compared to prednisolone.

| Study Number | Arm | Adverse events | Timing of adverse events (days) | Additional corticosteroids |
|---|---|---|---|---|
| AN01 | P | Acne | 56 | No |
| AN03 | MP | Acne | 77 | Yes |
| AN04 | P | Glaucoma | 305 | Yes |
| AN05 | MP | Moon face | 138 | Yes |
| AN06 | P | Acne | 14 | Yes |
| AN07 | MP | Moon face | 85 | No |
| AN08 | P | Acne | 57 | Yes |
| AN09 | MP | Acne | 14 | Yes |
| | | Fungal infection | 14 | |
| | | Moon face | 57 | |
| | | NPP | 204 | |
| AN10 | P | NPP | 7 | Yes |
| | | Gastric pain | 29 | |
| | | Moon face | 57 | |
| AN13 | MP | NPP | 4 | No |
| | | Moon face | 29 | |
| AN14 | MP | Acne | 27 | No |
| | | Moon face | 55 | |
| AN16 | P | Gastric pain | 26 | Yes |
| | | Moon face | 55 | |
| AN17 | MP | Moon face | 83 | Yes |
| AN21 | P | NPP | 7 | No |
| | | Weight gain | 259 | |
| AN22 | MP | Acne | 54 | No |
| AN24 | P | Acne | 56 | Yes |
| AN29 | MP | Acne | 3 | Yes |
| | | Gastric pain | 3 | |
| AN32 | P | Acne | 83 | Yes |
| AN33 | P | Gastric pain | 21 | Yes |
| AN36 | MP | Infected ulcers | 170 | No |
| AN37 | MP | Gastric pain | 3 | No |
| AN39 | P | Gastric pain | 13 | No |
| AN42 | P | Gastric pain | 243 | Yes |

**Table 3.09. Timing of adverse events for each individual**

Table 3.09 shows the timing of adverse events experienced by each individual. AN36 was randomized to the methylprednisolone arm of the study and developed infected neuropathic ulcers. This adverse event occurred at day 170. AN04 developed glaucoma at day 305 following additional courses of prednisolone for ENL.

There was no significant association between the occurrence of adverse events and the prescribing of additional prednisolone.
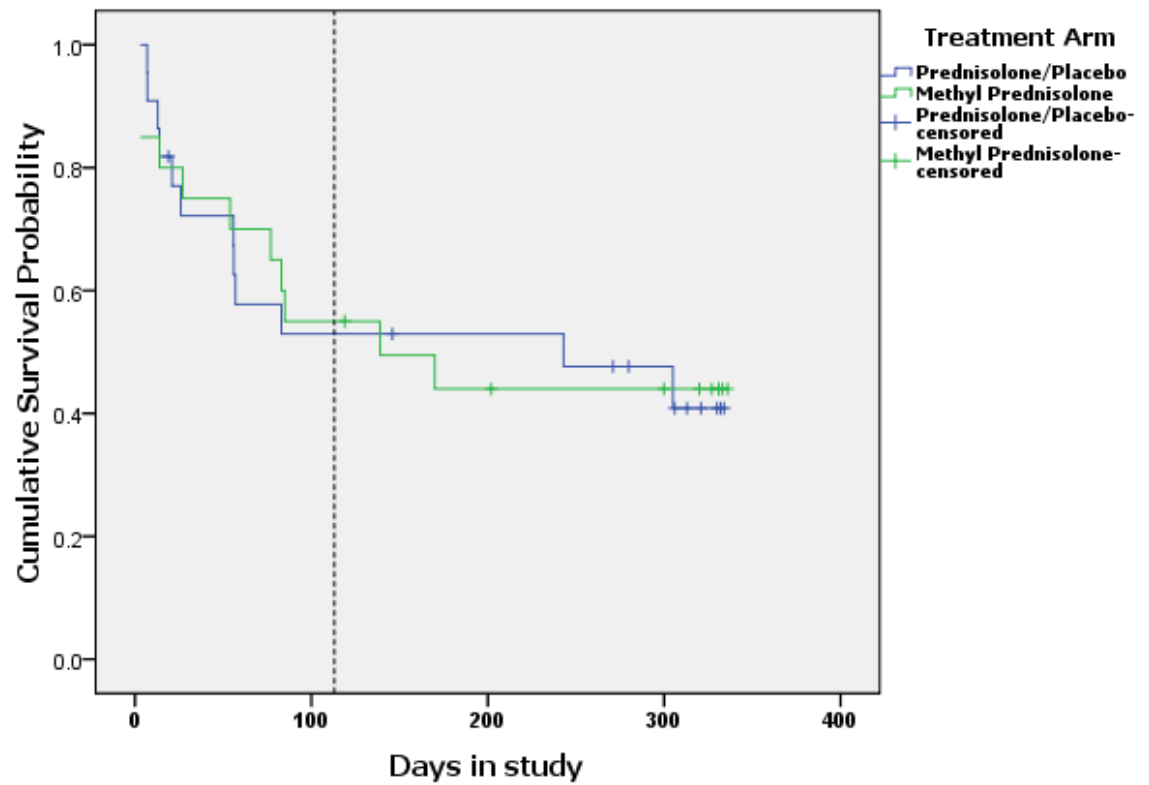


**Figure 3.04 Time to first adverse event**

**Figure 3.05 Tinea cruris (AN9)**

Individuals were most likely to experience an adverse event whilst taking the first course of corticosteroids between days 1 and 112. Fig 3.04 is a Kaplan-Meier survival curve showing the cumulative "survival" probability (i.e. not having an adverse event) for individuals in each group. There was no significant difference between the two groups (Log Rank (Mantel-Cox) 0.945).

Four individuals had their first adverse event after the initial study intervention had been completed (post day 112). Two others had a new adverse event after the intervention period. AN21 and AN36 were the only two to experience an adverse event, weight gain and infected neuropathic ulcers respectively, whilst not taking corticosteroids.

Baseline visual acuity was recorded in 24 individuals. Fifteen individuals had unchanged or normal visual acuity at the end of the study. There were five individuals (two had received MP) who experienced deterioration in their visual acuity. Four of these individuals had received additional prednisolone and of these two had ENL.

Twelve individuals did not have their visual acuity measured during the study. In 22 individuals it was not possible to determine change in visual acuity because data were missing.

The only individual who reported weight gain on direct questioning was AN21. However 22 individuals had objective evidence of weight gain during the study (11 from each group).

The mean weight gain was 0.8 kg. There was no significant difference between the two groups. AN04 who was randomized to the prednisolone only arm had the largest weight gain of 7.5 kg after receiving a total of 4.08 g of prednisolone.

There was no correlation between weight gain and the total quantity of corticosteroid prescribed during the study ($r^2 = 5.89 \times 10^{-6}$).
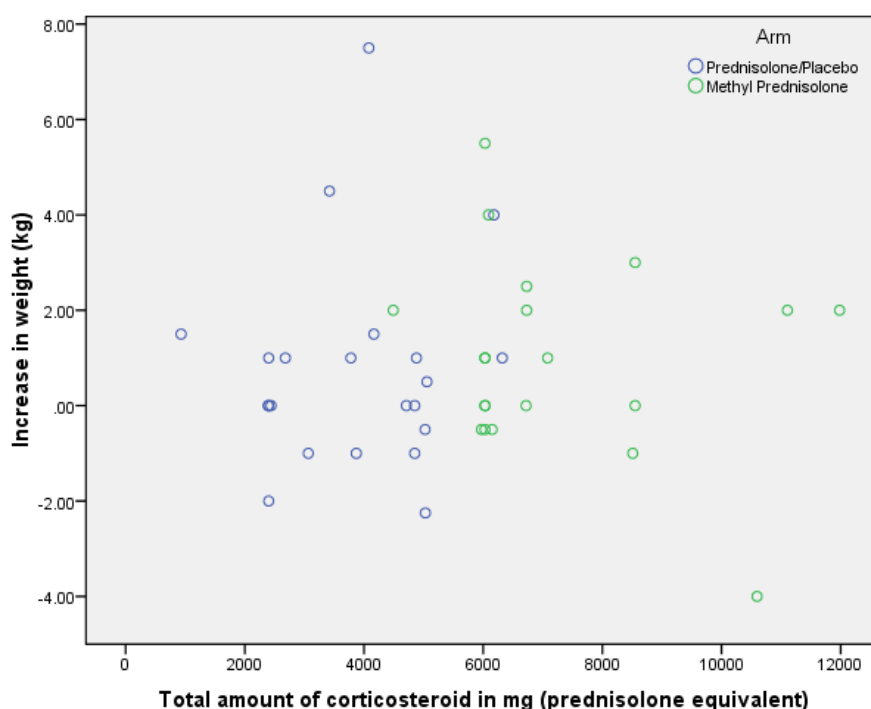


**Figure 3.06. Increase in weight plotted against total amount of corticosteroid prescribed**

Five individuals had abnormal urinalysis. Two individuals had a trace of albumin detected at baseline prior to the administration of steroids. The serum creatinine of both were normal. Another individual had a trace of albumin detected by urinalysis at day 237. Her serum creatinine was normal and all subsequent urinalysis was normal.

AN6 had glycosuria of +2 at day 309, this had resolved at the following visit. AN24 had an episode of +1 glycosuria and albuminuria at day 287. His serum creatinine was normal and subsequent urinalysis did not demonstrate any abnormality. Both these individuals were asymptomatic, neither complained of nocturia, polyuria and polydipsia.

One individual had a dermatophyte fungal infection.

### 3.4.5 Change in clinical status during the study

The total clinical severity scores, calculated using the validated scale, for each arm of the study at day 1 (enrolment) and days 4, 29,113 and 337 are shown using boxplots in fig.3.07.

There is a downward trend in the total clinical severity scores of both groups. There were no statistically significant differences between the prednisolone and methylprednisolone groups at any time point.
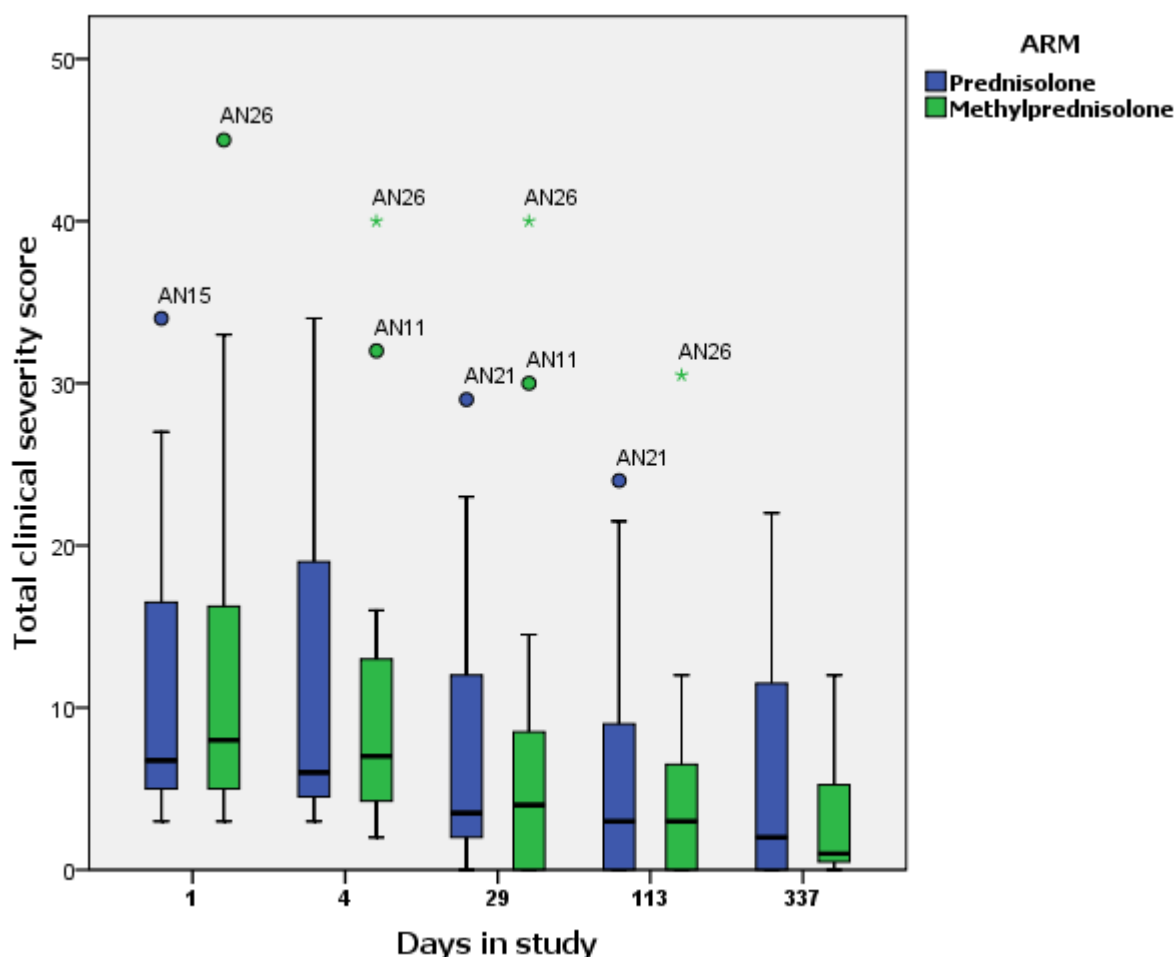


**Figure 3.07. Total severity score at days 1, 4, 29, 113, 337**

### 3.4.5.1 Skin signs

There was a significant difference between skin scores at baseline (p=0.014). This occurred despite allocation to the groups being randomized. The median skin score at baseline in the MP group was 4 (IQR =8) and in the P group was 0.5 (IQR =7).

The median skin scores were zero at the end of the study for both groups.

The Kaplan-Meier in fig.3.10 shows the cumulative probability of no deterioration in skin at a given time point. There is no statistical difference between the two groups (Log Rank (Mantel-Cox) = 0.838).
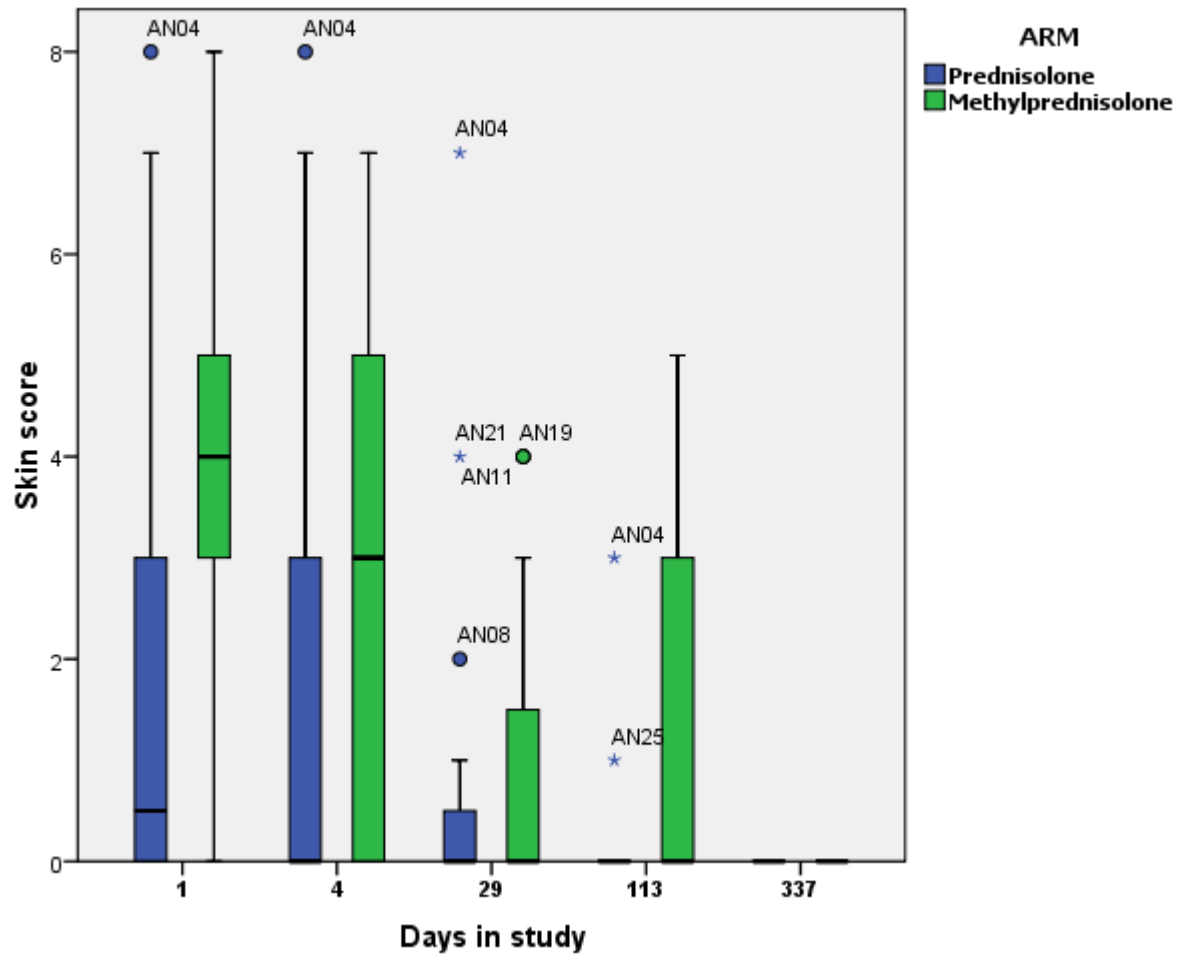
**Figure 3.08 Skin score at days 1, 4, 29, 113, 337**.



**Figure 3.09 Facial T1R a. before and b. after corticosteroid treatment (AN19) (Image 3.09a Dr R A Hawksworth)**

118
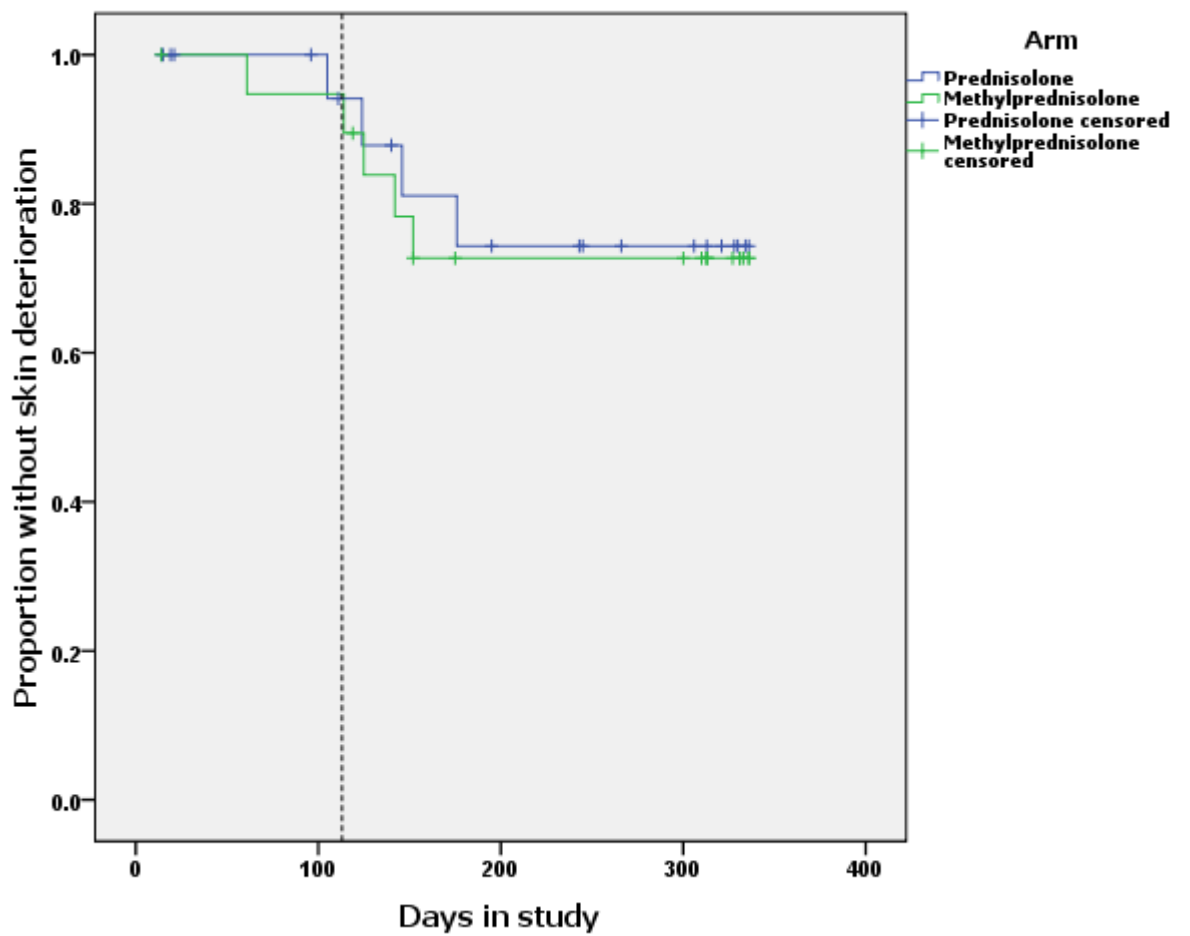
**Figure 3.10 Time to deterioration of the skin**

### 3.4.5.2 Sensory scores

There was no significant difference between the sensory scores (corrected for impairment > 6 months) of the two groups at baseline. A downward trend is visible for both groups but there are no differences at any of the pre-specified time points.
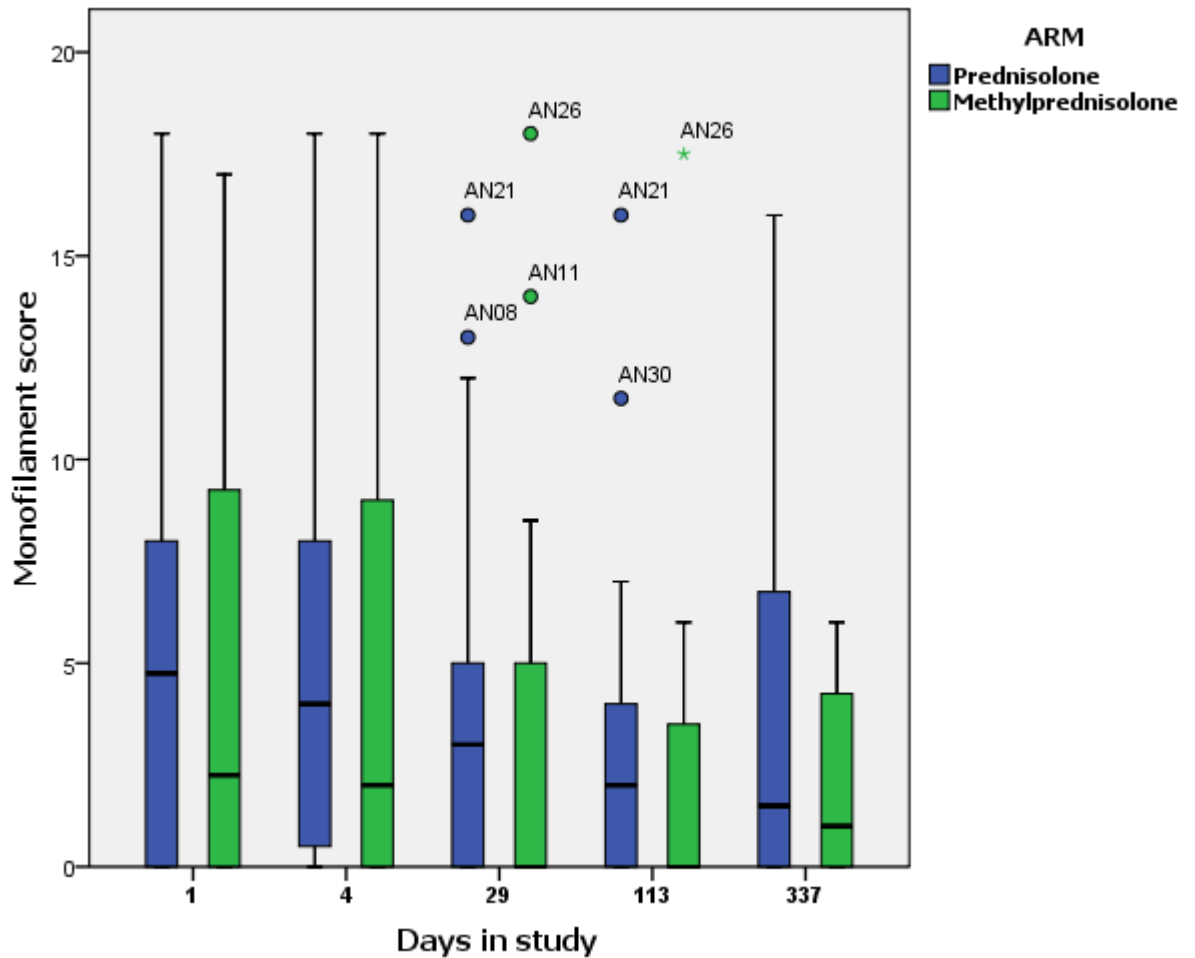
**Figure 3.11 Monofilament score at days 1, 4, 29, 113, 337**

The Kaplan-Meier survival analyses of deterioration in sensory score during the study to days 29, 113 and 337 demonstrate that there is no difference between the groups at day 29 but at day 113 there is a significant difference in the probability of monofilament deterioration between MP and P arms (p=0.046). The prednisolone group were more likely to experience deterioration in sensation. This effect is not maintained at the end of the study follow-up period at day 337.
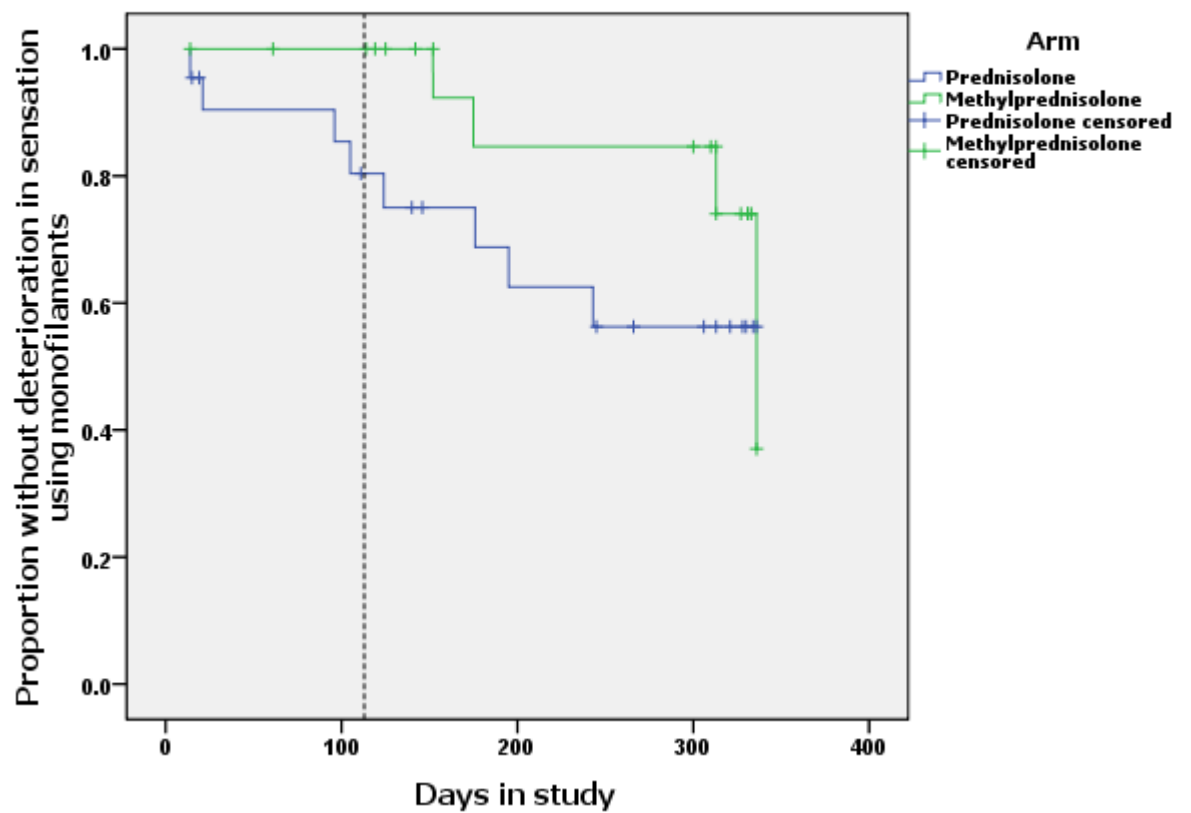
**Figure 3.12 Time to deterioration of sensory function**

### 3.4.5.3 Motor scores

The motor scores of the two groups at baseline are not significantly different. They show a downward trend during the course of the study. There are no significant differences between the scores of the group at any of the time points.

There were no significant differences between the groups in the probability of an individual experiencing a deterioration in motor function at days 29, 113 or 337.
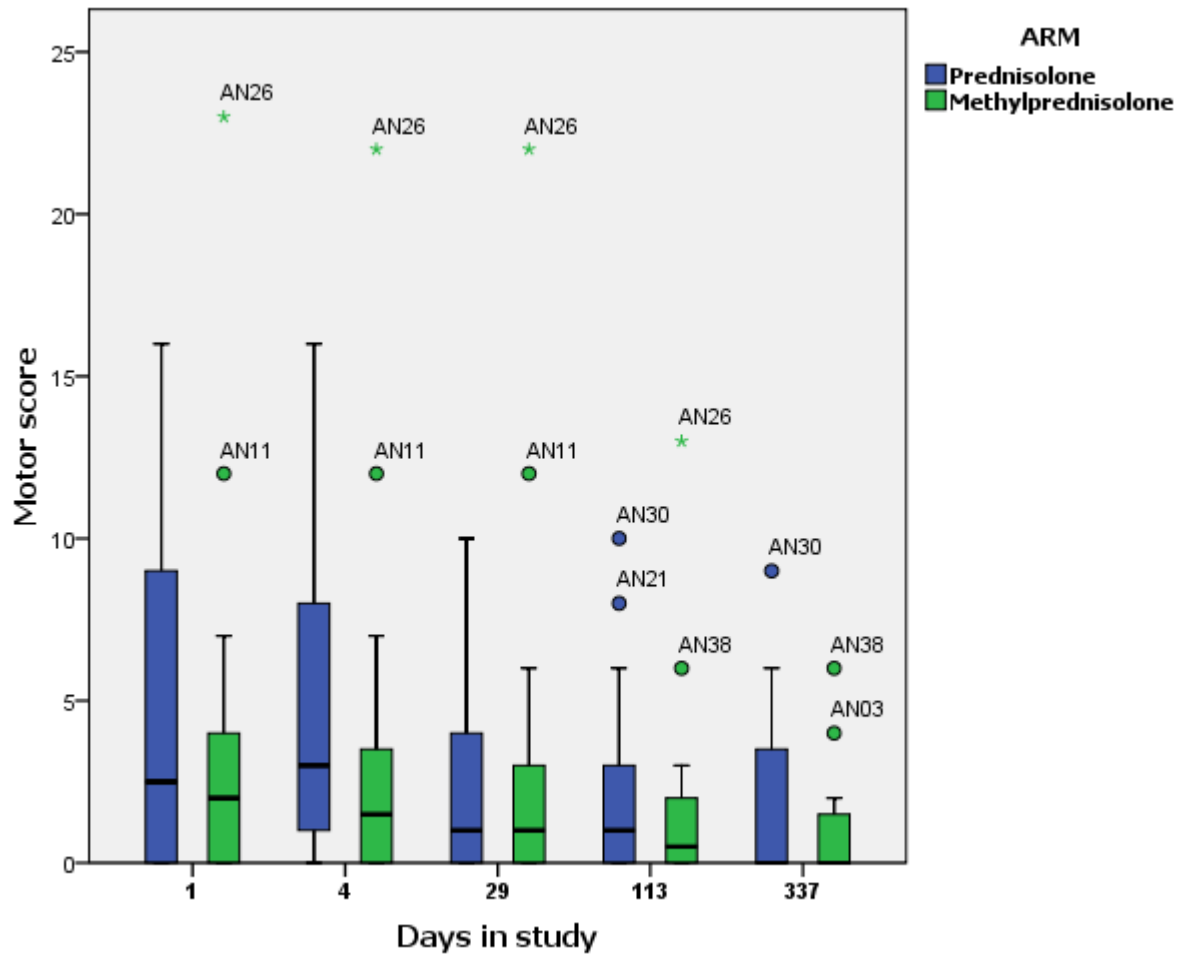
**Figure 3.13 Motor score at days 1, 4, 29, 113, 337**
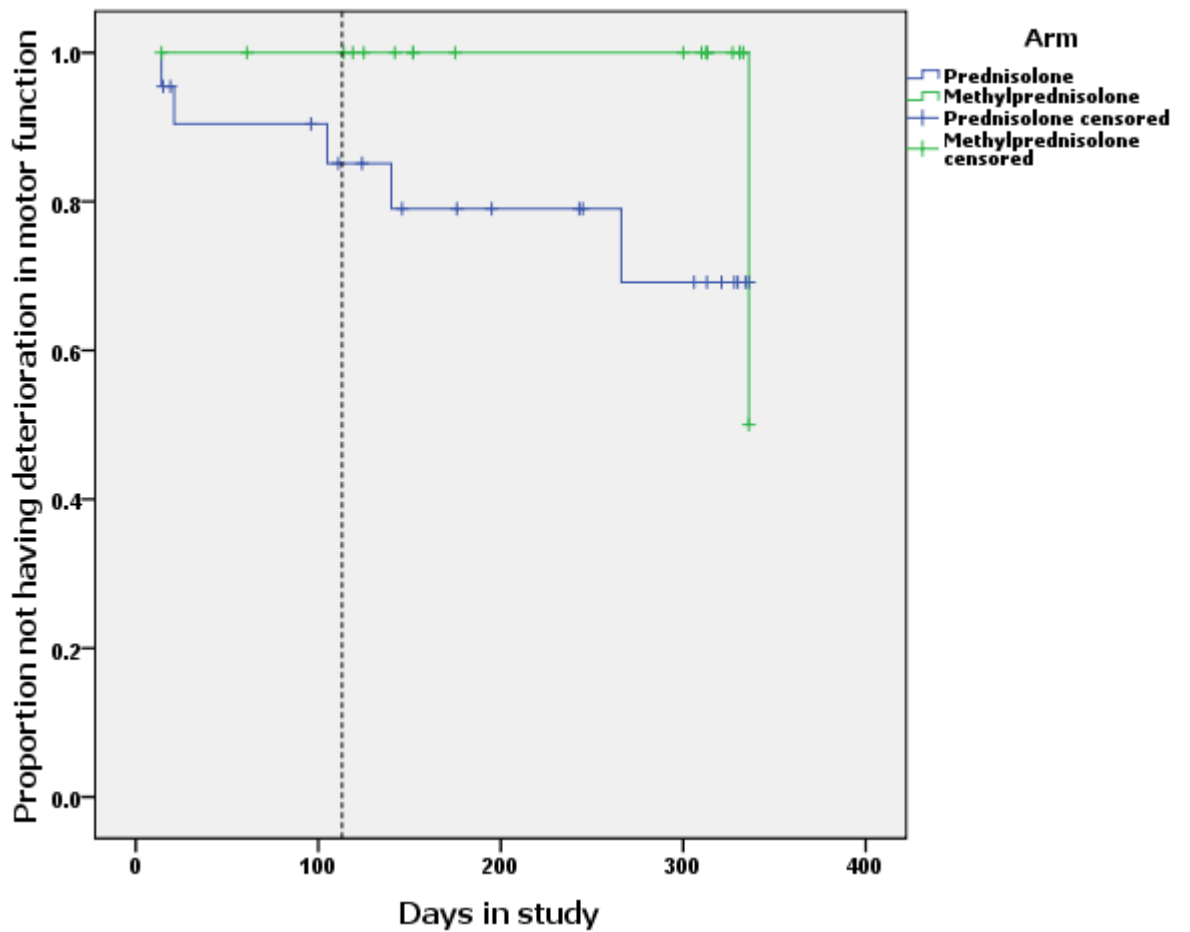
**Figure 3.14 Time to deterioration of motor function**

### 3.4.6 Additional prednisolone requirements of participants

Figure 3.14 is a Kaplan-Meier estimate of the survival curve for the two groups showing the event when additional steroid was prescribed and censoring individuals who were unavailable for further assessment or who received prednisolone either inappropriately or for ENL. There was no significant difference in the probability of being prescribed additional prednisolone between the two groups (Log Rank (Mantel Cox) $p=0.126$).
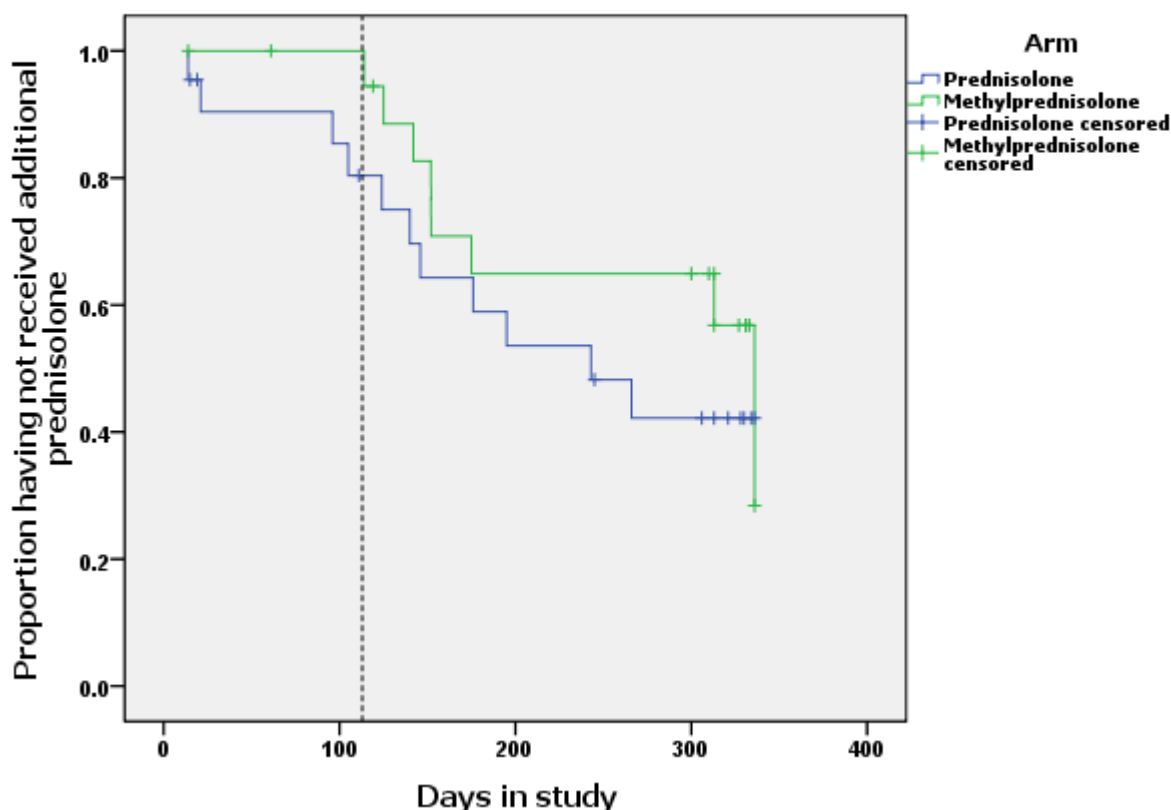
**Figure 3.15 Time to requiring first course of additional prednisolone**

The events that resulted in additional prednisolone being prescribed are shown in Table 3.09 for each individual. Four individuals subsequently required a further course of prednisolone.

The amount of additional prednisolone required by individuals randomized to either the MP or P alone treatment group did not differ significantly. The mean amount of additional prednisolone prescribed during the study was 1252.5 mg (SD±1862.0) for the MP group and 1432.7 mg (SD±1245.9) for the P group (p=0.718).

**Individuals and events leading to the prescribing of additional prednisolone**

Twenty individuals (47.6%) required additional prednisolone because they experienced a deterioration of nerve function (n= 11) or a recurrence of a T1R (n= 6) or both (n= 3). In addition two individuals received additional prednisolone inappropriately and two (AN04 and AN40) developed ENL requiring prednisolone.

| Study Number | Arm | Time of event (Days after enrolment) | Event | Appropriate | Further additional prednisolone required |
|---|---|---|---|---|---|
| AN03 | MP | 336 | Deterioration in sensation right and left median and right and left ulnar power | Yes | No |
| AN04 | P | 111 | ENL | Yes | Yes |
| AN05 | MP | 313 | Deterioration both posterior tibial nerves | Yes | No |
| AN06 | P | 266 | Deterioration right lateral popliteal power | Yes | No |
| AN08 | P | 176 | Deterioration in skin and right and left median sensation | Yes | No |
| AN09 | MP | 175 | Deterioration left ulnar sensation | Yes | No |
| AN10 | P | 140 | Left facial, right and left ulnar weakness | Yes | Yes, day 183 for skin flare |
| AN15 | P | 15 | No change in skin or nerves | No | No |
| AN16 | P | 96 | Deterioration left ulnar sensation | Yes | No |
| AN17 | MP | 152 | Deterioration both posterior tibial nerves | Yes | No |
| AN18 | P | 105 | Deterioration in skin, sensation both ulnar and median nerves and power both ulnar nerves | Yes | No |
| AN19 | MP | 125 | Skin flare | Yes | No |
| AN20 | P | 21 | Deterioration right posterior tibial sensation | Yes | Yes, day 160 for skin flare |
| AN23 | MP | 142 | Skin flare | Yes | No |
| AN24 | P | 14 | Deterioration in sensation and power right ulnar | Yes | Yes, day 147 deterioration sensation and power right ulnar |
| AN25 | P | 245 | No change in skin or nerves | No | No |
| AN27 | P | 124 | Skin flare and deterioration left posterior tibial | Yes | Yes, day 280 for skin flare and deterioration sensation left ulnar, right and left median and left posterior tibial |
| AN28 | MP | 61 | Skin flare | Yes | Yes developed ENL |
| AN29 | MP | 114 | Skin flare | Yes | No |
| AN32 | P | 195 | Deterioration right ulnar sensation | Yes | No |
| AN33 | P | 146 | Skin flare | Yes | No |
| AN35 | MP | 152 | Skin flare | Yes | No |
| AN40 | P | 306 | ENL | Yes | No |
| AN42 | P | 243 | Left ulnar weakness | Yes | No |

**Table 3.10. Indication for and timing of additional prednisolone**

Five of the 20 individuals (appropriately prescribed additional prednisolone for a trial indication) required prednisolone before day 112, the last day of the intervention period.

The median time to requiring additional prednisolone was 61 days when individuals were receiving prednisolone 20 mg daily.

The other 75% had finished the prednisolone before experiencing a deterioration requiring further treatment. The median number of days between finishing the study intervention and requiring additional prednisolone was 63 days (range = 2-224).

**Physician assessment of neurological outcome**

The physician assessment of neurological outcome demonstrated that 7 (20.6%) individuals who had nerve damage at baseline (of less than six months duration) and completed a 16 week course of corticosteroid therapy recovered (fig. 3.15). Seventeen individuals of 34 (50%) had an improvement in their nerve function. However nine participants (26.5%) had nerve function that was unchanged and one individual's nerve function had deteriorated.

Six individuals were excluded from this analysis because throughout the study they only had cutaneous involvement and two individuals did not complete the 16 week course of corticosteroid therapy.



Figure 3.16. Comparison of nerve impairment at final recorded assessment in individuals (n=34) who completed initial steroid course
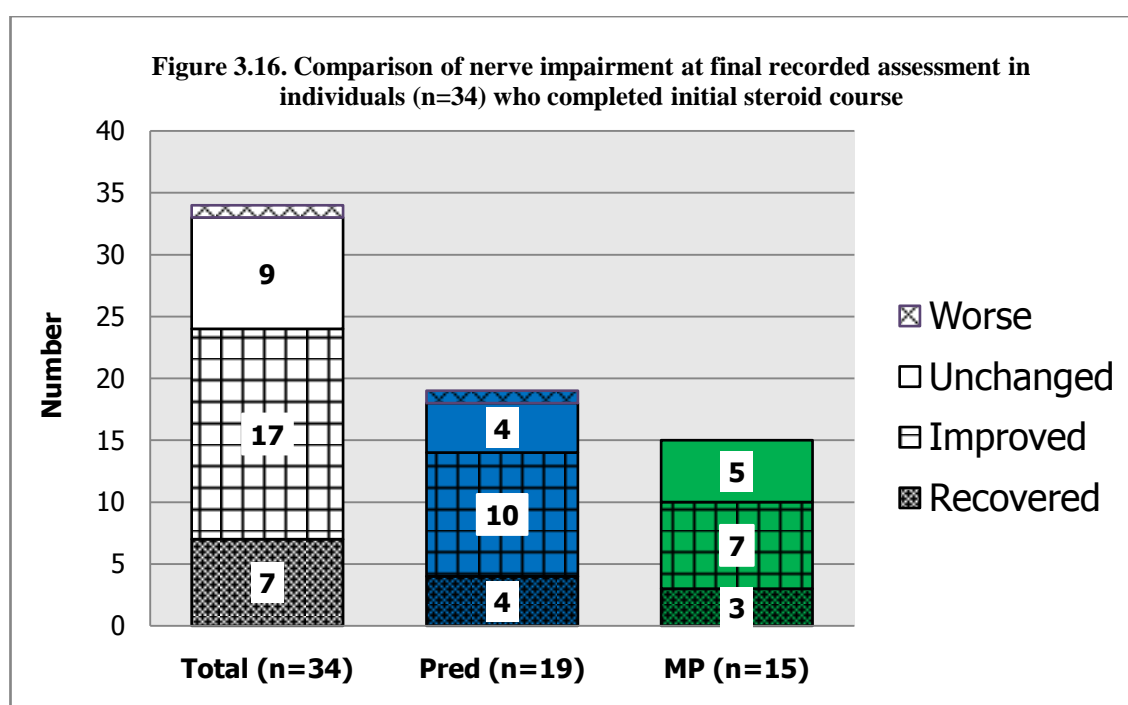
Table 3.11 shows the physician assessment of neurological outcome by individual and the difference between the neurological components of the clinical severity score at their last recorded assessment and baseline. A negative value indicates that the value of the neurological component of the clinical severity score increased.

| Study Number | Outcome | Arm | Change in nerve score |
|---|---|---|---|
| AN20 | Recovered | P | 5.5 |
| AN42 | Recovered | P | 3 |
| AN39 | Recovered | P | 1 |
| AN40 | Recovered | P | 1 |
| AN05 | Recovered | MP | 10 |
| AN07 | Recovered | MP | 3 |
| AN13 | Recovered | MP | 2 |
| AN08 | Improved | P | 10.5 |
| AN25 | Improved | P | 10 |
| AN01 | Improved | P | 5 |
| AN32 | Improved | P | 5 |
| AN21 | Improved | P | 4 |
| AN24 | Improved | P | 4 |
| AN34 | Improved | P | 3 |
| AN16 | Improved | P | 1.5 |
| AN06 | Improved | P | 1 |
| AN10 | Improved | P | 0 |
| AN11 | Improved | MP | 21.5 |
| AN22 | Improved | MP | 12.5 |
| AN03 | Improved | MP | 9 |
| AN09 | Improved | MP | 2.5 |
| AN17 | Improved | MP | 2.5 |
| AN02 | Improved | MP | 1.5 |
| AN19 | Improved | MP | 1 |
| AN04 | Unchanged | P | 1.5 |
| AN30 | Unchanged | P | 1.5 |
| AN15 | Unchanged | P | 0 |
| AN18 | Unchanged | P | 0 |
| AN26 | Unchanged | MP | 9.5 |
| AN14 | Unchanged | MP | 1.5 |
| AN36 | Unchanged | MP | 0.5 |
| AN38 | Unchanged | MP | 0.5 |
| AN23 | Unchanged | MP | 0 |
| AN27 | Worse | P | -2.5 |
| AN33 | Skin only | P | 0 |
| AN41 | Skin only | P | 0 |
| AN28 | Skin only | MP | 0 |
| AN31 | Skin only | MP | 0 |
| AN29 | Skin only | MP | 0 |
| AN35 | Skin only | MP | 0 |
| AN12 | Lost | P | 1 |
| AN37 | Lost | MP | 0 |

**Table 3.10. Physician assessment of neurological outcome and change in nerve score. (Shaded area indicates participants not included in this assessment)**
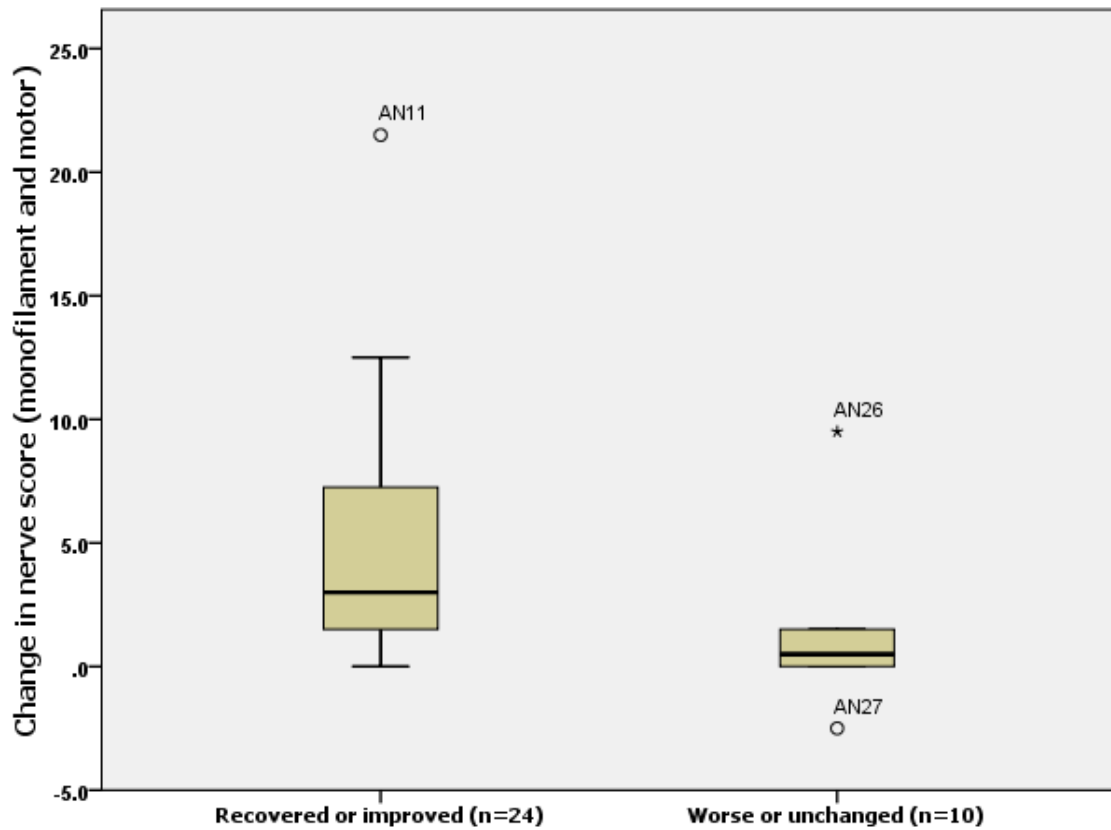
**Figure 3.17 Change in nerve score and clinical outcome in those completing corticosteroid course (n=34)**

Individuals were grouped according to their status with respect to the physician assessment of neurological outcome as shown in fig.3.17. The median change in nerve score between the baseline and the final recorded assessments were significantly different (Mann Whitney p=0.003).

## 3.5 Discussion

### 3.5.1 Adverse events

In this small study the occurrence and timing of minor and major adverse events did not differ significantly between the prednisolone and the methylprednisolone treated groups. Twenty-one (50%) individuals experienced at least one minor adverse event and two (4.8%) a major adverse outcome.

In the TRIPOD trials 8.4% (14/167) of the prednisolone treated Nepali cohorts experienced a minor adverse event. This was not significantly different from the placebo treated group. The individuals in these groups were treated with either 1.96 or 2.52 g of prednisolone depending on which of the three trials they were enrolled into.

The commonest minor adverse event in this study of methylprednisolone was acne. Ten (23.8%) participants developed acne which was relatively uniform, a characteristic feature of corticosteroid-induced acne (Monk *et al.*, 1993).

The TRIPOD cohorts included people from Bangladesh as well as Nepal. Two per cent of the 401 corticosteroid treated participants had acne. Interestingly only 89 individuals in these trials received 2.52 g of prednisolone (the same amount as the prednisolone only arm in this study) and none developed acne. In contrast nine (of 312) of those who received the smaller total dose of 1.96 g and three (of 414) who received placebo did. These differences were not statistically significant overall. The involvement of dermatologists in the methylprednisolone study may have facilitated the diagnosis of acne.

The self-reported prevalence of acne was between 10-20% in 2040 respondents to a survey of individuals in the United States of America who were taking long-term ($\geq$ 60days) oral glucocorticoid therapy (Curtis *et al.*, 2006).

Conn and Poynard performed a large meta-analysis of adverse events during corticosteroid therapy (Conn and Poynard, 1994). The purpose of this study was to assess the putative link between peptic ulceration and systemic corticosteroid therapy.

The meta-analysis analysed 93 double blind randomised controlled trials in which more than 8700 patients had participated. These patients received a mean daily dosage of prednisolone 35 mg (or its equivalent) for a mean duration of 64 days. The mean total dose received was 2.2 g.

In the meta-analysis acne, moon face, buffalo hump and truncal obesity were grouped together as "cosmetic" and "dermatologic" adverse events. They are reported as being four times more common in the corticosteroid treated group.

Increased fat deposition causes the "moon face" associated with Cushing's disease and Cushing's syndrome. This adverse event affected 19% of individuals in the study. In the TRIPOD cohorts 3% of corticosteroid treated individuals developed this problem (Richardus *et al.*, 2003a).

A French prospective study examined 88 patients started on corticosteroid therapy ($\geq$ 20 mg) for longer than three months. The mean dose of prednisone was 42 mg/day in the first three months. Ten of these individuals received pulse therapy. Sixty-one per cent ($\pm$8%) were adjudged to have corticosteroid-induced lipodystrophy (CIL) of the face or dorsocervical region at three months (Fardet *et al.*, 2007b).

Shubin reported the adverse effects of triamcinolone 4-12mg in 47 patients with pulmonary disease treated for a period of 5-8 years. Sixty-six per cent developed moon facies (Shubin, 1965).

Fardet and colleagues also demonstrated that individuals with CIL were more likely to exhibit features suggestive of the metabolic syndrome such as higher fasting blood glucose, triglyceride levels and total cholesterol concentrations (Fardet *et al.*, 2007a).

The metabolic syndrome is a group of risk factors including insulin resistance, hyperinsulinaemia, hypertension, increased triglycerides, reduced high-density lipoprotein cholesterol, abdominal obesity and hypercoagulability. The metabolic syndrome confers an increased risk of Type 2 diabetes mellitus and cardiovascular disease. This would suggest that CIL is not simply a cosmetic problem.

Only one individual complained of weight gain despite the fact that five individuals had gained weight by the end of the study of 4 kg or more.

One individual (AN09) experienced two episodes of tinea cruris. The initial episode occurred at day 14 and was successfully treated with oral fluconazole and topical 1% clotrimazole cream. The second episode (diagnosed on day 113) responded to clotrimazole cream alone. This man's tinea cruris was symptomatic. It caused pruritus and so likely to be reported early. It is possible that either a pre-existing infection deteriorated or he acquired the infection after starting corticosteroid therapy. The full physical examination at enrolment makes the latter possibility more likely. The second episode is likely to have been a further infection as he had complete resolution of his symptoms following oral and

topical treatment of the first episode and because of the considerable time interval between the two events.

Fungal infections were uncommon in the TRIPOD studies (Richardus *et al.*, 2003a). Five individuals (1.2%) who were treated with prednisolone developed a fungal infection, which were defined as "severe fungal skin infections". All of these infections occurred in the prednisolone treated group of TRIPOD 1 who received 1.96 g of prednisolone over 16 weeks (Smith *et al.*, 2004).

Seven (16.7%) participants experienced gastric pain. There were no cases of peptic ulceration detected in this study. Gastric pain occurred in 18% of the prednisolone treated individuals in the TRIPOD studies.

Conn and Poynard found an increased number of peptic ulcers in the corticosteroid treated group but this did not reach statistical significance.

The symptoms of nocturia, polyuria and polydipsia were reported by four (9.5%) of individuals. The two individuals who had glycosuria did not complain of these symptoms. Their glycosuria was not persistent and therefore not considered to be clinically significant. The two individuals were both receiving additional prednisolone at the time but neither had received methylprednisolone. There were no individuals in the study diagnosed with diabetes mellitus.

TRIPOD 1 study reported one individual from the prednisolone treated group who developed glycosuria. This was considered a major adverse event in this study but the authors did comment whether this patient was diagnosed with diabetes mellitus (Smith *et al.*, 2004). Three individuals in the steroid treated groups of the three TRIPOD studies developed diabetes mellitus compared with one in the placebo group but this difference was not significant (Richardus *et al.*, 2003a).

The meta-analysis found a rate of diabetes mellitus four times greater in the steroid treated group which was statistically significant (Conn and Poynard, 1994).

One individual developed glaucoma. He had concomitant ENL which like corticosteroid therapy is a recognised cause of secondary glaucoma. This man developed glaucoma at day 305 of the study. He required additional prednisolone at day 111 because he developed painful, tender skin lesions typical of ENL. He required continuous oral prednisolone (receiving a total additional dose of 2.87 g of prednisolone between days 111 and 305) despite treatment of his ENL with high dose (300 mg daily) clofazimine. The majority of

individuals who develop ENL require long term treatment and many become corticosteroid dependent (Pocaterra *et al.*, 2006).

There were no cases of glaucoma in any of the TRIPOD participants. The meta-analysis does not contain any reference to glaucoma. The methylprednisolone study and TRIPOD studies would have been unable to detect any asymptomatic elevations in intra-ocular pressure. There are no good data concerning the incidence of glaucoma in individuals treated with systemic corticosteroids. Thirty-four per cent of individuals taking systemic corticosteroids in an Israeli study had intra-ocular pressure >20 mmHg compared with 6% of those not taking corticosteroids. However 71.7% of these individuals had received corticosteroid for longer than one year (Godel *et al.*, 1972).

Infected neuropathic ulcers affected one individual treated with methylprednisolone. This occurred 58 days after this man (AN36) completed the trial intervention.

Two individuals in the TRIPOD studies (one from the prednisolone treated group) developed infected ulcers. It is not reported whether the prednisolone treated person was taking the drug at the time the infection was diagnosed.

There were no episodes of hypertension in this cohort which was also the case in the TRIPOD studies. The meta-analysis of Conn and Poynard found that the frequency of hypertension was increased in patients treated with corticosteroids and that this difference was significant.

There were no episodes of mental illness reported by the participants or identified by the study physicians. This was also the case in the TRIPOD studies. The reported findings of Conn and Poynard are somewhat conflicting. The difference between the numbers of steroid treated individuals was significant using the sign test but not when odds ratio methods (fixed and random effects models) were employed.

Tuberculosis or other severe infections were not observed in the study. It is possible that the individuals who did not complete follow-up may have been unable to do so because of a severe illness such as TB. It is also possible that the duration of follow-up was not sufficient to identify any infections that might have occurred after 48 weeks. The TRIPOD studies included 300 individuals treated with corticosteroids who were followed for 24 months; none of these individuals were diagnosed with TB (Richardus *et al.*, 2003a).

TB was a rare occurrence in the meta-analysis with only five cases reported in 2056 individuals treated with steroids. However there were none in the placebo group. The odds ratio for this adverse event was not significant.

The size of the study limited our ability to detect rare adverse events however a much higher rate of acne and moon face was recorded than the TRIPOD studies. Another factor that might have reduced our estimation of adverse events is the duration of follow-up which may have been too short, however most studies have assumed that adverse events will occur during the treatment phase predominantly. We were also unable to examine the effect of our interventions on bone density which may be significantly affected by corticosteroid therapy in the doses and durations commonly used to manage leprosy T1R and NFI.

Very large randomised trials would be required to identify accurately the risk of rare adverse events such as peptic ulceration in individuals receiving corticosteroids. This is unlikely to be possible due to financial and logistical constraints. However it remains important to monitor individuals for adverse events. The establishment of registries of steroid treated patients at specialised centres such as Anandaban could facilitate the collection of reliable data without the need to resort to more costly randomised controlled trials.

### 3.5.2 Clinical outcomes

The use of a validated scale to measure leprosy Type 1 reactions and nerve function impairment allows the comparison of the two groups in this study. There are no significant differences in terms of the total severity score or the sensory or motor scores between the prednisolone and methylprednisolone treated groups at any of the pre-defined time points.

The difference in the total scores of all the participants taken together were significantly different at the pre-defined time points (ANOVA p=0.003) compared with baseline. This appears to be due to the change in the skin scores with time. The sensory and motor scores did not differ significantly at later time points from baseline. However there was a trend towards improvement in sensory and motor scores during the study. This is demonstrated by the change in nerve score between baseline and last recorded assessment in Table 3.10.

Participants in the prednisolone treated group were significantly more likely to have a decrease in sensory function than the methylprednisolone treated group by the end of the 16 week course of treatment (fig. 3.12). However this difference is not sustained to the end of the study. This phenomenon is similar to the outcome in the TRIPOD 1 study of prophylactic prednisolone to prevent the occurrence of reactions and nerve function impairment. It demonstrated a protective effect of prednisolone (total dose 1.96 g) compared with placebo during the 16 weeks of treatment which was lost by 48 weeks. The

higher dose may have a greater effect whilst an individual is receiving corticosteroids but not once they are no longer taking the drug.

This effect may have occurred by chance as it was not reproduced in the skin or in motor function. The number of participants contributing to all of the survival analyses towards the end of the study is small and the results therefore less reliable.

The physician assessment of neurological outcome although a less stringent outcome reveals the high rates of neurological impairment even after individuals have completed at least one prolonged course of steroids. Overall in this cohort only 70.6% (24/34) of those treated with at least 16 weeks of corticosteroid improved or recovered. This is consistent with data from Bangladesh where 67% of nerves improved after a 16 week course of prednisolone (Croft *et al.*, 2000b). The small study conducted in Nepal by Marlowe *et al* of prednisolone and a combination of azathioprine and prednisolone reported improvement in sensory function in 57.1% of individuals with sensory impairment present for less than six months (Marlowe *et al.*, 2004). The figure was identical for those with motor impairment before the start of treatment.

There was no significant improvement in NFI present for longer than six months at enrolment. Nineteen per cent of sensory nerves improved or recovered. The improvement was modest at best with a median decrease in monofilament score of 0.5 which equates to an individual sensing a finer monofilament at a single test site.

In the TRIPOD 3 study of treatment of longstanding nerve function impairment there was no significant difference between prednisolone (total dose of 2.52 g) treatment of longstanding nerve function impairment compared to placebo (Richardus *et al.*, 2003b). It is striking that 51% of individuals in the placebo arm (n=52) experienced improvement in longstanding nerve impairment compared with 54% in the prednisolone treated group (n=40). The authors do not report the change in the scores for the nerves and even if they did it would be difficult to compare the scoring system they used with that used as part of the validated Clinical Severity Scale. Five monofilaments were used in the TRIPOD studies compared to two in this study. The sensory thresholds that were considered acceptable were also different.

### 3.5.3 Additional prednisolone

Forty-five per cent of the methylprednisolone group and 50% of the prednisolone group were prescribed additional prednisolone appropriately. Of the 20 individuals that required additional prednisolone 12 did not do so until at least 28 days after completing the trial intervention. The clinical nature of the deterioration (skin or nerves or both) did not differ significantly between those who experienced it whilst receiving the study intervention and those who experienced deterioration after completing it ($\chi^2$=0.292).

The delay in deterioration in the majority of individuals requiring additional prednisolone is similar to that seen in the TRIPOD 1 study when prednisolone 20 mg daily for 12 weeks was used as a prophylaxis to try and prevent nerve damage in newly diagnosed leprosy patients (Smith *et al.*, 2004).

The requirement for extra prednisolone was used as the sole outcome measure in the multi-centre double blind randomised controlled trial of three different prednisolone regimens conducted in India (Rao *et al.*, 2006). The proportion of individuals requiring additional prednisolone in the three groups was 24%, 31% and 46% respectively. Individuals who received prednisolone for five months were significantly less likely to require additional steroid. However this does not necessarily reflect clinical improvement.

The inclusion criteria were individuals with evidence of severe T1R which was defined as nerve tenderness or any motor or sensory impairment of less than three months duration or severely inflamed skin lesions. These criteria are broadly similar to those of the methylprednisolone study.

A pragmatic design was used by Rao and colleagues. The decision to use additional prednisolone was left to the individual clinician's judgement at each of the six centres. It is not clear how consistency was ensured between individual physicians or at different stages of the trial.

The shorter duration of nerve function impairment compared with the other studies in Table 3.12 may in part account for the lower rates of additional prednisolone being prescribed to individuals.

| Study | Inclusion criteria | Arm | Number in arm | %age requiring extra prednisolone at 48 weeks |
|---|---|---|---|---|
| TRIPOD 2 | Mild sensory impairment of the ulnar or post tib nerves <6 months | Prednisolone 2.52 g 16 weeks | 41 | 27 |
| TRIPOD 2 (van Brakel *et al.*, 2003) | | Placebo 16 weeks | 34 | 18 |
| Marlowe | Severe Type 1 reaction | Prednisolone 1.68 g 12 weeks | 19 | 37 |
| Marlowe (Marlowe *et al.*, 2004) | | Prednisolone 1.33 g* 8 weeks | 21 | 48 |
| Rao | Severe Type 1 reaction or NFI | Prednisolone 3.5 g 20 weeks | 113 | 24 |
| Rao | | Prednisolone 2.31 g 20 weeks | 113 | 31 |
| Rao (Rao *et al.*, 2006) | | Prednisolone 2.94 g 12 weeks | 108 | 46 |
| Methylprednisolone | Severe Type 1 reaction or NFI | Prednisolone 2.52 g 16 weeks | 22 | 50 |
| Methylprednisolone | | MP (Prednisolone equivalent = 6.15 g) 16 weeks | 20 | 45 |

*this group received azathioprine as well for a period of 12 weeks

**Table 3.12 Studies of T1R and NFI (including current study) and the requirement for extra prednisolone**

The protocol of the MP study was stringent in treating NFI. Mild deterioration in NFI and NFI of short duration were both treated. This may in part account for the high proportion of individuals who received additional prednisolone. Any sustained (as little as one week) deterioration in monofilament testing at even a single test site. On the hand deterioration in sensation, at a single point, from being able to sense the 2 g monofilament to only being able to sense the 10 g would score 0.5 using the validated Clinical Severity Scale and be equivalent to a change of two points using van Brakel's sensory scoring system (TRIPOD 2) (van Brakel *et al.*, 2003). In the TRIPOD 2 study a two point increase in the score of a particular nerve was classified as "unchanged". However an individual unable to feel the 10g at a single site on the hand would score two using the Clinical Severity Scale but at least three using the TRIPOD system which the authors defined in those studies as deterioration. This illustrates the lower threshold used in the methylprednisolone for defining deterioration.

It is possible that some of the change labelled as deterioration was due to test response variability.

In the TRIPOD 2 cohort 27% of prednisolone treated individuals with mild sensory impairment experienced deterioration necessitating additional prednisolone. A group with mild isolated sensory impairment would be expected to require less additional prednisolone than a group that included severe nerve impairment both sensory and motor and marked skin involvement.

The results of this small study should be interpreted with caution but it would appear that given the available data methylprednisolone does not result in an increase in the number or severity of adverse events in individuals with leprosy in Nepal. However close detailed adverse event recording would still be required in any future studies of methylprednisolone in this setting.

The clinical outcome of patients in the two arms of this study was not significantly different in terms of the validated clinical severity scale or a physician assessment of neurological outcome. The methylprednisolone treated group had significantly less deterioration in sensory function during the 16 weeks of corticosteroid therapy but this was not maintained to the end of the 48 week follow-up period. This may be a reflection of the small numbers in the study, particularly towards the end of follow-up. A much larger study would be required to examine this potential effect further.

The study has also highlighted that corticosteroid treatment for T1R and NFI is sub-optimal even when given in large doses for 16 weeks. It adds further support to the argument that treatment should be given for longer durations. At present there is convincing evidence for at least 20 weeks but some would argue for 24 (Walker and Lockwood, 2008) and even longer (Naafs, 2003). The development of more prolonged treatment protocols would require further monitoring of adverse events and in particular the long term sequelae of corticosteroid therapy.

## 3.6 Summary

- There were no significant differences in the rate of minor or major adverse effects between the methylprednisolone and prednisolone treated groups.
- 20.6% of individuals with NFI recovered, 50% improved and 26.5% were unchanged. One individual was worse.
- All eight individuals who only had a cutaneous T1R recovered but 50% required additional prednisolone.

- The methylprednisolone group were significantly less likely to have a deterioration in sensory function during the 16 week treatment period but this was not sustained to the end of the follow-up period

- The proportion of individuals receiving additional prednisolone was high in both groups. In the MP group it was 45% and in the prednisolone group 50%. There was no significant difference between the two groups in the requirement for additional prednisolone.

- There was no significant improvement in longstanding NFI in either group.

# CHAPTER FOUR

## The expression of toll-like receptors in individuals with leprosy Type 1 reactions at diagnosis and during corticosteroid therapy

## 4.1 Introduction

The role of the innate immune system in the immunopathology of leprosy skin lesions has received much less attention than that of the adaptive immune system. The role of TLRs in the initiation and promotion of inflammatory and infectious disease in a wide variety of systems has been investigated but there is a paucity of *in vivo* data from experiments performed with skin.

The intracellular niche occupied by *M. leprae* and the immunological inflammatory complications associated with leprosy provide evidence for the role of pattern recognition receptors in both primary infection and its sequelae. There are few *in vivo* data concerning the expression of TLRs in patients with leprosy and leprosy reactional states. Krutzik *et al* used immunoperoxidase and immunofluorescence methods to stain skin biopsies from leprosy patients (Krutzik *et al.*, 2003). The skin biopsies from 10 patients reported to have T-lep and 10 L-lep disease. This is a nomenclature this group uses in their published reports. Patients are classified using the Ridley-Jopling classification and they report that these groups of patients, T-lep and L-lep, have tuberculoid and lepromatous leprosy respectively. Using murine monoclonal antibodies and the avidin-biotin method they reported that patients with T-lep had greater amounts of TLR1 and TLR2 staining than individuals with L-lep. They used confocal microscopy to show these two TLRs were expressed together on cells predominantly of the macrophage/monocyte lineage.

*In vitro* data also support the hypothesis that TLRs play a role in leprosy immunopathology. Transfected human embryonic kidney 293 cells that have been made to transiently express TLRs are activated by killed *M. leprae* via TLR1/2 heterodimer or TLR2 *in vitro*. Peritoneal macrophages from TLR2 knockout mice do not produce TNF following stimulation with killed *M. leprae*. The TNF production by these cells from TLR1 knockout mice is reduced compared to wild type mice (Krutzik *et al.*, 2003). The TLR1/2 heterodimer recognises bacterial triacylated lipopeptides including the mycobacterial 19-kDa lipoprotein. Mycobacterial lipomannan has been shown to activate the secretion of TNFα by murine macrophages events via TLR2 and TLR4 (Doz *et al.*, 2007). TLR9 recognises bacterial CpG motifs. TLR9 has been shown to contribute to the recognition of *Mycobacterium bovis* BCG by showing that TNFα, IL12p40 and IL6 was not produced by bone marrow derived dendritic cells from TLR2/4/9 deficient mice but was by TLR2/4 deficient mice (but at a lower level than cells from wild type mice) (von

Meyenn *et al.*, 2006). The TLR9 deficient murine dendritic cells did not up-regulate costimulatory molecules such as CD86 either.

Individuals with polymorphisms in *TLR1* have a reduced risk of acquiring leprosy (Johnson *et al.*, 2007; Schuring *et al.*, 2009). Two SNPs in the *TLR4* gene have also been shown to be associated with a lower risk of leprosy (Bochud *et al.*, 2009a). A polymorphism in *TLR1* has been shown to be associated with protection against T1Rs (Misch *et al.*, 2008). A SNP in *TLR2* is associated with protection against T1R and a microsatellite polymorphism with increased risk (Bochud *et al.*, 2008). This further implicates TLRs in the immunopathology of T1Rs.

Activation of TLR leads to cytokine production and the expression of co-stimulatory molecules which result in activation of adaptive immune system cells. This results in an increased cellular immunity which is the hallmark of T1Rs (Job, 1994).

TLR2 has been studied in leprosy skin lesions and Schwann cells and so was felt to be an interesting focus for its expression during T1R in this study. TLR2 forms a heterodimer with TLR1 and so this receptor was included for study. The expression of TLR4 is increased in TB, sarcoid and experimental neuropathy and so its role in T1Rs which may exhibit cutaneous and neural pathology is warranted. T1R often occur after the initiation of MDT. The killing of *M. leprae* may release bacterial DNA which is recognised by TLR9 and lead to the activation of inflammatory pathways.

## 4.2 Aims

- To compare the expression of TLRs according Ridley-Jopling classification in non reactional control patients and patients with T1R.
- To compare the cellular infiltration during T1Rs and nerve function impairment in the skin of leprosy patients with that of untreated non-reactional leprosy controls.
- To compare the expression of selected TLRs during T1Rs and NFI in the skin of leprosy patients with that of untreated non-reactional leprosy controls.
- To assess the effect of corticosteroid therapy on the cellular infiltration in skin lesions in individuals with leprosy T1Rs or NFI.
- To assess the effect of corticosteroid therapy on the expression of TLRs in skin lesions in individuals with leprosy T1Rs or NFI.
- To determine the cellular sub-types expressing TLRs in skin lesions of individuals with T1R.

- To validate a quantitative method of gene expression in corticosteroid treated individuals.
- To determine the gene expression of TLRs in skin lesions of individuals with T1R and NFI.

## 4.3 Participants, materials and methods

### 4.3.1 Participants

The study subjects were leprosy patients with evidence of T1R or NFI of less than six months duration enrolled in the randomised controlled trial of IV methylprednisolone versus prednisolone at Anandaban Hospital in Nepal (see Chapter 3).

Control subjects were untreated newly diagnosed leprosy patients who presented to Anandaban Hospital during the period of recruitment and follow-up of the methylprednisolone study.

### 4.3.2 Ethical Approval

The study was approved by the Nepal Health Research Council and the Ethics Committee of the London School of Hygiene and Tropical Medicine (Number 4022).

### 4.3.3. Consent

Informed consent was obtained by a native Nepali speaker after s/he had fully explained the trial and answered any questions. The trial consent forms and information leaflets were available in Devanagari script. The consent forms were signed by all participants (if they were unable to sign, a mark or thumb print was used instead and witnessed by the person obtaining the consent). Control subjects gave similar written informed consent before a study biopsy was performed.

### 4.3.4 Diagnosis and classification

Study and control subjects were diagnosed in accordance with the method described in Chapter 3. The Ridley-Jopling classification was used to classify both study subjects and controls. All participating individuals had a skin biopsy taken from a typical skin lesion. This was fixed in formalin and sent for histopathological examination for confirmation of diagnosis and Ridley-Jopling classification (Ridley and Jopling, 1966). This was performed at one of two Leprosy Mission Hospitals in India by either Dr Lakshmi Rajan (Delhi) or Dr Joyce Ponnaiya (Karigiri).

### 4.3.5 Study specimens

All participants in the randomised controlled trial provided a skin biopsy (6mm punch) at baseline, day 4 or day 29 and day 113. The three biopsies were taken from the same site. A skin biopsy was taken from control subjects at the time of diagnosis prior to starting MDT.

The specimens were bisected. One half was snap frozen in liquid nitrogen. The other half was immersed in RNA*later*$^{TM}$ (Ambion Inc., Austin, Texas, USA) and kept at 4°C overnight. The following day the RNA*later*$^{TM}$ was discarded and the skin sample stored at -80°C.

These samples were transported to the UK in a liquid nitrogen Dewar flask (CP100 Jencons (Scientific) Ltd. Lutterworth, UK) and stored at the London School of Hygiene and Tropical Medicine in liquid nitrogen.

### 4.3.6 Immunohistochemistry (IHC)

Skin biopsies were embedded in FSC 22$^{TM}$ (Surgipath, Richmond, Illinois). Cryosections (6μm) were cut on a Leica CM1100 Cryostat (Leica Microsystems, Germany) and adhered to a polysine coated glass slides (VWR International Ltd**,** Lutterworth, United Kingdom). The sectioning of skin biopsies was performed in a Category 3 laboratory.

The slides were stored at -20ºC.

The antibodies used in the study were directed against human CD1a, CD3, CD68 and human TLR1, TLR2, TLR4 and TLR9.

Table 4.01 shows the source, isotype and dilutions of the primary antibodies. Immunohistochemistry was performed using the peroxidise-anti-peroxidase (PAP) and the labelled streptavidin-biotin (LSAB) horseradish peroxidise methods. The staining protocols were performed in a humidified chamber at room temperature.

| Antibody Specificity | Source | Isotype | Immunoglobulin concentration (mg/ml) | Working dilution | Supplier (Cat. No.) | Method |
|---|---|---|---|---|---|---|
| CD1a | Mouse | $IgG_1$ | 1.383 | 1:100 | Dako (M3571) | PAP |
| CD3 | Mouse | $IgG_1$ | 0.273 | 1:100 | Dako (M7254) | PAP |
| CD68 | Mouse | $IgG_1$ | 0.53 | 1:200 | Dako (M0718) | PAP |
| TLR1 | Mouse | $IgG_1$ | 0.5 | 1:20 | eBioscience (14-9911) | LSAB |
| TLR2 | Mouse | $IgG_{2a}$ | 0.5 | 1:200 | eBioscience (14-9029) | LSAB |
| TLR4 | Rabbit | Polyclonal | 0.2 | 1:200 | Santa Cruz (SC-10741) | LSAB |
| TLR9 | Mouse | $IgG_{2a}$ | 0.1 | 1:500 | Hycult Biotechnology (HM2087) | LSAB |

**Table 4.01. Primary antibodies, source and isotype**

### 4.3.6.1 Peroxidase-anti-peroxidase method

Slides were defrosted for 60 minutes and then fixed in acetone for 15 minutes and air dried. Each section was circumscribed using a hydrophobic barrier pen (ImmEdge™ Pen, Vector Laboratories Inc., Burlingame, California).

The slides were washed in phosphate buffered saline (PBS) for five minutes. Non-specific background staining was minimised by incubating each section with PBS containing 10% rabbit serum (Dako, X0902, Glostrup, Denmark), the same species as the secondary antibody for 30 minutes.

The primary antibody was diluted in antibody diluent (Dako, S3022) and applied to the sections for 60 minutes. Negative controls were performed using the same antibody isotype directed against non-human antigens (Mouse $IgG_1$ Dako, X0931).

The slides were then washed in PBS before addition of the secondary antibody (polyclonal rabbit anti-mouse immunoglobulins Dako, Z0259) diluted to 1:100 in antibody diluent for 30 minutes. This was followed by a wash in PBS for 10 minutes.

The sections were then incubated for 30 minutes with the antibody detection reagent mouse monoclonal PAP (Dako, P0850) at 1:100 dilution in antibody diluents.

The slides were then washed in PBS and the enzymatic reaction was developed using the chromagen 3, 3' diaminobenzidine (DAB) (Sigma, St. Louis, Missouri) as the substrate. The reaction was assessed using a light microscope and stopped in water.

The sections were then counterstained with Harris haematoxylin (Sigma) for two minutes followed by washing in running water. The sections were decolourised by brief immersion in 1% acid alcohol and washed in running water. The sections were dehydrated in an alcohol gradient of: 70% ethanol, 100% ethanol, 100% ethanol, xylene and xylene for two minutes each.

The slides were then mounted with a cover slip using DPX (Fluka, St. Louis, Missouri).

### 4.3.6.2 Labelled streptavidin-biotin horseradish peroxidise method

The sections were thawed, fixed and marked as in the PAP method. After the first PBS wash the sections were then incubated in 0.3% hydrogen peroxide in methanol for 10 minutes. This was followed by a further PBS wash.

A non-specific background blocking step was performed using PBS containing 10% goat serum (Dako, X0907 from the same species as the biotinylated secondary antibodies) for 30 minutes.

The sections were then incubated with primary antibodies diluted in antibody diluent for 60 minutes. Negative controls were used (Mouse IgG$_1$ Dako, X0931 and Mouse IgG$_{2a}$ Dako, X0943) depending on the isotype of the primary antibody. The negative control for TLR4 was PBS.

The sections were washed with PBS and then incubated with the biotinylated goat anti-mouse/rabbit secondary antibody (Dako Real™ Detection System, Peroxidase/DAB+, Rabbit/Mouse, Dako, K5001) for 15 minutes. A further PBS wash was performed.

The sections were then incubated with the streptavidin conjugated to horseradish peroxidase (Dako, K5001) for 15 minutes.

The slides were then washed in PBS and the enzymatic reaction was developed using the chromagen DAB (Dako, K5001) as the substrate. The reaction was assessed using a light microscope and stopped in water. Counterstaining, dehydration and mounting of the slides was identical to that used in the PAP method.

### 4.3.6.3 Optimisation of staining

Each primary antibody was tested on sections at concentrations ranging from 0.1μg/ml to 100μg/ml using the PAP method for CD1a, CD3 and CD68, the PAP and LSAB method for TLR2 and the LSAB method for TLRs 1, 4 and 9.

### 4.3.6.4 Assessment of staining

Sections were assessed using a light microscope (Nikon, Kingston-upon-Thames, UK) with x50, x200 and x400 magnification. Photographs of representative sections were taken using a Leica DFC420 digital camera (Leica, Milton Keynes, UK).

The degree of cellular infiltration of the sections and the intensity of staining were assessed. Positive staining cells were determined by the presence of brown intra- or extracellular staining due to the chromagen DAB. Negative cells lacked staining.

### 4.3.6.5 Quantification of staining

The degree of cellular infiltration and intensity of staining of sections was evaluated by two independent assessors once the slides had been re-coded with a unique number. This ensured that neither assessor knew which slides were from study participants and which from controls. It also blinded the assessors to the timing of the biopsies in study participants.

The percentage of positive staining cells was graded 0-5 using a semi-quantitative scoring system. The degree of cellular infiltration was scored from 0-3. The criteria used for scoring are shown in Table 4.02.

| Intensity of staining | | Cellular Infiltration | |
|---|---|---|---|
| Grade | %age of positive cells | Grade | Cellular infiltration |
| 0 | None | 0 | No cellular infiltrate |
| 1 | <10% | | |
| 2 | 10-30% | 1 | Few small granulomas/group of cells |
| 3 | 30-50% | 2 | Medium-sized granulomas/ moderate cellular infiltrate |
| 4 | 50-80% | | |
| 5 | 80-100% | 3 | Large granulomas/extensive cellular infiltration |

**Table 4.02 Criteria for grading of cellular infiltration and intensity of staining.**

The grading of the immunohistochemical staining was with a semi-quantitive scale and so non-parametric methods were used for analysis. The level of staining of biopsies from controls was compared with those of the study participants at baseline using the Mann-Whitney $U$ test. The analysis of the level of staining in biopsies from study participants at different time points was by the Wilcoxon signed rank test. The threshold for accepting statistical significance was $p < 0.05$.

### 4.3.7 Double Fluorescent Immunostaining

The primary antibodies used for double immunofluorescent staining were directed against the same target antigens as in the IHC experiments. In addition to the polyclonal rabbit anti-TLR4 antibody a mouse monoclonal anti-TLR4 IgG$_{2a}$ antibody (Santa Cruz, SC-13593) was also used. A fluorescein isothiocyanate (FITC) conjugated mouse monoclonal anti-TLR9 IgG$_{2a}$ antibody (Hycult HM2087F) was also used.

The fluorochrome conjugated secondary (or tertiary) antibodies used are shown in Table 4.03.

| Antibody | Fluorochrome | Laser (wavelength, nm) Excitation/Emission (nm) | Working dilution | Supplier (Cat.No.) | Target |
|---|---|---|---|---|---|
| Goat anti-mouse IgG$_1$ | Fluorescein | Argon (488) 494/519 | 1:200 | Molecular Probes (A10530) | CD1a, CD3, CD68, TLR1 |
| Goat anti-mouse IgG$_{2a}$ | Alexa Fluor® 546 | Helium-Neon-1 (543) 556/573 | 1:200 | Molecular Probes (A21133) | TLR2, TLR4, TLR9 |
| Goat anti-rabbit | Alexa Fluor® 350 | Diode (405) 346/442 | 1:200 | Molecular Probes (A11046) | TLR4 (polyclonal) |
| Streptavidin (Tertiary) | Alexa Fluor® 350 | Diode (405) 346/442 | 1:100 | Molecular Probes (S11249) | Biotin conjugated goat anti-mouse/rabbit (1:100) |

**Table 4.03. Labelled secondary and tertiary reagents used in immunofluorescence studies**

### 4.3.7.1 Double immunostaining methods

Skin biopsies were embedded in FSC 22™ (Surgipath, Richmond, Illinois). Cryosections (6µm) were cut on a Leica CM1100 Cryostat (Leica Microsystems, Germany) and adhered to polysine coated glass slides (VWR International Ltd, Lutterworth, United Kingdom). The sectioning of skin biopsies was performed in a Category 3 laboratory.

The staining protocols were performed in a humidified chamber at room temperature. Following the addition of fluorochrome conjugated antibodies all further incubations and washes were performed in the dark.

In all staining methods the sections were thawed at room temperature for 60 minutes and then fixed in acetone for 15 minutes and air dried. Each section was circumscribed using a hydrophobic barrier pen (ImmEdge™ Pen, Vector Laboratories Inc., Burlingame, California). The slides were washed in PBS for five minutes.

Sections were fluorescently double stained using one of the following protocols:

1. Primary murine antibody isotypes $IgG_1/IgG_{2a}$ staining. Non-specific background staining was minimised by incubating each section with PBS containing 10% goat serum (Dako, X0907 from the same species as the secondary antibody) for 30 minutes. The sections were then incubated with both primary antibodies diluted together in antibody diluent for 60 minutes. Negative controls were performed using isotype specific antibodies. The sections were then washed in PBS for 10 minutes. The sections were then incubated with a mixture of the fluorochrome conjugated isotype specific goat anti-mouse secondary antibodies in antibody diluent for 30 minutes.

2. Polyclonal rabbit anti-human TLR4 antibody staining. Non-specific background staining was minimised by incubating each section with PBS containing 10% rabbit serum (Dako, X0902, the same species as the secondary antibody) for 30 minutes. The sections were then incubated with the primary antibody diluted in antibody diluent for 60 minutes. Negative controls were performed using PBS. The sections were then washed in PBS for 10 minutes. The sections were then incubated with Alexa Fluor® 350 conjugated goat anti-rabbit secondary antibody in diluent for 30 minutes.

3. Primary murine antibody isotypes $IgG_1/IgG_{2a}$ staining. Non-specific background staining was minimised by incubating each section with PBS containing 10% goat serum (Dako, X0907 from the same species as the secondary antibody) for 30 minutes. The sections were then incubated with a primary antibody diluted in antibody diluent for 60 minutes. Negative controls were performed using isotype specific antibodies. The sections were then washed in PBS for 10 minutes. The sections were then incubated with the appropriate biotinylated secondary antibody, either goat anti-mouse $IgG_1$ (Molecular Probes, A10519, 1:100) or goat anti-mouse $IgG_{2a}$ (Caltag Laboratories M32215, 1:100). The sections were then washed in PBS for 10 minutes and then incubated for 30 minutes with Alexa Fluor® 350 conjugated streptavidin diluted in antibody diluent.

4. FITC-conjugated anti-TLR9 antibody staining. The sections were incubated with this antibody for 60 minutes.

In all of the fluorescent staining methods the sections remained shielded from light and were washed in PBS for 10 minutes and then equilibrated in water for 5 minutes. The slides were then mounted with a cover slip using Vectashield® Hard Set™ (Vector Laboratories Inc., Burlingame, California). All of the slides were stored at 4ºC in the dark and examined within 24 hours.

**4.3.7.2 Optimisation of fluorescent staining**

The optimisation of fluorescent staining was achieved by staining with each primary antibody in the concentration used in the IHC methods. If this concentration did not result

in adequate and specific fluorescence the concentration of primary antibody was increased, in some cases undiluted.

This approach was also used to achieve the optimal concentrations of the fluorochrome conjugated secondary antibodies and the biotinylated isotype specific secondary antibodies and Alexa Fluor® 350 conjugated streptavidin.

### 4.3.7.3 Confocal laser microscopy

Immunofluorescent labelled sections were examined using a confocal microscope (LSM510, Zeiss, Welwyn Garden City, UK) fitted with diode, argon and helium-neon lasers. The excitation wavelengths used are shown in Table 4.03. A 505-550 band-pass emission filter was used for fluorescein and FITC conjugated antibodies. A 585nm long-pass emission filter was used for Alexa Fluor® 546 conjugated goat anti-mouse $IgG_{2a}$ antibody. A 420-480 band pass filter was used for Alexa Fluor® 350 conjugated antibodies.

The images were superimposed for colocalisation analysis. Staining with negative control antibodies was recorded using the same settings as the test antibodies to check for non-specific fluorescence.

### 4.3.8 Real-time Polymerase Chain Reaction (PCR) Assays

### 4.3.8.1 RNA extraction

RNA was isolated from skin stored in RNA*later*™ (Ambion, Austin, Texas) using the RNeasy Fibrous Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Tissue from half of a 6 mm punch biopsy (approximately 10 mg) was disrupted using a disposable pellet pestle (Anachem, Luton, UK) and homogenised with a Qiashredder (Qiagen, Crawley, UK). DNA was digested with DNase I (Qiagen, Crawley, UK). The isolation of RNA was performed in a Category 3 laboratory. RNA samples were stored at -80 ºC.

RNA concentration and quality was confirmed using a NanoDrop 1000, spectrophotometer (Thermo Scientific, Epsom, UK). The concentration of each sample was calculated in triplicate according to the Beer Lambert Law. The mean 260/280 ratio was also calculated.

### 4.3.8.2 cDNA synthesis

cDNA was synthesised from RNA using the Omniscript Reverse Transcriptase Kit (Qiagen). Reactions were performed using 1x RT buffer, 0.5 mM dNTP, 1 µM Oligo-dT

primer (Invitrogen, Paisley, UK), 0.5 units/µl RNase inhibitor (Invitrogen, Paisley, UK), 0.2 units/µl reverse transcriptase, 80 ng template RNA and nuclease free water to a total volume of 20 µl. Reactions were incubated in an ABI 9700 Programmable Thermal Cycler (Applied Biosystems, Foster City, California) for 60 minutes at 37 ℃ followed by 5 minutes at 95 ℃ and cooling to 4 ℃ for 5 minutes and then 15 ℃.

### 4.3.8.3 Choice of the control gene

human Acidic Ribosomal Phosphoprotein P0 (hARP-P0) has been used previously in PCR experiments examining TLR gene expression *in vitro* (Renn *et al.*, 2006).

### 4.3.8.4 Primers

Primers for hARP-P0 and TLR1, TLR2, TLR4 and TLR9 were obtained from previously published reports (Renn *et al.*, 2006). Predicted primer binding specificities and fragment sizes were tested by PCR *in silico* using the AmpliFx software (Nicholas Jullien, Institut Jean Roche, Université de la Mediterranée Marseilles, France) against sequences obtained from the European Molecular Biology Laboratory (EMBL) Nucleotide databases.

Oligonucleotide primers were synthesised and desalted by Sigma-Genosys (Gillingham, UK). Working stocks of 12.5 µM were kept at 4 °C. These primers could not amplify genomic DNA targets and functioned with cDNA templates only as they spanned exon boundaries.

Table 4.04 shows the primer sequences, primer melting temperatures, amplicon size and melting temperature.

| Primer (F, sense; R, antisense) | Sequence | $T_m$ (ºC) | Amplicon size (bp) | Amplicon Melt (ºC) |
|---|---|---|---|---|
| hARP-P0(F) | 5′CCACgCTgCTgAACATgCT | 67.7 | 67 | 81.0 |
| hARP-P0(R) | 5′TCgAACACCTgCTggATgAC | 66.4 | | |
| TLR1(F) | 5′TCTAgTgTgCTgCCAATTgCTC | 66.5 | 102 | 79.8 |
| TLR1(R) | 5′AAAgTCTTgAAggCCCTCAgg | 65.6 | | |
| TLR2(F) | 5′CAT TCC CTC Agg gCT CAC Ag | 67.1 | 51 | 76.8 |
| TLR2(R) | 5′TTgTTggACAggTCAAggCTT | 65.8 | | |
| TLR4(F) | 5′AggATgAggACTgggTAAggAAT | 64.8 | 76 | 78.0 |
| TLR4(R) | 5′TgAAggCAgAgCTgAAATggA | 67.1 | | |
| TLR9(F) | 5′CTCTgAAgACTTCAggCCCAACT | 66.8 | 76 | 82.2 |
| TLR9(R) | 5′CACggTCACCAggTTgTTCC | 67.8 | | |

**Table 4.04. Primer sequences, melting temperatures ($T_m$) and amplicon size**

## 4.3.8.5 Real-time quantitative PCR

Real-time quantitative PCR of all the genes was performed on the Rotor-Gene™ 3000 programmable thermal cycler (Corbett Life Science (Qiagen), Crawley, UK) using the QuantiTect® SYBR® Green PCR Kit (Qiagen, Crawley, UK).

Reactions consisted of 1x QuantiTect SYBR Green PCR Master Mix, 0.3 µM forward primer, 0.3 µM reverse primer, 1 µl cDNA and nuclease free water to a total volume of 25 µl. The master mix contains HotStarTaq DNA polymerase, PCR buffer, dNTP mix, SYBR Green I, ROX (passive reference dye) and 5 mM magnesium chloride.

The Rotor-Gene conditions were as follows: polymerase activation was achieved by incubating at 95 ℃ for 15 minutes; and 45 cycles of denaturation at 94 ℃ for 10 seconds, annealing at 60 ℃ for 15 seconds, extension at 72 ℃ for 20 seconds, and fluorescence acquisition for five seconds at 72 ℃.

Melting point data were obtained by increasing the temperature from 72 ℃ to 95 ℃ by 1 ℃ on each step. The interval between increases in temperature was 45 seconds for the first step and then five seconds for subsequent steps.

Negative and no template controls were included in all experiments.

The size of the PCR products was confirmed for each gene by electrophoresis of the amplicon products in 3% agarose gel with ethidium bromide and photographed under UV transillumination. This was only done during standardisation and not performed on each specimen.

Relative gene expression was analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The $C_T$ value is the threshold number for the amplification of the target gene. The threshold cycles for the target gene and control gene at each time point are measured. The difference in the threshold, $\Delta C_T$, between the two genes was calculated. The difference between the $\Delta C_T$ of the second time point and that of baseline was calculated (the $\Delta\Delta C_T$). The fold change in gene expression from baseline is given by the expression $2^{-\Delta\Delta CT}$.

## 4.3.8.6 Validation of hARP-P0 as the control gene

The expression of the control gene in relative gene expression experiments must not vary significantly under the conditions being studied. Corticosteroids are well recognised to affect gene expression of most genes (Nishimura *et al.*, 2006). The choice of control gene was influenced by the effect of corticosteroids on gene expression. The control gene hARP-P0 was amplified.

RNA extracted from skin biopsies taken from three of the study individuals at each of the three time points was diluted to a concentration of 20 ng/µl and reversed transcribed to cDNA. These were then amplified in triplicate with hARP-P0 and the cycle threshold determined.

## 4.3.8.7 Validation of the $2^{-\Delta\Delta CT}$ method for hARP-P0

The $2^{-\Delta\Delta CT}$ method is only valid if the efficiency of amplification of the target gene does not differ significantly from that of the control gene (in this case hARP-P0). The $\Delta C_T$ was calculated for different dilutions (ranging from 1 to 1:128) of cDNA in triplicate for each TLR gene and for hARP-P0, the control gene.

## 4.3.8.8 Analysis

The data were analysed using the Statistical Package for the Social Sciences (SPSS version 16. SPSS Inc., Chicago, Illinois) or *GraphPad Prism* (version 4.02 for Windows, *GraphPad* Software, San Diego, California).

The threshold for accepting statistical significance was <0.05. The level of statistical significance between a group and its baseline results are indicated in figures as follows: $p< 0.05$ (*), $p\leq0.01$ (**), and $p\leq0.001$ (***).

## 4.4 Results

### 4.4.1 Study and control subjects

Forty-two individuals were enrolled into the randomized controlled trial and 23 control subjects were recruited. The clinical data for the controls and participants are shown in Tables 4.05 and 4.06 respectively.

| Non-reactional controls | Gender | Age | Ridley Jopling Classification | BI |
|---|---|---|---|---|
| C3 | Female | 41 | LL | 3.5 |
| C4 | Male | 34 | LL | 3.75 |
| C5 | Male | 29 | LL | 4.25 |
| C9 | Male | 20 | BT | 1 |
| C10 | Male | 20 | BT | 0 |
| C11 | Male | 16 | BT | 0 |
| C12 | Female | 67 | TT | 0.25 |
| C15 | Male | 26 | BL | 1.75 |
| C16 | Male | 24 | BL | 2.75 |
| C17 | Female | 45 | BT | 0 |
| C18 | Male | 25 | BT | 0 |
| C19 | Male | 21 | BT | 0 |
| C20 | Female | 47 | I | 0 |
| C22 | Female | 51 | BT | 0 |
| C23 | Male | 21 | BT | 0 |

**Table 4.05. Control subjects.**

Eight controls were rejected. One individual (Control 1) had a histological diagnosis of cutaneous leishmaniasis. The label on the vial of Control 2 became illegible and so this specimen was used in some of the optimisation experiments. Control 6 had no evidence of leprosy on her skin biopsy. Controls 7, 8 and 13 all received oral prednisolone immediately after their skin biopsy was performed. The biopsy of Control 14 did not stain appropriately with any antibody used in the immunohistochemical analysis and was felt to have been compromised during transit or storage. The specimen of Control 21 was too small to section.

| Study Number | Gender | Age | Ridley Jopling Classification | BI | MDT Status | Reaction Type | Study Arm |
|---|---|---|---|---|---|---|---|
| AN01 | Male | 36 | BL | 1.5 | Completed | Nerves Only | Prednisolone |
| AN02 | Male | 28 | BT | 0 | None | Skin and Nerves | MP |
| AN03 | Male | 23 | BT | 0 | None | Skin and Nerves | MP |
| AN04 | Male | 49 | BL | 0 | Started | Skin and Nerves | Prednisolone |
| AN05 | Male | 64 | LL | 0 | Completed | Skin and Nerves | MP |
| AN06 | Male | 24 | BL | 0 | Started | Skin and Nerves | Prednisolone |
| AN07 | Male | 16 | TT | 0 | Started | Skin and Nerves | MP |
| AN08 | Male | 24 | BT | 0.75 | Started | Skin and Nerves | Prednisolone |
| AN09 | Male | 42 | BT | 0 | Completed | Nerves Only | MP |
| AN10 | Male | 65 | BL | 2.5 | Completed | Nerves Only | Prednisolone |
| AN11 | Female | 17 | BL | 0 | Started | Skin and Nerves | MP |
| AN12 | Male | 55 | BL | 0 | Completed | Nerves Only | Prednisolone |
| AN13 | Male | 63 | BL | 0 | Started | Skin and Nerves | MP |
| AN14 | Female | 18 | BT | 0 | Completed | Skin and Nerves | MP |
| AN15 | Female | 35 | BT | 0 | Completed | Nerves Only | Prednisolone |
| AN16 | Female | 39 | BT | 0 | Completed | Nerves Only | Prednisolone |
| AN17 | Female | 42 | BB | 1 | None | Skin and Nerves | MP |
| AN18 | Male | 41 | BL | 2.5 | None | Skin and Nerves | Prednisolone |
| AN19 | Male | 54 | BL | 1.25 | Started | Skin and Nerves | MP |
| AN20 | Female | 54 | BT | 3.25 | Started | Skin and Nerves | Prednisolone |
| AN21 | Male | 53 | LL | 0 | Started | Nerves only | Prednisolone |
| AN22 | Male | 16 | BT | 0.5 | Started | Nerves Only | MP |
| AN23 | Male | 29 | BT | 0 | Completed | Skin and Nerves | MP |
| AN24 | Male | 33 | BT | 0 | Started | Nerves Only | Prednisolone |
| AN25 | Male | 44 | BT | 0 | Started | Skin and Nerves | Prednisolone |
| AN26 | Male | 28 | BT | 0 | None | Skin and Nerves | MP |
| AN27 | Male | 40 | BB | 0 | Started | Skin Only | Prednisolone |
| AN28 | Male | 59 | BL | 1.75 | None | Skin Only | MP |
| AN29 | Male | 28 | BT | 0 | Completed | Skin Only | MP |
| AN30 | Male | 35 | BT | 0 | None | Nerves Only | Prednisolone |
| AN31 | Male | 18 | BB | 3.75 | None | Skin Only | MP |
| AN32 | Male | 36 | BT | 0 | Started | Nerves Only | Prednisolone |
| AN33 | Female | 35 | BT | 0 | Started | Skin Only | Prednisolone |
| AN34 | Male | 40 | BL | 2 | Started | Nerves Only | Prednisolone |
| AN35 | Male | 27 | BT | 0 | Started | Skin Only | MP |
| AN36 | Male | 62 | BT | 0 | Started | Skin and Nerves | MP |
| AN37 | Female | 17 | BT | 0 | Started | Skin and Nerves | MP |
| AN38 | Male | 55 | BT | 0 | Started | Nerves Only | MP |
| AN39 | Female | 40 | BT | 0 | Started | Skin Only | Prednisolone |
| AN40 | Male | 41 | BL | 2.5 | Started | Skin and Nerves | Prednisolone |
| AN41 | Male | 23 | BL | 3 | Started | Skin Only | Prednisolone |
| AN42 | Male | 22 | BT | 0 | Started | Nerves Only | Prednisolone |

**Table 4.06. Study subjects.**

## 4.4.2 Immunostaining by Ridley-Jopling classification

The controls and study patients were grouped by Ridley-Jopling classification. Controls diagnosed with TT and BT leprosy were grouped together and those with BL or LL classifications were grouped together. The same was done for study participants. For the purposes of this analysis the control patient with indeterminate leprosy was excluded as were the three study participants with BB leprosy. This analysis compared the grade of staining of biopsies from control patients and biopsies from the study participants taken at enrolment.

There were no significant differences between the TT/BT and BL/LL control groups or between a control group and its corresponding study group in terms of cellular infiltration, dermal CD1a or CD68 staining. This was also true for CD3 staining with the exception of the TT/BT control group which had significantly higher staining ($p = 0.043$) than the corresponding study group with median grades of CD3 staining of 4 and 2.5 respectively.

The grade of TLR staining for the control and study groups is shown in fig. 4.01.

TLR1 staining was significantly higher in BL/LL controls compared to TT/BT controls ($p = 0.002$). The median grade of staining for the BL/LL control group was the maximum 5 and for the TT/BT control group 2. The BL/LL control group TLR1 grade of staining was also significantly higher than that of both the BL/LL and TT/BT study groups at baseline ($p = 0.005$ and $p = 0.01$ respectively). There were no significant differences TLR1 staining between the other groups.

The grade of TLR2 staining was not significantly different between any of the groups except the BL/LL control group and the TT/BT study group ($p = 0.037$). The median TLR2 grade of the BL/LL controls was the maximum 5 and that of the BL/LL study group 3 but this was not significant ($p = 0.057$).

The grade of TLR4 staining was significantly higher in BL/LL control subjects than in their TT/BT counterparts. The median score for the former was the maximum 5 and for the TT/BT controls 1.5 ($p = 0.045$). There were no other significant differences between the groups.

The results were not affected by excluding study subjects with only nerve involvement. TLR9 was not analysed in this way (see 4.4.11).

**Figure 4.01. TLR1, TLR2 and TLR4 staining for controls and study participants by Ridley-Jopling classification.**

p< 0.05 (*), p≤0.01 (**) and p≤0.001 (***)
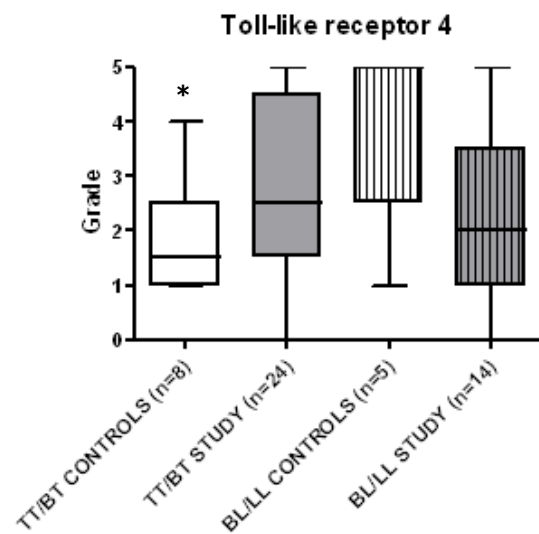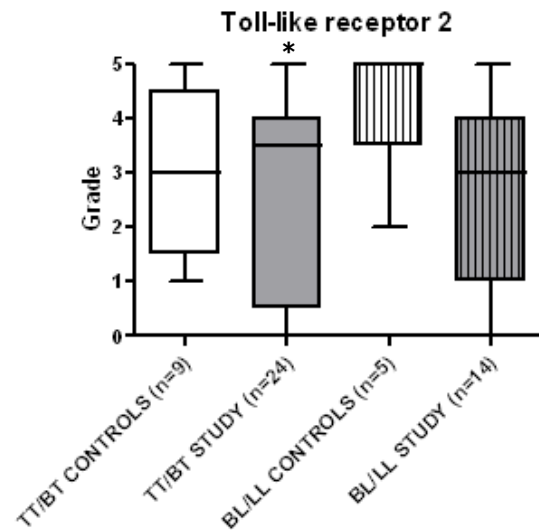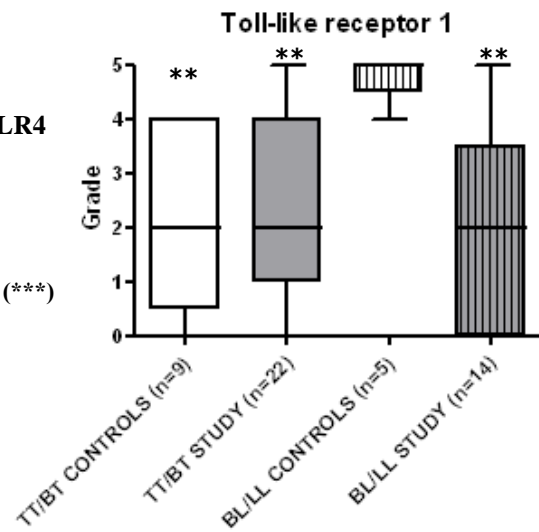
### 4.4.3 Cellular infiltration in the skin during corticosteroid treatment.

The degree of cellular infiltration was similar in both control subjects and study participants at baseline (p=0.053) and then was significantly reduced at days 4 and 133 (p=0.03 and p=0.001 respectively). There was no significant difference in the cellular infiltration in the skin of study participants at baseline and day 29.

The 14 individuals in the study who had nerve involvement only, had significantly lower cellular infiltration than controls (p=0.007) but not the 28 study participants with skin (with or without NFI) involvement (p=0.105).

### 4.4.4 CD3 staining in the skin during corticosteroid treatment.

The number of CD3 positive cells was significantly higher in control subjects compared to study participants (p=0.016). This is accounted for by the individuals with isolated nerve involvement who had significantly lower scores for CD3 staining than controls (p=0.003) but not those study participants with skin involvement (p=0.190). The individuals with clinical evidence of T1R in the skin did not have significantly different CD3 scores compared to controls (p=0.099).

The degree of CD3 staining was significantly reduced in study participants at all time points compared with baseline.

### 4.4.5 CD68 staining in the skin during corticosteroid treatment.

CD68 staining was similar in controls and study participants (p=0.356) and was significantly reduced in study participants by day 29 (p= 0.006) and this was maintained at day 113 (p < 0.001).

### 4.4.6 Dermal CD1a staining in the skin during corticosteroid treatment.

Overall the level of staining of dermal CD1a was less than that of CD3 or CD68. The maximum level of staining recorded was 3 regardless of an individual's status (control or study participant) or the timing of the biopsy.

There was no significant difference between the number of dermal CD1a positive staining cells in the controls and study participants (p=0.722). There were significant reductions in the dermal CD1a of study participants staining at days 4 (p=0.002) and days 29 (p=0.025) but not day 113 (p=0.171).
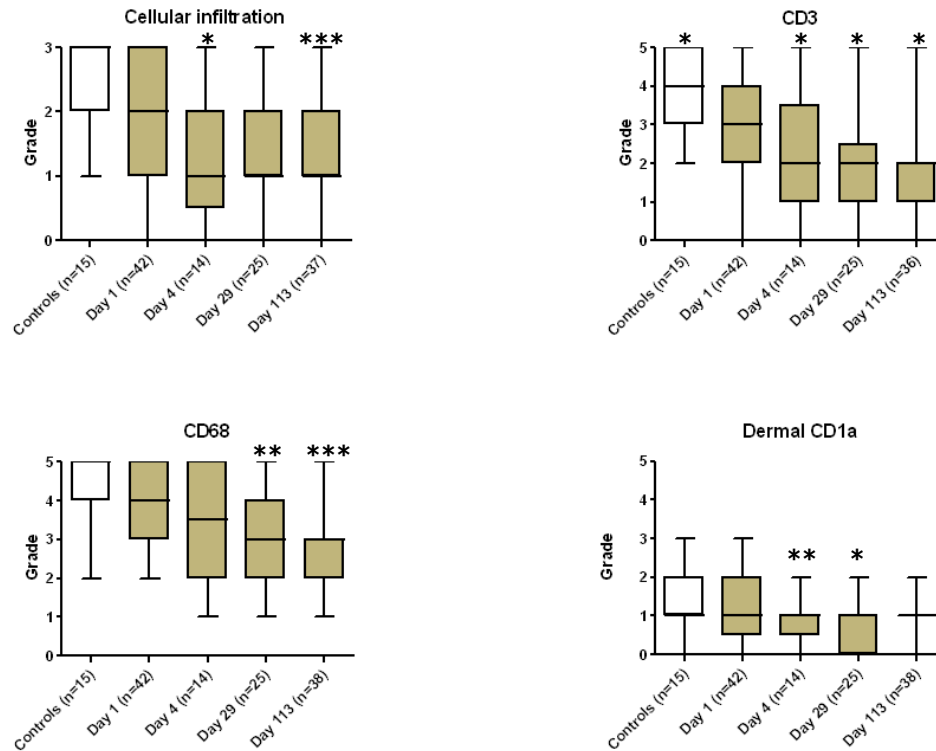
**Figure 4.02 Grade of cellular infiltration and staining for CD3, CD68 and CD1a**

## 4.4.7 The relationship between clinical skin inflammation and cellular infiltration and cellular expression of CD1a, CD3 and CD68 before treatment

There were no significant differences in the grade of cellular infiltration or the number of cells staining positive for CD1a, CD3 and CD68 between individuals with signs of T1R reaction cutaneous inflammation and those without prior to starting treatment.

**CD3**
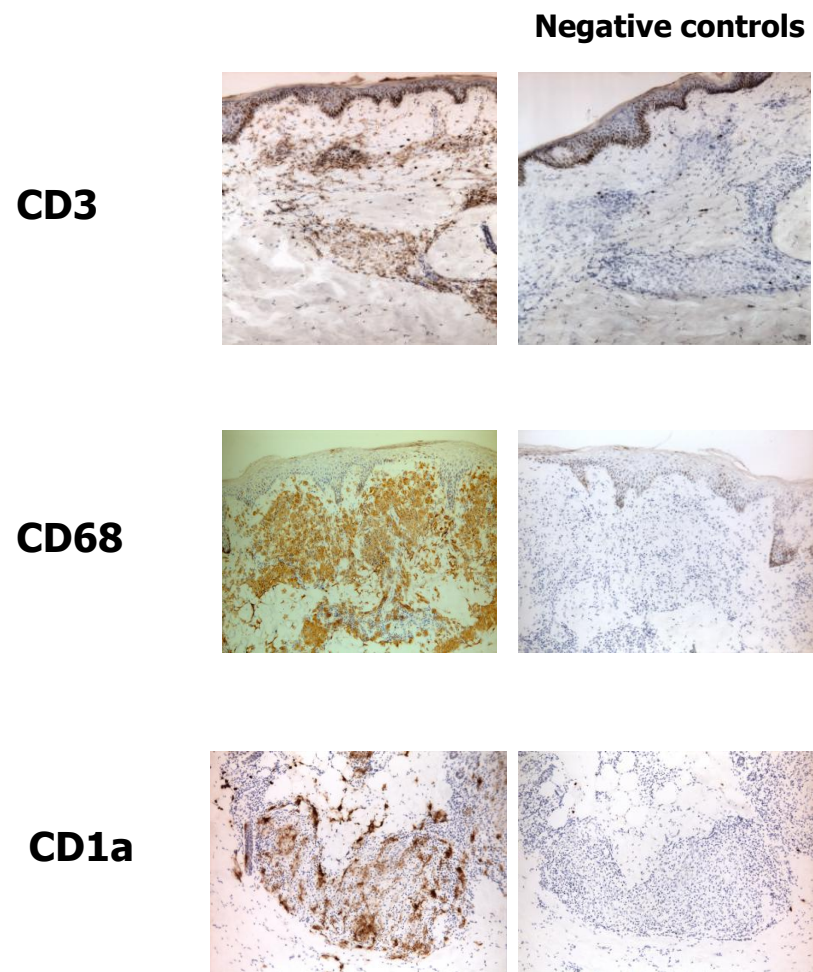
**CD68**

**CD1a**

**Figure 4.03. Representative staining of CD3, CD68 and CD1a in study subjects at baseline with their negative controls**

### 4.4.8 The cellular expression of toll-like receptor 1 in the skin during corticosteroid treatment

There was no statistically significant difference in the level of staining of TLR1 in controls compared to study participants at baseline (p=0.061). In the study participants the only significant decline, compared to baseline in staining for TLR1, occurred at day 113 (p=0.009).
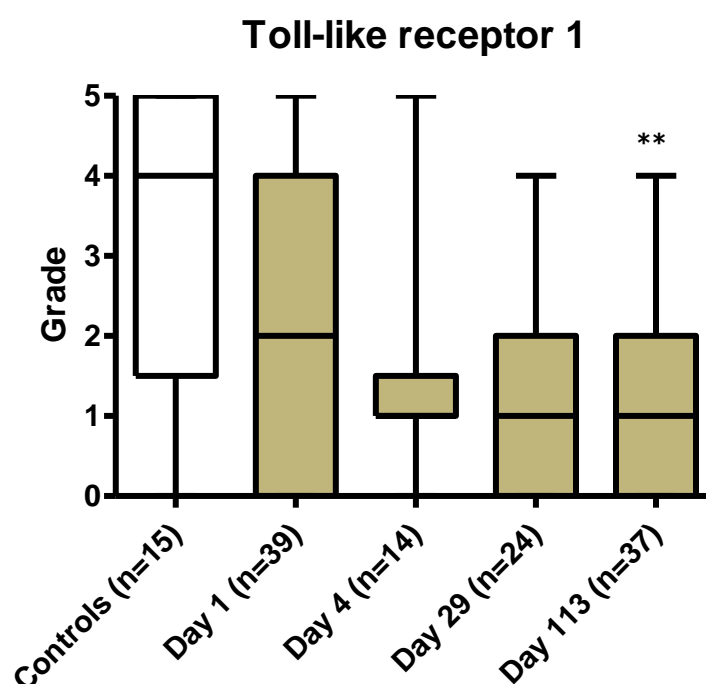


**Figure 4.04. Cellular expression of TLR1 by controls and MP study participants days 1, 4, 29 and 113**

### 4.4.9 The cellular expression of toll-like receptor 2 in the skin during corticosteroid treatment

There was no significant difference in the level of TLR2 staining between controls and study participants (p=0.349). The subgroup with only nerve involvement showed significant differences in the degree of staining for TLR2. This group not only had significantly less TLR2 staining than controls (p=0.043) but also significantly less than those individuals in the study with clinical features of skin inflammation due to T1R (p=0.015). There was no significant difference between the latter group and controls.

The level of TLR2 staining had declined significantly by day 29 ($p = 0.001$) and remained significantly reduced at day 113 ($p < 0.001$) compared to baseline. The reductions in TLR 2 staining during corticosteroid therapy at days 29 and 113 remained significant for the subgroup with cutaneous features of T1R.
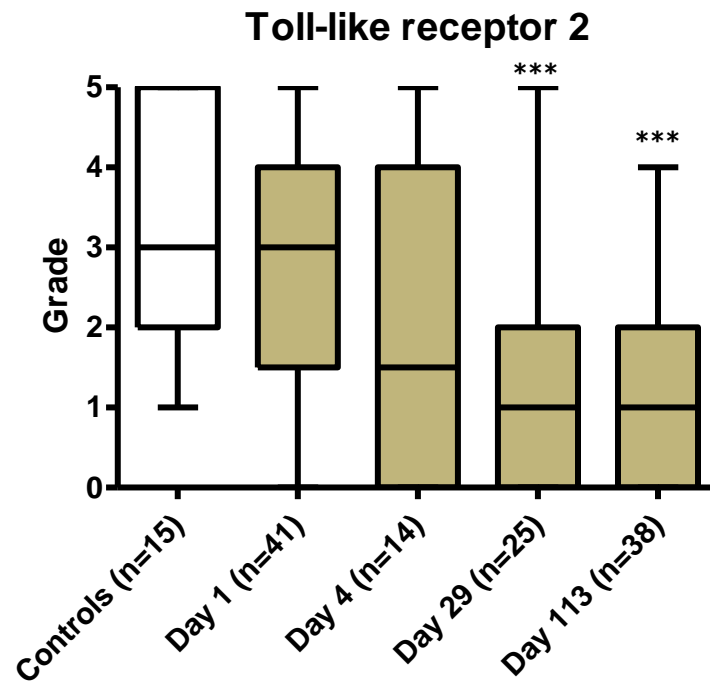


**Figure 4.05 Cellular expression of TLR2 by controls and MP study participants days 1, 4, 29 and 113**

**Toll-like receptor 1**

**Toll-like receptor 2**

**Toll-like receptor 4**

**Toll-like receptor 9**
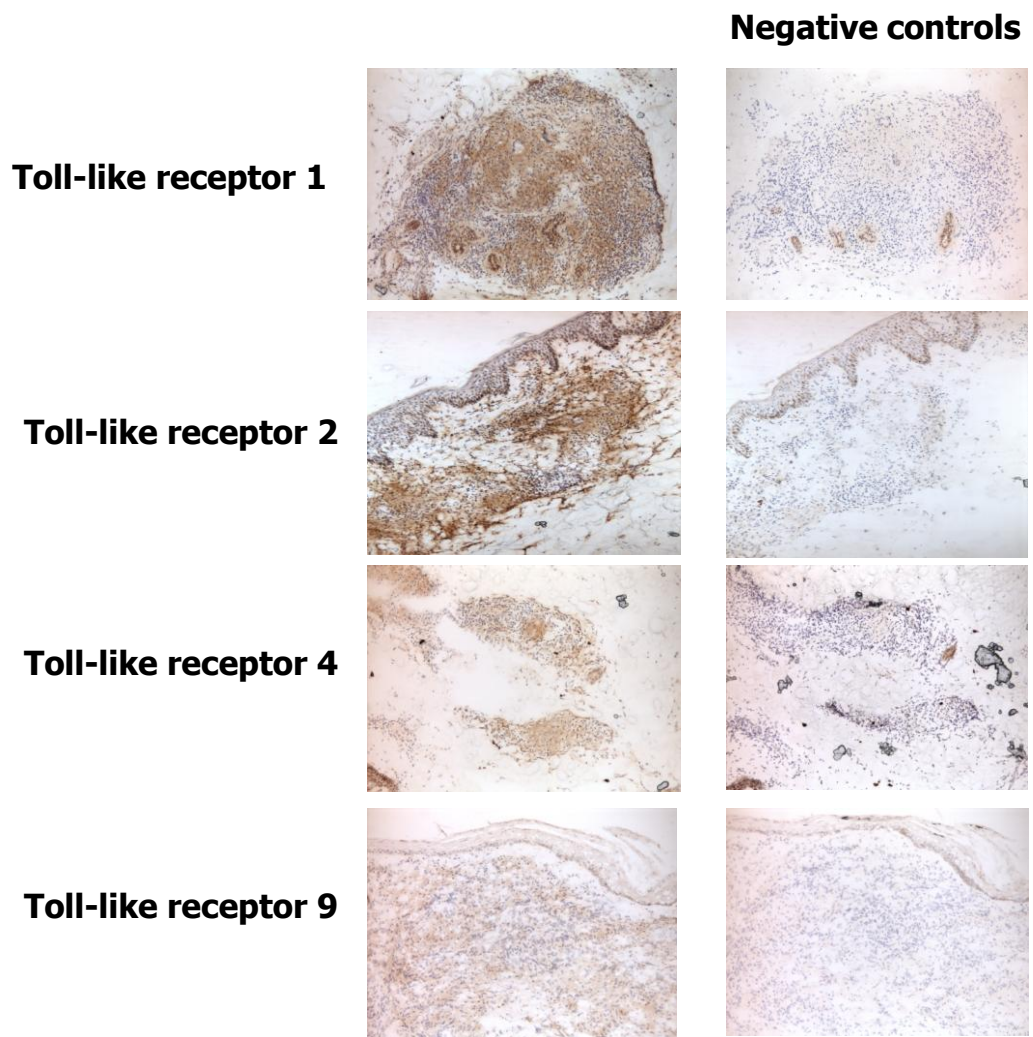


**Figure 4.06. Representative staining of TLR1, TLR2, TLR4 and TLR9 in study subjects at baseline with their negative controls.**

### 4.4.10 The cellular expression of toll-like receptor 4 in the skin during corticosteroid treatment

There was no significant difference in the level of staining for TLR4 between controls and study subjects (p = 0.797). TLR4 staining was significantly reduced at days 4 (p = 0.011) and days 133 (p = 0.01) compared to baseline.
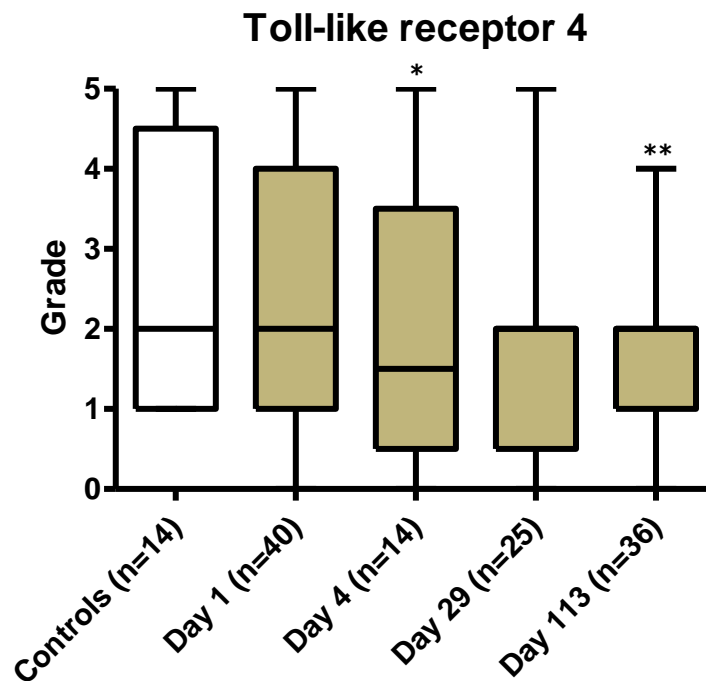


**Figure 4.07 Cellular expression of TLR4 by controls and MP study participants days 1, 4, 29 and 113**

Excluding individuals who received additional corticosteroids before day 113 did not significantly alter the findings of the analysis of the immunohistochemistry data for the cellular markers or the TLRs.

### 4.4.11 The cellular expression of toll-like receptor 9 in the skin during corticosteroid treatment

Skin sections TLR9 staining was less intense than other antibody stains. Sections were therefore assessed as either positive or negative. It was not possible to perform a positive control.

|  | Toll-like receptor 9 staining | |
|  | Positive | Negative |
| --- | --- | --- |
| Controls | 9 | 6 |
| Steroid treated individuals (Day 1) | 9 | 32 |

**Table 4.07. TLR9 staining**

The control subjects were more likely to have skin that stained positive for TLR9 (Chi-squared, Fisher's exact test p=0.011).

None of the nine corticosteroid treated individuals who had TLR9 positive staining cells in the baseline skin biopsy had evidence of it in subsequent biopsies. Two individuals AN07 and AN29 who had negative day 1 biopsies had TLR9 staining of skin sections at day 4 (AN07), day 28 (AN29) and day 113 (both AN07 and AN29).

### 4.4.12 The relationship between skin inflammation and cellular expression of toll-like receptor 1

The median grade for individuals with clinical signs of T1R in the skin was three. This was greater than the median scores of individuals who did not have clinical signs of skin reaction which was two but lower than controls whose median score was four. However these differences were not statistically significant.
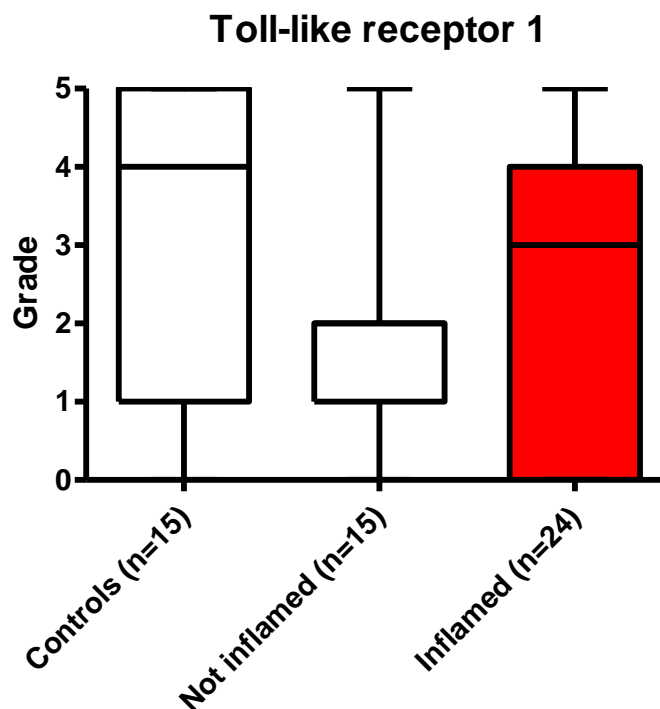


**Figure 4.08. TLR1 expression by controls and individuals with and without skin T1R at baseline**

### 4.4.13 The relationship between skin inflammation and cellular expression of toll-like receptor 2

Individuals with clinical evidence of cutaneous involvement due to T1R had significantly different scores from those individuals with no skin involvement (p = 0.015). The median score of the inflamed group was higher than that of the controls but this was not statistically significant.
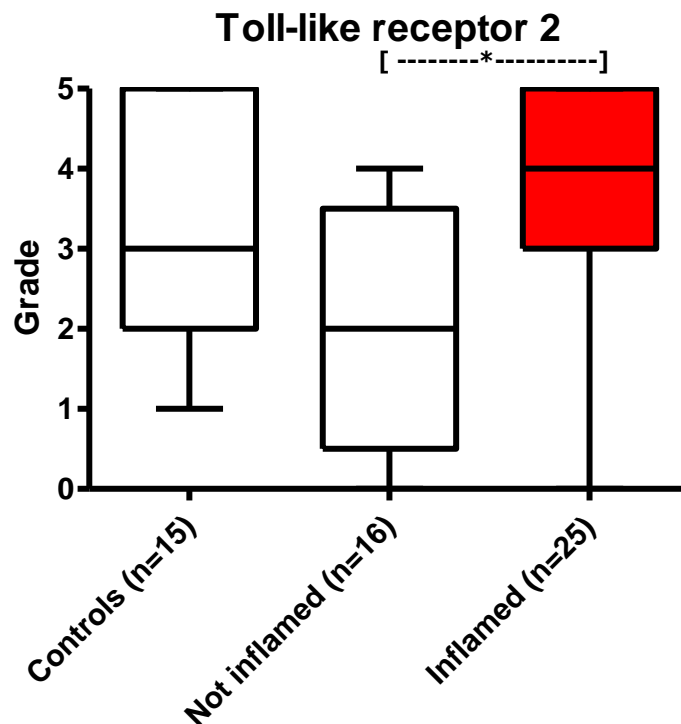


**Figure 4.09. TLR2 expression by controls and individuals with and without skin T1R at baseline**

### 4.4.14 The relationship between skin inflammation and cellular expression of toll-like receptor 4

There were no significant differences between any of the three groups with respect to TLR4 although the median score for the inflamed group was higher than that of the controls and individuals with no skin inflammation.
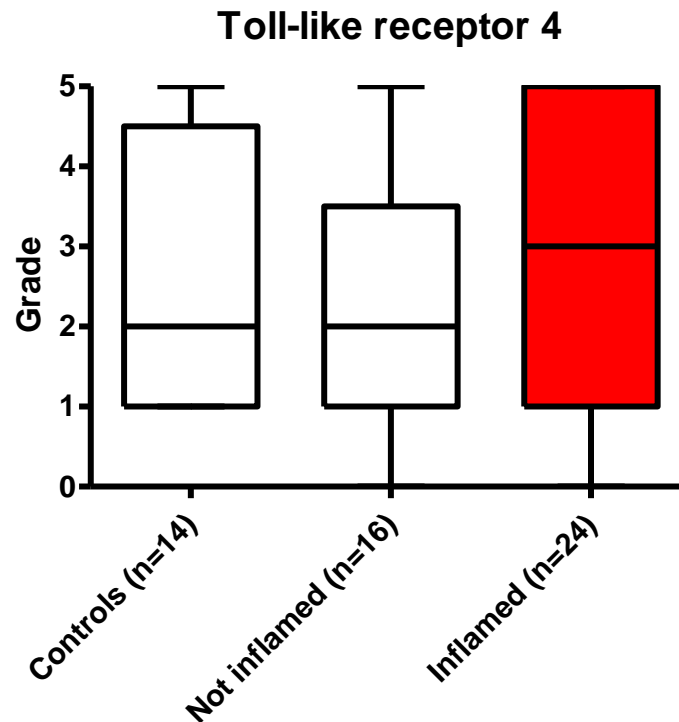
**Toll-like receptor 4**

## 4.4.15 Comparison of the effect of methylprednisolone and prednisolone on cellular infiltration in the skin

There were no significant difference between the cellular infiltration in biopsies from controls and those from either of the study arms. The degree of cellular infiltration did not differ significantly between the two study groups at any time point. However both the prednisolone and the methylprednisolone groups had significantly less cellular infiltration in biopsies taken at day 113 than was present at baseline ($p = 0.005$ and $p = 0.036$ respectively).

## 4.4.16 Comparison of the effect of methylprednisolone and prednisolone on CD3 staining in the skin

The level of staining for CD3 was significantly higher in the control group than the prednisolone treated group ($p = 0.008$). There was no significant difference between the control group and the methylprednisolone treated group. There were no significant differences in the level of CD3 staining between the two study groups at any of the time points.

The number of CD3 positive cells was significantly reduced at day 113 in the prednisolone treated group compared to baseline (p = 0.013). The methylprednisolone group had significant reductions in CD3 positive staining cells at day 4 (p = 0.041) and day 113 (p = 0.003).

### 4.4.17 Comparison of the effect of methylprednisolone and prednisolone on CD68 staining in the skin

There were no significant differences between the control group and either of the study groups, or between the study groups at any of the four time points. The number of CD68 positive cells was significantly reduced at day 29 (p = 0.031) and day 113 (p = 0.007) compared to baseline in the prednisolone treated group. The methylprednisolone group only had a significant reduction in CD68 positive staining cells on day 113 (p = 0.001).

### 4.4.18 Comparison of the effect of methylprednisolone and prednisolone on dermal CD1a staining in the skin

There were no significant differences between the control group and the study groups with respect to the dermal staining of CD1a positive cells. There were no significant differences between the study groups at any of the time points. The only significant reduction in CD1a positive staining compared to baseline was seen in the methylprednisolone group at day 4 (p = 0.005) but this significant reduction was not sustained.
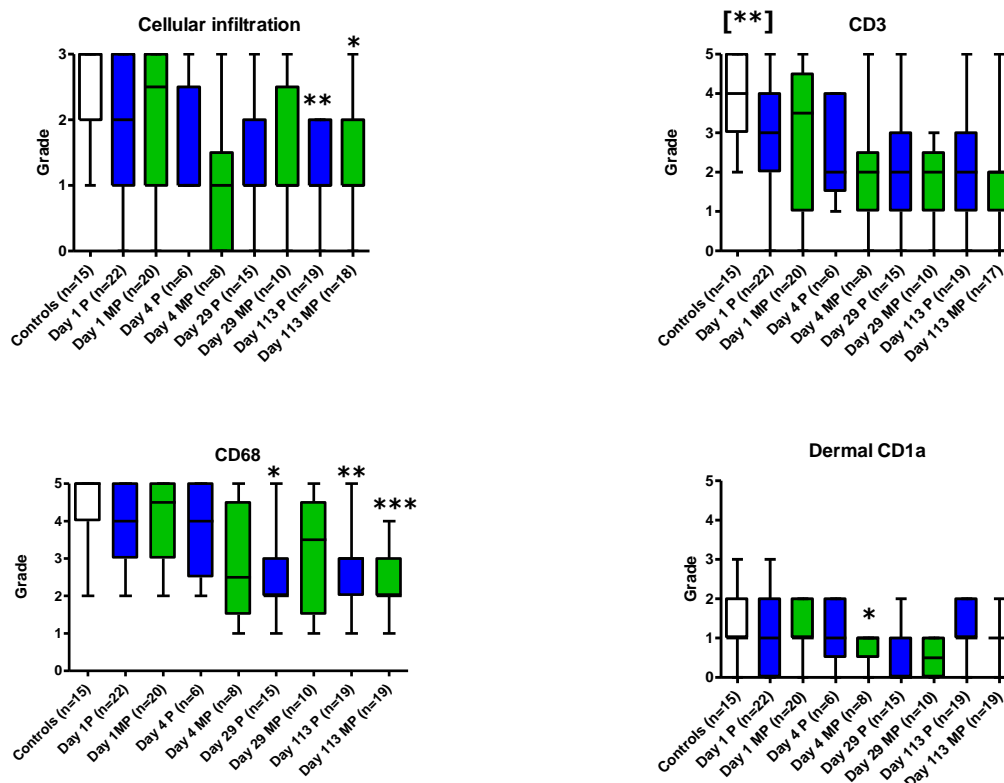
**Figure 4.11. Cellular infiltration and grade of staining of CD3, CD68 and CD1a for controls and individuals by study treatment arms. p< 0.05 (*), p≤0.01 (**) and p≤0.001 (***).**

## 4.4.19 Comparison of the effect of methylprednisolone and prednisolone on toll-like receptor 1 expression in the skin

There were no significant differences between the control group and either of the study groups with respect to cellular staining of TLR1. The level of staining of TLR1 was not significantly different between the study groups at any of the time points.

There were no significant changes in TLR1 staining in the prednisolone group at any of the time points. However the methylprednisolone group showed a significant reduction in TLR1 staining compared to baseline at day 4 (p = 0.041) and day 113 (p = 0.012).

# Toll-like Receptor 1
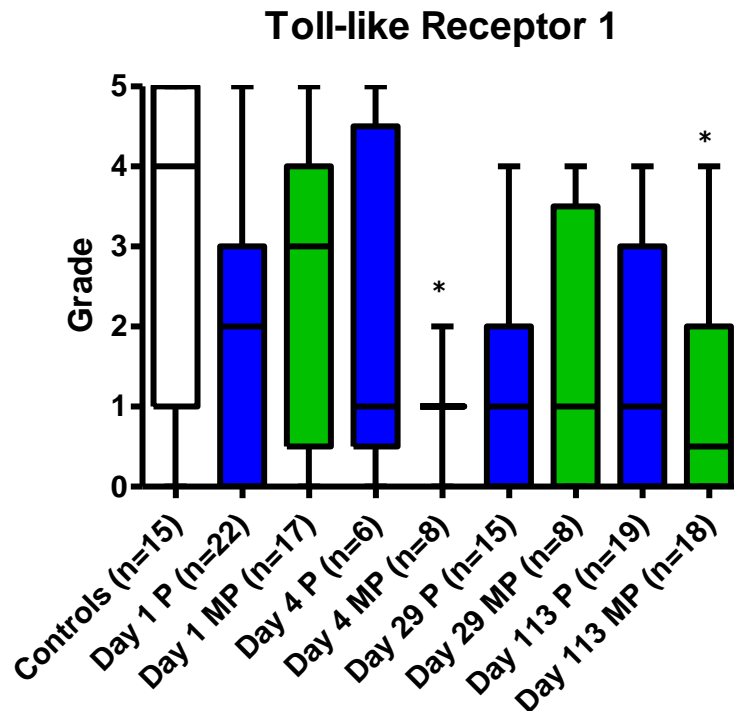


**Figure 4.12. TLR1 staining for controls and individuals in the study treatment arms**

## 4.4.20 Comparison of the effect of methylprednisolone and prednisolone on toll-like receptor 2 expression in the skin

The grading of TLR2 staining did not differ significant between the control group and the study groups. The study groups were not significantly different from each other.

The prednisolone group showed a significant reduction in TLR2 staining at day 113 (p = 0.004). At day 29 the level of staining was reduced but this was not statistically significant (p = 0.051). The methylprednisolone group showed a reduction in TLR2 staining at all time points. This was not statistically significant at day 4 (p = 0.066) but was at day 29 (p = 0.011) and day 113 (p = 0.001). These findings were not altered by analysing individuals with only nerve involvement and cutaneous involvement separately.

# Toll-like Receptor 2
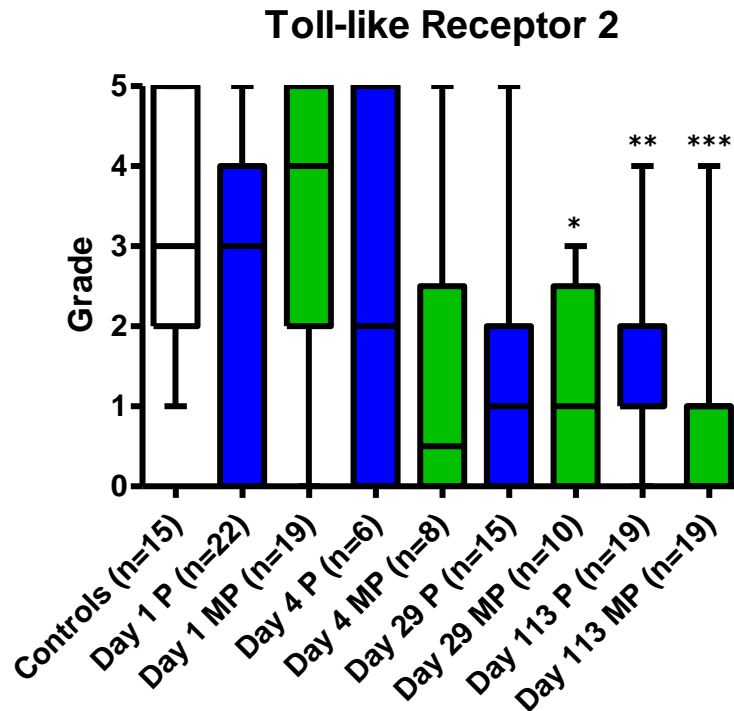


**Figure 4.13. TLR2 staining for controls and individuals in the study treatment arms**

## 4.4.21 Comparison of the effect of methylprednisolone and prednisolone on toll-like receptor 4 expression in the skin

There were no significant differences in the level of TLR4 staining between controls and either of the study groups. There were no significant differences between the two study groups at any of the time points.

The prednisolone group did not have a significant change in the level of TLR4 staining at any of the time points. The methylprednisolone group showed a reduction in TLR4 staining at all time points. This was significant at day 4 (p = 0.026) and day 113 (p = 0.008) but not at day 29 (p = 0.058).

**Toll-like Receptor 4**
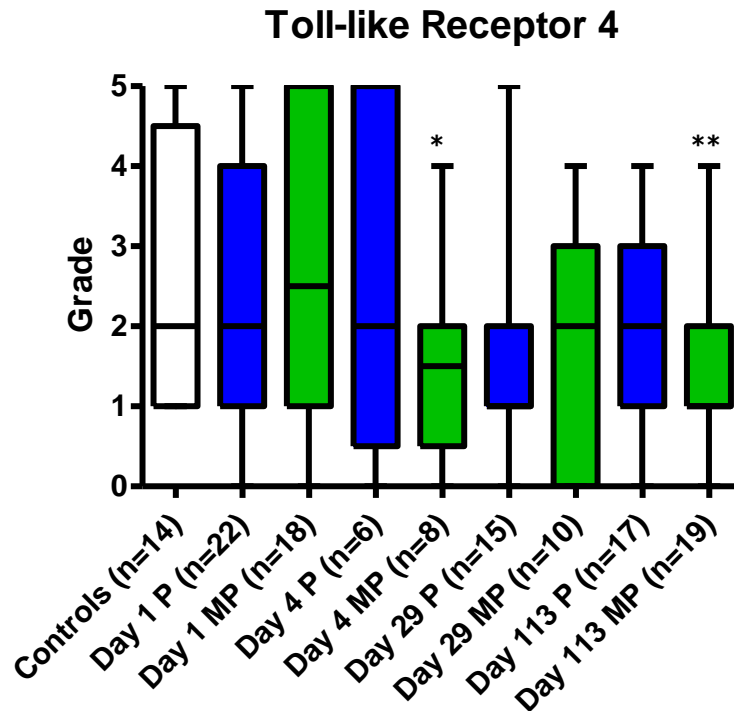
Figure 4.14. TLR4 staining for controls and individuals in the study treatment arms

## 4.4.22 Comparison of the effect of methylprednisolone and prednisolone on toll-like receptor 9 expression in the skin

Further sub-division and analysis of the small numbers of TLR9 positive biopsies was not performed.

## 4.4.23 Co-localisation of toll-like receptors and cell surface markers during Type 1 reaction and corticosteroid therapy

The labelling of CD1a, CD3, CD68 and TLR2 was successful. There were no differences in the pattern of staining between study subjects and BT or BL leprosy controls.

TLR2 was closely associated with both CD3 and CD68 positive cells and co-localised with these cells in some areas of the sections examined.

There is marked CD1a staining of the epidermis but there are also CD1a positive staining cells within the dermis which are closely associated with but not co-localised to cells expressing TLR2.
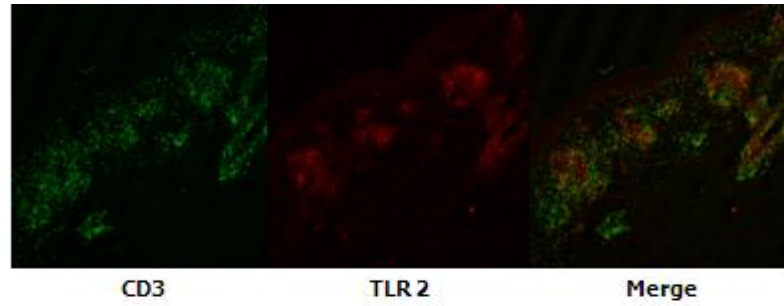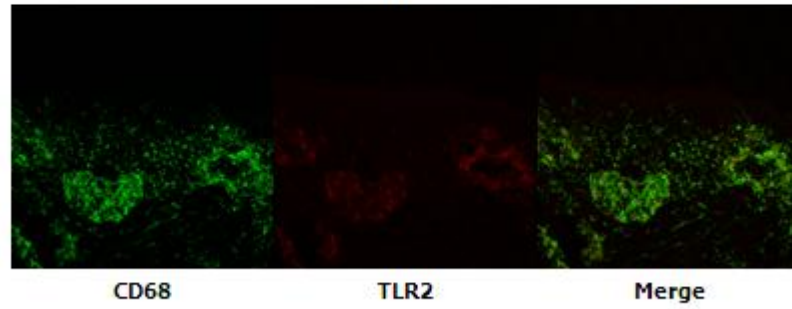
**Figure 4.15. Confocal microscopy images of fluorescent staining of TLR2 and a. CD3, b. CD68 and c. CD1a. The images are merged in the final panel**
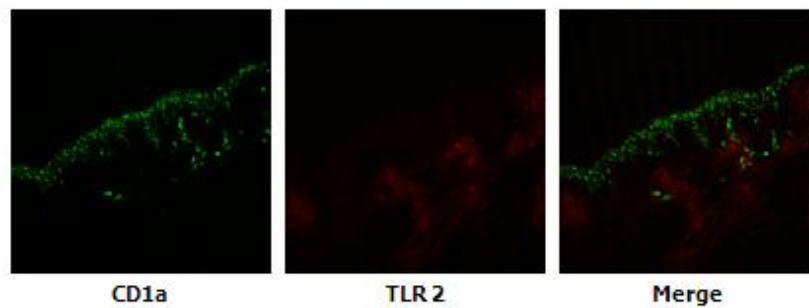
Despite using different techniques no positive staining was achieved for TLR1, TLR4 or TLR9. TLR1 primary antibody was used at various concentrations (including undiluted) with Alexa Fluor® 350 and fluorescein anti-IgG$_1$ secondary antibodies. Visualisation was also attempted using biotinylated secondary and an Alexa Fluor® 350 conjugated streptavidin tertiary antibody. Two different primary antibodies were used to try and label TLR4, the rabbit polyclonal antibody that was used in the immunohistochemical experiment and an IgG$_{2a}$ murine monoclonal (Santa Cruz SC-13593). These were used in various concentrations with appropriate secondary conjugated antibodies. The antibodies were also used with a biotinylated secondary and the Alexa Fluor® 350 conjugated streptavidin tertiary antibody. None of these were successful. TLR 9 was not visualised using immunofluorescent techniques. These included those outlined above for other IgG$_{2a}$ antibodies. The FITC conjugated anti-TLR9 antibody did fluoresce but background fluorescence was such that it was not possible to determine if the labelling was specific.

**4.4.24 Validation of control gene hARP-P0 for PCR assays**

There was no significant difference in the transcriptional activity of hARP-P0 before and during corticosteroid therapy (One way ANOVA p=0.058).

The primers for the reference gene hARP-P0 were used to amplify cDNA and compared with each pair of primers for the four TLR genes under investigation.

The $\Delta C_T$ ($C_{T, TLR\ gene}$- $C_{T, hARP-P0}$) was calculated for each of the eight dilutions of template cDNA (see Appendix 5.3).

The data fit using least squares linear regression. All four study TLR amplicons had similar efficiencies as hARP-P0. The slopes of $\Delta C_T$ plotted against log(concentration) are close to zero. $R^2$ is the proportion of the total variance in $\Delta C_T$ explained by the regression model i.e. relative concentration.

**Delta Ct(TLR1-hARP-P0) against log 10 (relative concentration)**

$y = 0.007\log_{10}(x) + 9.1078$
$R^2 = 0.0007$

Delta Ct (TLR1-36B4)

**Delta Ct (TLR2-hARP-P0) against log10 (relative concentration)**

$y = 0.07\log_{10}(x) + 10.89$
$R^2 = 0.04$

Delta Ct (TLR2-36b4)

**Delta Ct (TLR4-hARP-P0) against log10 (relative concentration)**

$y = 0.0901\log_{10}(x) + 16.217$
$R^2 = 0.2151$

Delta Ct (TLR4-36B4)

**Delta Ct (TLR9-hARP-P0) against log10 (relative concentration)**

$y = 0.0115\log_{10}(x) + 10.501$
$R^2 = 0.0008$

Delta Ct (TLR9-36B4)

**Figure 4.16. ΔCt for each TLR gene and hARP-P0 at dilutions ranging from 1 to 1:128**

**4.4.25 Toll-like receptor 1 gene expression during corticosteroid treatment**

## Toll-like receptor 1
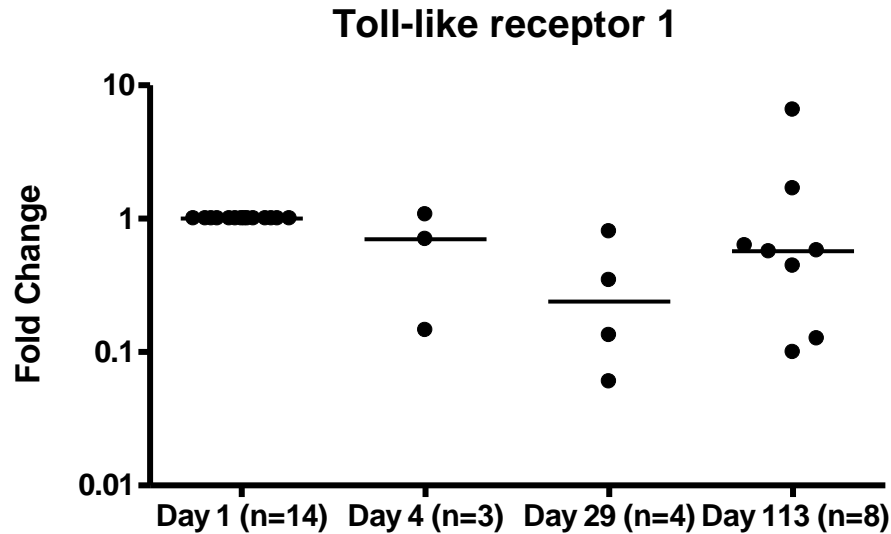


**Figure 4.17. TLR1 gene expression days 1, 4, 29 and 113**

The median fold change of TLR1 gene expression decreased at all time points after baseline but none were significant. At day 113 the methylprednisolone group had a significant reduction in gene expression ($p = 0.043$).

**4.4.26 Toll-like receptor 2 gene expression during corticosteroid treatment**

## Toll-like receptor 2
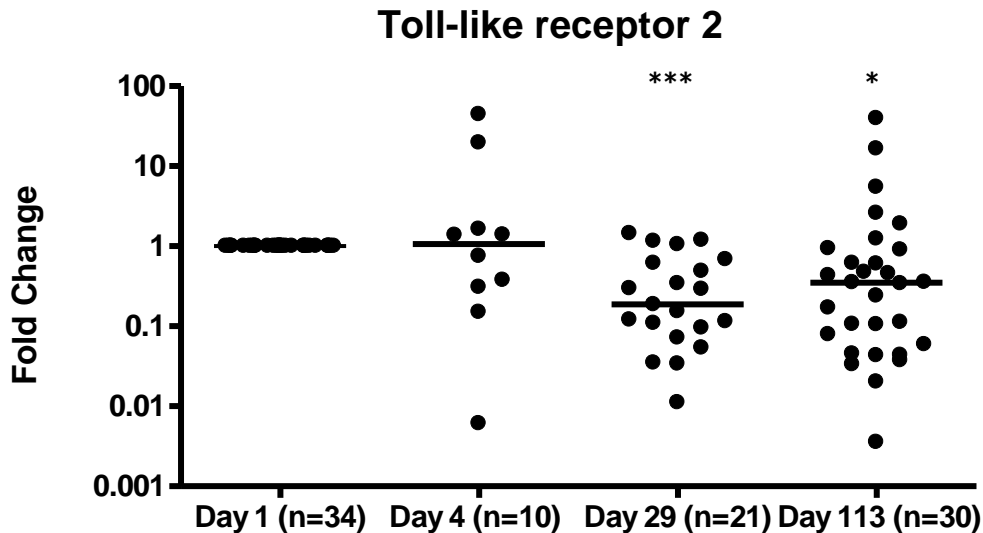


**Figure 4.18. TLR2 gene expression days 1, 4, 29 and 113**

There was a significant fall in TLR2 gene expression at day 29 (p < 0.001) and day 113 (p = 0.043) compared to day 1. There was no significant difference between the study groups.

The prednisolone (p = 0.004) and methylprednisolone (p = 0.037) groups showed significant decreases in gene expression at day 29 from their respective day 1 levels. The fold change difference was not significant at day 113 for either group.

### 4.4.27 Toll-like receptor 4 gene expression during corticosteroid treatment
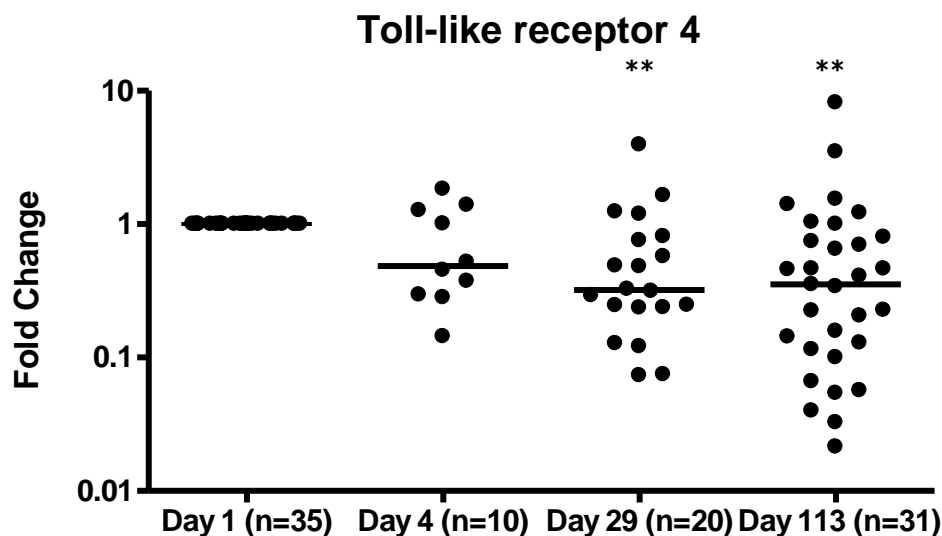


**Figure 4.19. TLR4 gene expression days 1, 4, 29 and 113**

There was a significant reduction in TLR4 gene expression at day 29 (p = 0.008) and day 113 (p = 0.002). There was no significant difference between the study groups. The prednisolone group showed a significant decrease in TLR 4 gene expression at day 29 (p = 0.013) compared to day 1. The methylprednisolone group did not (p = 0.173). At day 113 the methylprednisolone group showed a significant reduction in TLR 4 gene expression compared to baseline (p = 0.009) but the prednisolone did not (p = 0.075).

**4.4.28 Toll-like receptor 9 gene expression during corticosteroid treatment**

## Toll-like receptor 9
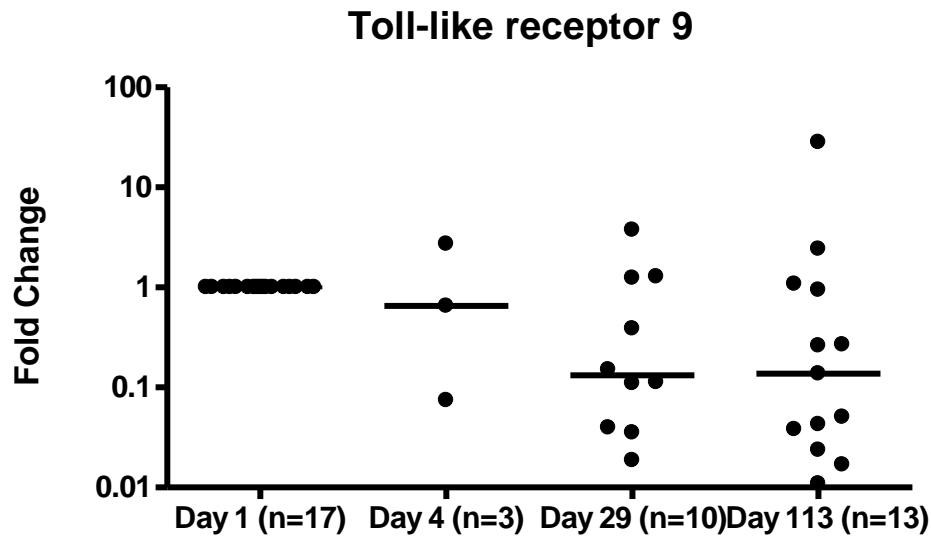


**Figure 4.20. TLR9 gene expression days 1, 4, 29 and 113**

There were no significant changes in TLR9 gene expression in the steroid treated group as a whole at any of the time points. However the methylprednisolone treated group showed a significant decrease in gene expression at day 113 (p = 0.018).

**4.4.29 The relationship between the clinical outcome and gene expression of toll-like receptors at day 113 compared to baseline**

There was no pattern observed in the level of gene expression of TLR1 and TLR9 for the various clinical outcomes. The only individual in the recovered category whose day 113 sample cDNA amplified satisfactory had an eight fold reduction in TLR1 gene expression compared to day 1.

TLR2 showed successive decreases in the median fold change of TLR2 gene expression as one moves from the individual who deteriorated to those who recovered. In the recovered group there was a median tenfold reduction in TLR2 gene expression compared to baseline. The individual who deteriorated had almost doubled the amount of TLR2 gene expression. There were no statistical differences between the groups but the pattern is striking.

The pattern of TLR4 gene expression at day 113 compared to day 1 was similar to that of TLR2. The improved and unchanged groups had almost identical median fold changes in gene expression. There were no statistical differences between the clinical categories.

178
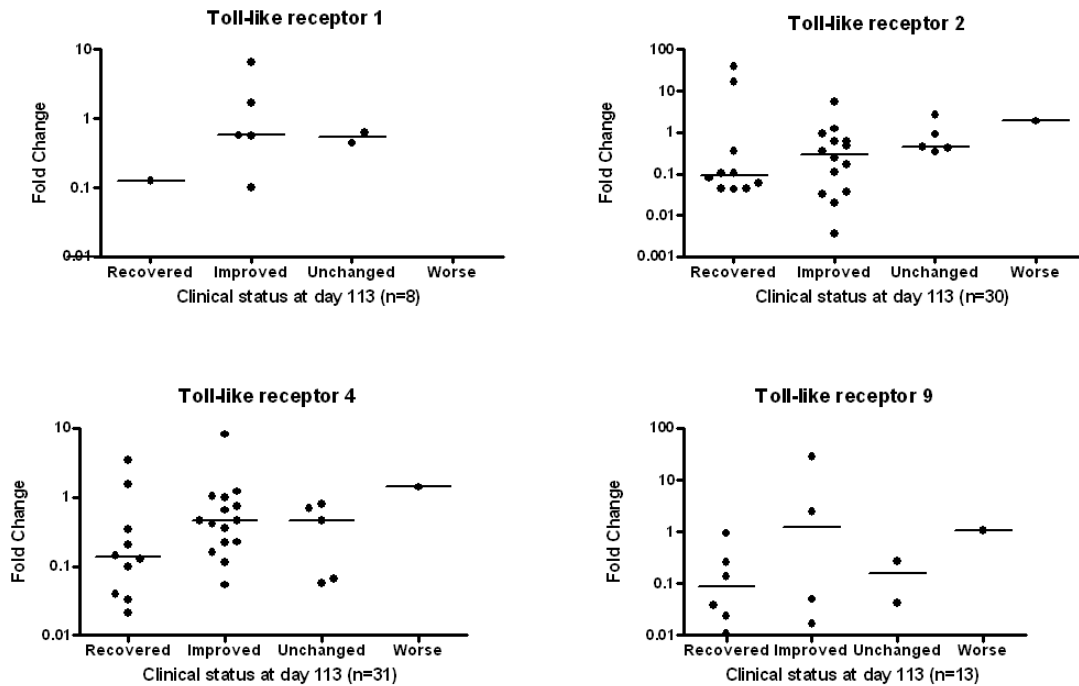
**Figure 4.21. TLR gene expression by clinical outcome at day 113 compared to baseline**

## 4.5 Discussion

In this study individuals with T1R do not have levels of cellular infiltration in skin lesions that are significantly different to that in skin lesions from untreated non-reactional leprosy control subjects. This is in contrast to the findings reported in the study by Andersson *et al* (Andersson *et al.*, 2005) in which 30 patients with T1R had significantly increased levels of cellular infiltration and CD68 positive staining cells than untreated leprosy control patients. In this study Andersson used the same method for grading cellular infiltration as used for the current experiments.

There are some differences between the current study and Andersson's Indian cohort. Andersson only included individuals with T1R affecting the skin. The control groups differed in that 50% of Andersson's 12 controls had BL leprosy or LL whereas only 33% did so in this study.

The findings are broadly in agreement with the studies of both Andersson and Little (Little *et al.*, 2001) in demonstrating a reduction in cellular infiltration and CD68 positive staining cells during corticosteroid therapy.

Andersson showed that both cellular infiltration and CD68 staining were significantly reduced at 28 days and 228 days after starting treatment with prednisolone 30 mg reduced by 5mg every month.

In this study cellular infiltration was significantly reduced at day 4 and day 113 compared to baseline. There was no significant difference in cellular infiltration between samples taken at baseline and those taken at day 29. The reduction seen at day 4 probably reflects the change in the MP treated group. The median cellular infiltration score of the corticosteroid group as a whole was two at baseline and that of the MP group was one at day 4, although the change of the median scores for the MP group went from 2.5 at baseline to one this was not statistically significant. The numbers in the day 4 group are small.

CD68 staining in this study was reduced compared to baseline at days 29 and 113 as in Andersson's study.

Little and colleagues showed that cellular infiltration in skin lesions declined during treatment with prednisolone. Fifteen patients with T1R affecting the skin had biopsies performed at baseline and day 7, day 28 and day 180 after starting treatment. In this study, which used the same methodology as Andersson, there was a significant reduction in cellular infiltration at day 28 but not days 7 and 180.

There is no consistent effect seen during the early stages of corticosteroid therapy and this may reflect the relatively smaller numbers compared to baseline and the final time point.

T lymphocyte cells have been demonstrated in skin lesions in all forms of leprosy and form a cuff around dermal granulomas (Van Voorhis *et al.*, 1982). CD3 positive cells have been demonstrated to be closely associated with macrophages and CD1a positive cells containing *M. leprae* antigen in both reactional and non-reactional skin lesions (Rambukkana *et al.*, 1992). The number of mature T-lymphocytes in skin lesions as indicated by those staining with anti-CD3 antibody were significantly reduced compared to controls and probably reflects the effect of individuals with nerve only involvement. The difference between controls and those with skin involvement was not significant. Narayanan *et al* reported an increase in CD2 T-lymphocytes in reactional skin lesions (using immunofluorescent technique with FITC labelled OKT11 monoclonal antibodies) from both BT (n=6) and BL leprosy (n=5) patients compared to matched controls (Narayanan *et al.*, 1984).

There were no statistical differences in the percentage of CD4 positive cells in skin biopsies of patients with T1R or TT or BT leprosy or LL in a study from Thailand (Mahaisavariya *et*

*al.*, 1999). In the same study there was no significant difference in the CD4/CD8 ratio in skin biopsies from BT patients and leprosy patients with T1R.

Dermal dendritic cells (CD1a positive staining cells) have been previously shown to be present in areas of granulomatous inflammation in leprosy. Sieling *et al* reported strong expression of CD1 proteins by dendritic cells in the skin of individuals with tuberculoid but not lepromatous leprosy (Sieling *et al.*, 1999). This finding has been repeated recently by Brazilian workers (Simoes Quaresma *et al.*, 2009). In a small series (n=7) from China the only comment concerning dermal OKT6 (CD1a) positive cells was that there was "no obvious change" seen in the dermis (Liu *et al.*, 1984).

In the two individuals reported by Sieling who were experiencing a T1R CD1 proteins were also strongly expressed. The authors suggest that the appearance of CD1 protein expressing cells is temporally related to the onset of a T1R.

In the current study there was no significant difference in the expression of CD1a between the controls and study participants. This must be interpreted with caution because no internal controls were available (i.e. a patient who was biopsied before and at the time of reaction) and also the preponderance of BT control patients.

It was interesting that the study participants showed a significant decrease in the expression of dermal CD1a at day 4 and day 29 but not day 113. In studies of the effect of topical corticosteroids on CD1a epidermal (Langerhans) cells *ex vivo* there was a significant reduction in the number of CD1a positive cells (Ashworth *et al.*, 1988). The fact that oral prednisolone, albeit at a dose of 5mg per day, does not continue to suppress expression of CD1a by dermal cells after four months of corticosteroid treatment may be an insight into the possible role of CD1a positive cells in individuals who deteriorate.

TLR1 and TLR2 exist as heterodimers and one would expect their expression at different time points to be similar. In the current study both showed a reduction in median staining scores during corticosteroid therapy but this was only significant for both of them at day 113. The reduction in the expression of both receptors is not mirrored in the two treatment groups. This is likely to be a reflection of the small numbers involved.

The significant reduction in TLR2 gene expression at days 29 and 113 is accompanied by a significant reduction in the level of TLR2 protein expression in the skin lesions.

The expression of TLR1 and TLR2 in the skin of patients with T-lep or L-lep leprosy has been demonstrated previously (Krutzik *et al.*, 2003). The expression of both receptors was greater in tuberculoid patients compared to patients with lepromatous leprosy. This is not the

case with the control patients in this study. The BL/LL control group were small in number (n = 5) and so the current results need to be interpreted with caution. This group had significantly greater staining of TLR1 than the TT/BT control group and both study groups. The staining of TLR2 was significantly higher than that of the TT/BT study group. This is the opposite of the findings of Krutzik and colleagues for the non-reactional control patients. The patients in the study by Krutzik are likely to have been a genetically more heterogeneous group than the Nepali patients in the current study which might possibly explain the difference in the results. It is also possible that in the BL/LL control patients subclinical T1Rs were occurring which were not identified using standard histopathological techniques. This seems unlikely. A further more plausible explanation is that this group were very active immunologically and this accounts for the high levels of TLR expression. It is possible that the greater quantity of *M. leprae* antigen present in the skin of patients with BL leprosy and LL results in increased expression which by the time clinically evident T1R occurs TLR expression has down regulated. However the significantly different level of staining of TLR2 in individuals with T1R affecting the skin compared to those with isolated NFI is somewhat at variance with this argument but the groups were too small to further subdivide by Ridley-Jopling classification.

TLR2 is highly expressed in the epidermis in normal skin and in non-lesional skin from individuals with psoriasis and from psoriatic lesions. Epidermal pathology results in different patterns of TLR2 staining. In cells obtained from patients with sarcoidosis using bronchoalveolar lavage there is reduced IFN-$\gamma$ production following incubation with an anti-TLR2 antibody (Oswald-Richter *et al.*, 2009). *Mycobacterium ulcerans* significantly increases the cell surface expression of TLR2 and TLR4 by primary human keratinocytes (Lee *et al.*, 2009a). *Propionibacterium acnes* activates TLR2 which may lead to inflammation in acne (Kim *et al.*, 2002), interestingly Shibata and colleagues have shown that there is a four-fold increase in TLR2 gene expression in cultured keratinocytes incubated with *P. acnes* and dexamethasone compared to *P. acnes* alone (Shibata *et al.*, 2009).

The corticosteroids dexamethasone, methylprednisolone and prednisone all resulted in an increase in gene expression of TLR2, TLR3 and TLR4 by dendritic cells. Despite this increased expression the dendritic cells produced significantly less TNF$\alpha$ and IL12 (Rozkova *et al.*, 2006). Prednisolone has also been shown to inhibit the function of plasmacytoid dendritic cells in liver transplant recipients.

In Ethiopian patients with leprosy, polymorphisms in the TLR2 gene are associated with different frequencies of T1R. The polymorphisms were examined in stored samples taken

from the AMFES cohort (n=441) (Bochud *et al.*, 2008; Saunderson *et al.*, 2000b). The study reported that the single nucleotide polymorphism resulting in the substitution of thymidine for cytosine at position 597 was associated with a lower risk of T1R (OR, 0.34 95% CI, 0.17-0.68). A 280 bp allelic length microsatellite was associated with an increased risk of T1R in this cohort (OR, 5.83 95% CI, 1.98-17.15). Sixty six patients had a T1R and only 150 were reported as having no reaction and so 225 individuals were excluded from the analysis. The findings therefore should be interpreted with caution.

A further finding supporting the role of TLR2 in T1R pathology is the lower gene expression in individuals with better clinical outcomes. In a study of 45 individuals with bacterial sepsis recurrence of disease was strongly associated with the level of monocyte expression of TLR2 at the time of discontinuation of antimicrobial therapy (Orihara *et al.*, 2007).

The three individuals (AN18, AN23 and AN29) who developed new skin inflammation at day 113 did not show a consistent pattern in the TLR2 gene expression or protein staining. AN23 had increased TLR2 gene expression compared to baseline whereas in AN29 it was decreased. AN29 had no TLR2 positive staining cells at any time point. Larger numbers of subjects with late skin deterioration would be required to investigate whether clinical deterioration in the skin is associated with increased TLR2 gene expression and TLR2 staining cells. The confounding effect of paradoxical increase in TLR2 gene expression secondary to corticosteroid therapy would also need to be taken into account.

Individuals with a SNP in TLR1 resulting in the substitution of thymidine with guanine at position 1805 may have a lower risk of T1R (Misch *et al.*, 2008). Individuals who are homozygous for the guanine allele at this position do not express TLR1 (Johnson *et al.*, 2007). The study by Misch was conducted in Nepal in patients recruited from Anandaban Hospital. However the primary aim of the project was to investigate associations with the different clinical types of leprosy and not associations with T1R. A study of patients in Bangladesh did not show a significant association between the presence of the S248 allele in the TLR1 gene and having a T1R (Schuring *et al.*, 2009).

It is possible that TLR polymorphisms may have affected the effectiveness of the primers used to amplify these genes in the current experiments. Sequence polymorphisms in herpes simplex virus significantly affected the performance of real time PCR used to identify the presence of the virus in clinical samples (Stevenson *et al.*, 2005). This problem has also been identified in diverse multicellular organisms such as the Pacific oyster (Taris *et al.*, 2008).

TLR4 staining was reduced during corticosteroid treatment and this was reflected in TLR4 gene expression. However unlike TLR2 the significant reduction in TLR4 gene expression at

day 29 is not accompanied by a significant decrease in TLR4 positive staining cells. At day 113 the reduction of both gene expression and protein staining are significant. At day 4 the reduced median fold change in TLR4 gene expression is not significant but it is associated with a significant reduction in positive staining cells. TLR4 gene expression also shows a similar pattern to that of TLR2 when individuals are categorised by clinical status at the end of corticosteroid treatment. The differences between the groups are not significant but there is a trend of increased gene expression in individuals who are unchanged or worse compared to those who have recovered.

The TLR4 SNPs (TLR4 896G→A and TLR4 1196C→T) have been reported to protect against developing leprosy in the control subjects of the study of Ethiopian patients with leprosy (Bochud *et al.*, 2009a). There have been no reported associations of TLR4 polymorphisms with T1Rs.

TLR9 is an intracellular molecule and this may make it more difficult to visualise using immunohistochemical techniques. There are few reports of TLR9 being successfully stained in human skin using immunohistochemistry. One study of Mycobacterium ulcerans disease used a mouse monoclonal antibody. Another study used a goat polyclonal in skin affected by viral warts or molluscum contagiosum. A third study used a rabbit polyclonal anti-human TLR9 antibody in skin biopsies from individuals with lichen planus. Jarrousse *et al* demonstrated TLR9 staining using a polyclonal donkey antibody in biopsies from patients with cutaneous T-cell lymphoma. The staining in all four studies was visualised using a biotin-streptavidin technique with either 3-amino-9-ethylcarbazole or DAB as the chromagen. In the current study positive controls were not used, however reliable TLR9 staining was demonstrated in 18 out of 56 samples using a murine monoclonal primary antibody. The lack of a positive control means that the statistically significant difference between controls and study participants should be interpreted with caution.

 All but one of the nine study participants had skin involvement. Six had BT leprosy but two had BL leprosy and one LL. The control patients had a similar distribution of Ridley-Jopling classifications. It is not possible to draw any firm conclusions from the immunohistochemical experiments of TLR9.

The TLR9 gene expression did not significantly decrease during corticosteroid therapy but the smaller numbers compared to TLR2 and TLR4 may be responsible. The lack of robust TLR9 immunostaining data makes interpreting the gene expression data problematic.

CD1a positive dermal dendritic cells did not express TLR2 nor did their epidermal counterparts. This is in keeping with the findings of Angel and colleagues who showed that

normal skin did not have dermal CD1a positive cells expressing TLR2 however some CD14 positive dermal APCs did (Angel *et al.*, 2007).

The same workers also demonstrated that CD14 positive dermal APCs express CD68 in normal skin. CD68 is largely a macrophage marker but phenotypic heterogeneity has been reported in atopic dermatitis in which cells were CD68/CD1a positive (Kiekens *et al.*, 2001).

Ochoa and colleagues have used immunohistology to demonstrate that there are two distinct populations of cells in the dermis of normal human skin which have the morphology of dermal dendritic cells. One group of cells express CD1 proteins. The other group expressed CD209 (DC-SIGN) which is a macrophage marker. This latter group of cells also expressed CD68 (Ochoa *et al.*, 2008).

The activation of human monocytes with TLR ligands *in vitro* leads to differentiation into macrophages or dendritic cells (Krutzik *et al.*, 2005). Different cell lineages express different TLRs.

In the dermal granulomatous inflammation that characterises leprosy and leprosy T1Rs the proportion of CD68 positive cells greatly outweighs those that are CD1a positive but concomitant expression of both markers was not investigated.

CD68 positive cells expressed TLR2 in the granulomas of individuals with T1R and BT and BL leprosy controls. This is in agreement with the findings of Krutzik *et al* in leprosy patients and also in individuals with acne inversa (Hunger *et al.*, 2008). This demonstrates that the expression of TLR2 is closely associated with some of the abundant cells forming the inflammatory infiltrate in individuals with T1R. However in these double immunofluorescence experiments it was not possible to replicate the findings of Krutzik in which five percent of cells in the granuloma were CD1a positive and expressed TLR2. CD1c positive epidermal Langerhans cells have been shown to express TLR2 *ex vivo* (Peiser *et al.*, 2008).

The CD3 positive lymphocytes were closely associated with TLR2 positive staining cells but did not appear to express TLR2 themselves. This too is in agreement with the work of Krutzik *et al*.

Krutzik and colleagues were able to demonstrate colocalisation of TLR1 and TLR2. In the current experiment it is likely that there was insufficient anti-TLR1 antibody bound to be visualised with the goat anti-mouse fluorochrome conjugated secondary antibodies. These secondary antibodies do not provide the same degree of signal amplification as the biotin-streptavidin method used in immunohistochemical techniques because they do not complex

with each other. There is less signal amplification when bound to small amounts of primary antibody compared to techniques based on biotin-streptavidin methods. The protocol using biotin-Alexa Fluor® 350 conjugated streptavidin may not have worked because the biotinylated secondary used was from the Dako K5001 kit which may have been incompatible with the fluorochrome labelled streptavidin used.

It should be noted that in the experiment described by Krutzik both primary antibodies against TLR1 and TLR2 were of the $IgG_1$ isotype which raises the possibility that each of the secondary fluorescent antibodies recognised the same primary antibody. This problem can complicate the method used by Krutzik in which each primary and secondary antibody pair are incubated sequentially (Jones and Westmacott, 2007).

There are no reports of immunofluorescent studies of TLR4 and TLR9 in human skin. It is possible that other workers have experienced difficulties with fluorescent techniques involving these targets.

## 4.6 Summary and conclusions

- The small BL/LL control group had consistently higher staining of TLR1, TLR2 and TLR4 than TT/BT control groups and reactional study patients.
- The findings of the immunohistochemical studies suggest that individuals with T1R have similar levels of cellular infiltration as non-reactional leprosy controls. There are no significant differences in the cells of the cellular infiltrate. The level of cellular infiltrate and the number of cells of each subtype studied falls during corticosteroid therapy.
- TLR2 is highly expressed by cells in skin lesions of individuals with T1R. TLR2 is expressed by CD68 positive cells in these skin lesions but not CD1a positive dermal dendritic cells.
- TLR2 expression in skin lesions of T1R is significantly higher than in the normal appearing skin of individuals with NFI.
- Corticosteroid therapy is associated with a significant reduction in TLR2 expression in skin lesions.
- hARP-P0 is a suitable control gene to use in MDT and corticosteroid treated individuals.
- TLR2 gene expression is reduced during corticosteroid therapy. Individuals with a favourable clinical outcome have lower TLR2 gene expression at the end of corticosteroid treatment compared to baseline than those who do less well.

- TLR4 protein expression and TLR4 gene expression in the skin is reduced during corticosteroid therapy.
- Individuals who received a higher dose of corticosteroid did have significantly different changes in TLR staining or TLR gene expression compared to those who received less.

# CHAPTER FIVE

## Discussion

## 5.1 Measuring the severity of Type 1 reactions and nerve function impairment

The Clinical Severity Scale is the first validated scale for measuring leprosy T1R and NFI. It provides a means of assessing the severity of an individual's reaction using clinical examination. The score generated by application of the scale allows the comparison of the severity of reaction between individuals and in a given individual during treatment.

The score could be used to categorize participants in future clinical trials. This would enable investigators to determine whether there was any significant difference in the severity of T1R between individuals in the different arms of an intervention study.

The Clinical Severity Scale does require the use of SWM and it is important that researchers using the scale ensure that adequate training in their use has been undertaken.

The Global Strategy for Further Reducing the Leprosy Burden and Sustaining Leprosy Control Activities (2006-2010) (WHO, 2006) recommends that individuals with T1Rs and/or NFI should be managed in referral centres. If staff in referral centres could be trained in the use of the SWM then the scale could be used to monitor an individual patients response to treatment.

It would be important to ensure that the scale was validated in the particular setting in which it was being employed. The greatest burden of leprosy remains in India and Brazil from where 69.5% of cases are reported (WHO, 2009). It is likely that any large intervention studies of T1Rs are likely to include centres from these countries and so validation of the scale in India should be made a priority. A validation of the scale in India should also be designed so that the impact of NFI present for longer than six months could be assessed. This would require a larger number of people to be recruited. The scale is currently being tested in Ethiopia where it is being validated and the MID in Ethiopian patients is also being assessed.

The change in score might be useful as an outcome measure in clinical trials. At present the range of scores that represent the clinical categories of mild or moderate or severe T1R have been determined using the receiver operating characteristic curves. Further work is needed to assess what a change in unit score represents in terms of clinical improvement or deterioration to someone affected by a T1R and/or NFI.

The scale needs to be assessed in terms of MID which will allow any change in severity score to be interpreted in clinically meaningful ways.

There are various methods for determining MID of a clinical measurement device. A study could be designed to apply the Clinical Severity Scale to individuals presenting with T1R and/or NFI in a setting where the scale had already been validated. The scale is applied again after four weeks of treatment. The individual is asked whether their condition is: much better, somewhat better, the same, somewhat worse or worse (Coeytaux *et al.*, 2006). It would be important to ensure that these terms were unambiguous in the first language of the participants in the study. The category "somewhat better" is assumed to be an improvement in clinical status which is discernible by the patient.

One could then calculate the mean change in score for individuals in the "somewhat better" group. To allow for variation of the score with time the mean change in score of the group that report their symptoms as the "same" could be subtracted from that of the "somewhat better" group. An alternative method would be to compare the mean scores at the four week assessment between the groups "somewhat better" and "same". The MID is calculated by subtracting one from the other. Another method assumes that the MID of "somewhat better" is the same as "somewhat worse" and the mean of the change in the scores of the two groups can be used to calculate MID.

Calculating MID using all of these methods will provide a more accurate assessment of MID.

A knowledge of the magnitude of the change in score required to achieve a MID would facilitate power calculations for clinical trials.

The significant difference in the change in nerve score between individuals who were better or improved and those who were unchanged or worse in the Nepali cohort, although a preliminary finding, suggests that the scale reflects clinically relevant change. The small numbers in the cohort and the criterion used to determine improvement (post hoc physician assessment) make this finding less reliable than a study designed using the methodologies outlined above.

It could be argued that the scale remains too complex. Studies could be undertaken to try and further reduce the number of items. However if neurological items (such as particular peripheral nerves) were removed then some other way of assessing the functional status of these nerves might be required.

The demonstration that it is possible to develop a valid, reliable scale to measure the severity of T1R and NFI will encourage researchers to turn their attention to developing and validating a scale for ENL. ENL has more clinical components than T1Rs and a scale might

be somewhat more complex and require larger study populations. The development of a scale would help to monitor those individuals with chronic disease and those whose ENL was becoming corticosteroid resistant. The multisystem nature of ENL makes the design of clinical trials difficult and a valid reliable scale would be useful in defining outcomes.

## 5.2 The treatment of Type 1 reactions

This study is the first to systematically assess pulse IV methylprednisolone for the management of T1Rs. The rationale for this investigation was that administration of this drug has been associated with clinical improvement in MS and RA. The drug is also affordable and available in leprosy endemic settings.

This study was too small to determine small differences in the effect of high dose pulse methylprednisolone and oral prednisolone compared to oral prednisolone alone. During the inception of this study the study by Rao and colleagues was published suggesting that the duration of treatment rather than total dose of corticosteroid used may be more beneficial in controlling the symptoms and signs of T1R (Rao *et al.*, 2006).

In the Rao double-blind placebo controlled study clinicians were less likely to prescribe additional prednisolone to individuals who received prednisolone for 20 weeks compared to those who received prednisolone for 12 weeks. In this study participants were treated with corticosteroids for 16 weeks despite this 47.6% of individuals required extra prednisolone. This is comparable to the 46% of individuals treated with the 12 week course of prednisolone by Rao. It should be noted that it is likely that the current study had a lower threshold for prescribing additional prednisolone than that of Rao. It is not clear from the Rao study how nerve function was assessed. Another difference between the studies is that the current study included individuals with NFI who did not have skin signs. The treatment of isolated NFI and T1R with NFI is the same and there is no evidence to suggest that the two syndromes differ in their response to treatment.

It is likely that future clinical trials will be designed to examine the effect of longer courses (24-36 weeks) of corticosteroids on leprosy T1Rs and NFI rather than larger cumulative doses. This pilot study has provided reasonable evidence to suggest that pulse methylprednisolone treatment is not associated with significantly greater adverse events than oral prednisolone but I do not think this provides sufficient evidence for a larger study of pulsed methylprednisolone given the data from the Rao study. I would advocate the design of a large multi-centre study of three oral prednisolone regimens of 20, 28 and 36 weeks duration. It would be important to maintain follow up for 48 weeks after cessation of corticosteroid treatment. Other agents such as azathioprine and ciclosporin are currently

being studied in clinical trials and these too should be subjected to the same design standards as trials of corticosteroids.

There are no agreed criteria for prescribing additional prednisolone to individuals in whom symptoms or NFI has deteriorated. This hampers the comparison of clinical trials. The development of the Clinical Severity Score should allow researchers to standardise criteria for clinical deterioration, particularly with respect to NFI, once the MID of the scale has been determined. An agreed standard is urgently needed to improve the design of future studies.

The recruitment of participants was not as high as had been hoped. The alteration of the entry criteria after 9 months of the study is not ideal but should not have affected the primary objective of the study to determine the adverse effect profile in individuals who received methylprednisolone compared to individuals who were treated with prednisolone alone.

The IV administration of methylprednisolone requires an individual to be hospitalised. This factor may have affected the willingness of potential participants to enrol as they have to be away from family and work for a greater period of time than would be the case with self-administered oral therapy.

Women are underrepresented in the studies of T1Rs. The under representation of certain groups affects many clinical trials worldwide (Bolen *et al.*, 2006). The results of trials may not be applicable if the study population is not representative. The lack of recruitment of women is a cause for concern. Gender inequalities may be more significant in leprosy as it is a highly stigmatizing disease (Le Grand, 1997).

All the prospective studies outlined in Tables 1.08 and 1.09 (Chapter 1) have recruited more men than women with rates of female recruitment varying from 13-36%. The rate of T1R in women with borderline forms of leprosy at Anadaban Hospital (where this study was performed) is 28.7% (Roche *et al.*, 1991). In this study 21.4% of the participants were female which makes this study reasonably representative.

The methylprednisolone study has provided data which has some implications for future policy. It demonstrates that even using 16 weeks of prednisolone there is a large proportion of people who require further prednisolone. Until recently WHO advocated the use of a 12 week standard tapering course of prednisolone for T1R and NFI. The current "The Global Strategy for Further Reducing the Leprosy Burden and Sustaining Leprosy Control Activities (2006–2010)" states that "Severe reversal reactions should be treated with a course of steroids, usually lasting 3–6 months" (WHO, 2006). This study and that of Rao

should be used by policy makers as evidence that 12 weeks oral prednisolone is insufficient treatment and the minimum should probably be 20 weeks.

As we move towards longer courses of corticosteroids we need to consider ways of identifying those individuals at risk of deteriorating as the dose of prednisolone is reduced. Identifying risk factors might also minimise exposing individuals unnecessarily to prolonged courses of prednisolone and hopefully reduce the risk of them experiencing an adverse event.

## 5.3 Toll-like receptors and Type 1 reactions

The publication of the paper by Lockwood concerning the histological diagnosis of T1R has an impact on future studies in this area (Lockwood *et al.*, 2008). The histological criteria including HLA-DR staining should be used to provide further support of the clinical diagnosis of T1R following enrolment. I think there are even grounds for arguing that two experienced histopathologists review all the skin biopsies from patients in clinical and immunological studies.

The experiments performed in this research might be further strengthened by categorizing participants into two groups depending on whether their skin sections are positive for epidermal staining of HLA-DR.

The results of semi-quantitative scales to assess the staining need to be interpreted cautiously. The expression of inflammatory cytokines and cell surface receptors in the skin does fall during corticosteroid therapy however it is not possible to determine whether subsequent staining is the result of identifying further antigen expression or a delay in clearance.

The expression of the markers of interest may not be proportional to the level of inflammation observed clinically, itself a difficult factor to quantify. Many biological systems rely on cascade mechanisms and once a pathway has been activated the expression of individual receptors or mediators may be amplified to such an extent that it bears little relationship to the initial trigger.

The main role of immunohistochemical techniques is to identify a target antigen in a tissue to show that it is being expressed and might be playing a role in the process being studied. It is of interest in studies in a chronological series of specimens to determine any change in expression but this does not necessarily reflect changes in level of function of the molecule of interest. The finding of increased TLR1, TLR2 and TLR4 in the skin of BL/LL control

patients is worthy of further research. The current findings are contrary to the existing concepts of TLR expression in skin lesions of a small number of leprosy patients.

Gene expression is a fundamental indication of the processes involved in initiating or propagating a pathological process. The data in this study allowed the analysis of TLR gene expression during treatment with corticosteroids and also MDT. The identification of a suitable control gene for use in this group of patients is of great use for future gene expression studies. The expression of hARP-P0 was not significantly affected by MDT or corticosteroids.

 The $\Delta\Delta C_T$ method is an approximation method and only provides relative expression data compared to the baseline sample. One could use the absolute standard curve method to calculate the input copy number of the gene of interest.

SYBR Green is a fluorescent dye that binds double-stranded DNA it is less specific than probe based technologies which employ oligonucleotides containing fluorescent reporter and quencher dyes. The quencher dye acts by the principle of Fluorescence (or Förster) Resonance Energy Transfer to inhibit fluorescence of bound reporter dye. Once the polymerase replicates a template the reporter dye of the probe is unbound and its fluorescence no longer inhibited by the quencher dye. The increased specificity makes the probe based approach more accurate.

The data on both protein and gene expression was limited because a pre-reaction sample was not available. Ideally experiments such as these would be designed as part of a cohort study in which individuals were enrolled and specimens obtained prior to the onset of the problem under investigation. This was done in the INFIR Cohort study and 38% (115 out of 303) had either a reaction or new NFI at presentation which shows how large such studies would need to be.

The data obtained from the current set of experiments is interesting in that it gives further *in vivo* evidence to the role of TLR2 in the pathophysiology of T1Rs. There have not been any reports of *in vivo* experiments of TLR4 in leprosy or T1R. The gene expression data for both demonstrated reduced expression during treatment but also suggested lower expression of these genes in individuals who had a better clinical outcome. This warrants further investigation with a larger sample of patients preferably nested within a cohort study. A cohort study of this nature would need to be large. The results of these studies should be widely disseminated and if possible repeated in other regions of the world.

The leprosy non-governmental organisations which support the care of leprosy patients and leprosy research should be encouraged to develop infrastructure within their organisations (particularly dedicated leprosy hospitals) so that an open-ended cohort study can operate where data and samples are collected prospectively and stored appropriately with relevant consent obtained. As leprosy services in endemic countries continue to be integrated into general health services leprosy research has a lower priority and this needs to be addressed.

The results obtained for immunostaining of TLR9 were disappointing because staining occurred much less frequently than with the other anti-TLR antibodies. This receptor warrants further investigation. Adaptations to staining techniques should be explored such as alternative fixatives. The role of heat-induced antigen retrieval has been explored in frozen sections using other antibodies (Yamashita and Okada, 2005). The experiments should also be performed with positive control tissue.

The experiments provide further evidence of the activation of the innate immune system during T1R and other receptors such as the nucleotide-binding oligomerization domain-like receptors warrant investigation in this condition.

The functional role of TLRs in the immunopathology of leprosy and leprosy reactional states could be further investigated *in vivo* using TLR agonists and antagonists (Kanzler *et al.*, 2007). These agents could be injected intradermally into reactional and non-reactional leprosy skin lesions in a manner similar to that employed by Kaplan in her experiments with recombinant IFN-γ (Kaplan *et al.*, 1989). Experiments such as this with suitable controls might provide functional data which would help in understanding the mechanisms of T1R and granulomatous inflammation in leprosy.

The improved management of T1Rs requires larger robust clinical trials to provide more evidence on the most appropriate treatment. These trials need to be well designed with clear outcome measures. An improved understanding of the mechanisms underlying T1R may indicate the potential usefulness of other treatments to control this damaging complication of leprosy.

The work in this thesis contributes to a better understanding of T1Rs by improving our ability to assess the severity of T1R and provide better outcome measures for clinical studies. This research has added to the evidence that the outcomes following prolonged systemic corticosteroid therapy for T1R are not satisfactory. This is the first study to examine TLR expression in leprosy T1Rs and has identified and validated a control gene for future gene expression studies of T1R.

## 5.4 Summary of future work

- The severity scale should be validated and MID determined in other leprosy endemic settings particularly India and Brazil.
- A more concise scale might be developed for use in descriptive studies of T1R severity.
- A scale should be developed and validated for ENL.
- The study has highlighted the need to agree criteria for the prescribing of additional corticosteroids to individuals who experience re-reaction. These criteria should be evidence based and evaluated in clinical studies.
- Further clinical trials and cohort studies of corticosteroids and other immunosuppressants are urgently needed.
- Data collection on the features and outcome of T1R in established leprosy centres should be improved to provide good cohort data.
- Case-control studies to identify risk factors for re-reaction should be undertaken.
- The mechanisms underlying the pathophysiology of T1R are difficult to determine without good control specimens. The best control specimens are provided by the individual experiencing T1R. This requires tissue to be collected and stored prior to reaction. A cutaneous (and neural) tissue biobank would facilitate current and future biological research. This could be established with suitable ethical oversight but would require financial support.
- Experiments should pay close attention to the MDT status of the patient and the cutaneous and neural involvement at the time of onset of the reaction.
- *In vivo* experiments with TLR agonists and antagonists might provide useful functional data.
- Optimisation of TLR9 staining and dual immunofluorescent staining for bacterial DNA should be undertaken.
- Microdissection of granulomas from reactional and non-reactional lesions may reduce the "background noise" of whole skin specimens in PCR assays.

# REFERENCES

Aaron, L., Saadoun, D., Calatroni, I., Launay, O., Memain, N., Vincent, V., Marchal, G., Dupont, B., Bouchaud, O., Valeyre, D., and Lortholary, O. (2004). Tuberculosis in HIV-infected patients: a comprehensive review. Clin Microbiol Infect *10*, 388-398.

Abraham, A., Sharma, V. K., and Kaur, S. (1990). Assessment of testicular volume in bacilliferous leprosy: correlation with clinical parameters. Indian J Lepr *62*, 310-315.

Ahn, J. H., Park, T. J., Jin, S. H., and Kang, H. Y. (2008). Human melanocytes express functional Toll-like receptor 4. Exp Dermatol *17*, 412-417.

Akashi-Takamura, S., and Miyake, K. (2008). TLR accessory molecules. Curr Opin Immunol *20*, 420-425.

Algood, H. M., Lin, P. L., and Flynn, J. L. (2005). Tumor necrosis factor and chemokine interactions in the formation and maintenance of granulomas in tuberculosis. Clin Infect Dis *41 Suppl 3*, S189-193.

American Diabetes Association (2008). Standards of medical care in diabetes--2008. Diabetes Care *31 Suppl 1*, S12-54.

Anderson, A. M., and Croft, R. P. (1999). Reliability of Semmes Weinstein monofilament and ballpoint sensory testing, and voluntary muscle testing in Bangladesh. Lepr Rev *70*, 305-313.

Anderson, A. M., and van Brakel, W. H. (1998). Agespecific normal thresholds for sensibility testing with monofilaments in a Nepali population. Int J Lepr *66*, 69A.

Anderson, K. V., Jurgens, G., and Nusslein-Volhard, C. (1985). Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. Cell *42*, 779-789.

Andersson, A. K., Atkinson, S. E., Khanolkar-Young, S., Chaduvula, M., Jain, S., Suneetha, L., Suneetha, S., and Lockwood, D. N. (2007). Alteration of the cortisol-cortisone shuttle in leprosy type 1 reactions in leprosy patients in Hyderabad, India. Immunol Lett *109*, 72-75.

Andersson, A. K., Chaduvula, M., Atkinson, S. E., Khanolkar-Young, S., Jain, S., Suneetha, L., Suneetha, S., and Lockwood, D. N. (2005). Effects of prednisolone treatment on cytokine expression in patients with leprosy type 1 reactions. Infect Immun *73*, 3725-3733.

Angel, C. E., Lala, A., Chen, C. J., Edgar, S. G., Ostrovsky, L. L., and Dunbar, P. R. (2007). CD14+ antigen-presenting cells in human dermis are less mature than their CD1a+ counterparts. Int Immunol *19*, 1271-1279.

Ashworth, J., Booker, J., and Breathnach, S. M. (1988). Effects of topical corticosteroid therapy on Langerhans cell antigen presenting function in human skin. Br J Dermatol *118*, 457-469.

Atkinson, A. J., Jr., Sheagren, J. N., Rubio, J. B., and Knight, V. (1967). Evaluation of B.663 in human leprosy. Int J Lepr Other Mycobact Dis *35*, 119-127.

Azulay-Abulafia, L., Pereira Spinelli, L., Hardmann, D., Kawa Kac, B., Levy, R. A., Talhari, C., and Ruzicka, T. (2006). [Lucio phenomenon. Vasculitis or occlusive vasculopathy?]. Hautarzt *57*, 1101-1105.

Babu, S., Bhat, S. Q., Kumar, N. P., Anuradha, R., Kumaran, P., Gopi, P. G., Kolappan, C., Kumaraswami, V., and Nutman, T. B. (2009). Attenuation of toll-like receptor expression and function in latent tuberculosis by coexistent filarial infection with restoration following antifilarial chemotherapy. PLoS Negl Trop Dis *3*, e489.

Bafica, A., Scanga, C. A., Feng, C. G., Leifer, C., Cheever, A., and Sher, A. (2005). TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. J Exp Med *202*, 1715-1724.

Baker, B. S., Ovigne, J. M., Powles, A. V., Corcoran, S., and Fry, L. (2003). Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. Br J Dermatol *148*, 670-679.

Baker, R. W., Walker, B. R., Shaw, R. J., Honour, J. W., Jessop, D. S., Lightman, S. L., Zumla, A., and Rook, G. A. (2000). Increased cortisol: cortisone ratio in acute pulmonary tuberculosis. Am J Respir Crit Care Med *162*, 1641-1647.

Ballanger, F., Tenaud, I., Volteau, C., Khammari, A., and Dreno, B. (2008). Anti-inflammatory effects of lithium gluconate on keratinocytes: a possible explanation for efficiency in seborrhoeic dermatitis. Arch Dermatol Res *300*, 215-223.

Barnes, P. F., Chatterjee, D., Brennan, P. J., Rea, T. H., and Modlin, R. L. (1992). Tumor necrosis factor production in patients with leprosy. Infect Immun *60*, 1441-1446.

Barnes, P. J. (2006). How corticosteroids control inflammation: Quintiles Prize Lecture 2005. Br J Pharmacol *148*, 245-254.

Barnes, P. J., and Adcock, I. M. (2009). Glucocorticoid resistance in inflammatory diseases. Lancet *373*, 1905-1917.

Baroni, A., Orlando, M., Donnarumma, G., Farro, P., Iovene, M. R., Tufano, M. A., and Buommino, E. (2006). Toll-like receptor 2 (TLR2) mediates intracellular signalling in human keratinocytes in response to Malassezia furfur. Arch Dermatol Res *297*, 280-288.

Barreiro, L. B., Quach, H., Krahenbuhl, J., Khaliq, S., Mohyuddin, A., Mehdi, S. Q., Gicquel, B., Neyrolles, O., and Quintana-Murci, L. (2006). DC-SIGN interacts with Mycobacterium leprae but sequence variation in this lectin is not associated with leprosy in the Pakistani population. Hum Immunol *67*, 102-107.

Barton, R. P. (1976). Clinical manifestation of leprous rhinitis. Ann Otol Rhinol Laryngol *85*, 74-82.

Becx-Bleumink, M., and Berhe, D. (1992). Occurrence of reactions, their diagnosis and management in leprosy patients treated with multidrug therapy; experience in the leprosy control program of the All Africa Leprosy and Rehabilitation Training Center (ALERT) in Ethiopia. Int J Lepr Other Mycobact Dis *60*, 173-184.

Begon, E., Michel, L., Flageul, B., Beaudoin, I., Jean-Louis, F., Bachelez, H., Dubertret, L., and Musette, P. (2007). Expression, subcellular localization and cytokinic modulation of Toll-like receptors (TLRs) in normal human keratinocytes: TLR2 up-regulation in psoriatic skin. Eur J Dermatol *17*, 497-506.

Bernink, E. H., and Voskens, J. E. (1997). Study on the detection of leprosy reactions and the effect of prednisone on various nerves, Indonesia. Lepr Rev *68*, 225-232.

Berrebi, D., Bruscoli, S., Cohen, N., Foussat, A., Migliorati, G., Bouchet-Delbos, L., Maillot, M. C., Portier, A., Couderc, J., Galanaud, P., Peuchmaur, M., Riccardi, C., and Emilie, D. (2003). Synthesis of glucocorticoid-induced leucine zipper (GILZ) by macrophages: an anti-inflammatory and immunosuppressive mechanism shared by glucocorticoids and IL-10. Blood *101*, 729-738.

Berth-Jones, J., Grotzinger, K., Rainville, C., Pham, B., Huang, J., Daly, S., Herdman, M., Firth, P., and Hotchkiss, K. (2006). A study examining inter- and intrarater reliability of three scales for measuring severity of psoriasis: Psoriasis Area and Severity Index, Physician's Global Assessment and Lattice System Physician's Global Assessment. Br J Dermatol *155*, 707-713.

Bettoni, I., Comelli, F., Rossini, C., Granucci, F., Giagnoni, G., Peri, F., and Costa, B. (2008). Glial TLR4 receptor as new target to treat neuropathic pain: efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. Glia *56*, 1312-1319.

Beyene, D., Aseffa, A., Harboe, M., Kidane, D., Macdonald, M., Klatser, P. R., Bjune, G. A., and Smith, W. C. (2003). Nasal carriage of Mycobacterium leprae DNA in healthy individuals in Lega Robi village, Ethiopia. Epidemiol Infect *131*, 841-848.

Birke, J. A., and Sims, D. S. (1986). Plantar sensory threshold in the ulcerative foot. Lepr Rev *57*, 261-267.

Bland, J. M., and Altman, D. G. (1995). Comparing methods of measurement: why plotting difference against standard method is misleading. Lancet *346*, 1085-1087.

Bochud, P. Y., Hawn, T. R., Siddiqui, M. R., Saunderson, P., Britton, S., Abraham, I., Argaw, A. T., Janer, M., Zhao, L. P., Kaplan, G., and Aderem, A. (2008). Toll-like receptor 2 (TLR2) polymorphisms are associated with reversal reaction in leprosy. J Infect Dis *197*, 253-261.

Bochud, P. Y., Sinsimer, D., Aderem, A., Siddiqui, M. R., Saunderson, P., Britton, S., Abraham, I., Tadesse Argaw, A., Janer, M., Hawn, T. R., and Kaplan, G. (2009a). Polymorphisms in Toll-like receptor 4 (TLR4) are associated with protection against leprosy. Eur J Clin Microbiol Infect Dis *28*, 1055-1065.

Bochud, P. Y., Sinsimer, D., Aderem, A., Siddiqui, M. R., Saunderson, P., Britton, S., Abraham, I., Tadesse Argaw, A., Janer, M., Hawn, T. R., and Kaplan, G. (2009b). Polymorphisms in Toll-like receptor 4 (TLR4) are associated with protection against leprosy. Eur J Clin Microbiol Infect Dis.

Boerrigter, G., Ponnighaus, J. M., Fine, P. E., and Wilson, R. J. (1991). Four-year follow-up results of a WHO-recommended multiple-drug regimen in paucibacillary leprosy patients in Malawi. Int J Lepr Other Mycobact Dis *59*, 255-261.

Boivin, A., Pineau, I., Barrette, B., Filali, M., Vallieres, N., Rivest, S., and Lacroix, S. (2007). Toll-like receptor signaling is critical for Wallerian degeneration and functional recovery after peripheral nerve injury. J Neurosci *27*, 12565-12576.

Bojar, R. A., Eady, E. A., Jones, C. E., Cunliffe, W. J., and Holland, K. T. (1994). Inhibition of erythromycin-resistant propionibacteria on the skin of acne patients by topical erythromycin with and without zinc. Br J Dermatol *130*, 329-336.

Bolen, S., Tilburt, J., Baffi, C., Gary, T. L., Powe, N., Howerton, M., Ford, J., Lai, G., Wilson, R., and Bass, E. (2006). Defining "success" in recruitment of underrepresented populations to cancer clinical trials: moving toward a more consistent approach. Cancer *106*, 1197-1204.

Bornstein, S. R., Schumann, R. R., Rettori, V., McCann, S. M., and Zacharowski, K. (2004). Toll-like receptor 2 and Toll-like receptor 4 expression in human adrenals. Horm Metab Res *36*, 470-473.

Bornstein, S. R., Ziegler, C. G., Krug, A. W., Kanczkowski, W., Rettori, V., McCann, S. M., Wirth, M., and Zacharowski, K. (2006). The role of toll-like receptors in the immune-adrenal crosstalk. Ann N Y Acad Sci *1088*, 307-318.

Boucher, P., Millan, J., Parent, M., and Moulia-Pela, J. P. (1999). [Randomized controlled trial of medical and medico-surgical treatment of Hansen's neuritis]. Acta Leprol *11*, 171-177.

Brain (2000). Aids to the examination of the peripheral nervous system, 4th edn (Philadelphia, W.B. Saunders).

Brand, P. W. (1952). The orthopedic care of leprosy patients. Lepr Rev *23*, 50-62.

Brandsma, J. W., Yoder, L., and MacDonald, M. (2005). Leprosy acquired by inoculation from a knee injury. Lepr Rev *76*, 175-179.

Brandsma, W. (1981). Basic nerve function assessment in leprosy patients. Lepr Rev *52*, 161-170.

Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Bleharski, J. R., Maitland, M., Norgard, M. V., Plevy, S. E., Smale, S. T., Brennan, P. J., Bloom, B. R., Godowski, P. J., and Modlin, R. L. (1999). Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science *285*, 732-736.

Britton, W. J., and Lockwood, D. N. (2004). Leprosy. Lancet *363*, 1209-1219.

Bullock, W. E., Jr., Ho, M. F., and Chen, M. J. (1970). Studies of immune mechanisms in leprosy. II Quantitative relationships of IgG, IgA, and IgM immunoglobulins. J Lab Clin Med *75*, 863-870.

Burton, J. M., O'Connor, P. W., Hohol, M., and Beyene, J. (2009). Oral versus intravenous steroids for treatment of relapses in multiple sclerosis. Cochrane Database Syst Rev, CD006921.

Buttgereit, F., da Silva, J. A., Boers, M., Burmester, G. R., Cutolo, M., Jacobs, J., Kirwan, J., Kohler, L., Van Riel, P., Vischer, T., and Bijlsma, J. W. (2002). Standardised nomenclature for glucocorticoid dosages and glucocorticoid treatment regimens: current questions and tentative answers in rheumatology. Ann Rheum Dis *61*, 718-722.

Bwire, R., and Kawuma, H. J. (1994). Type 1 reactions in leprosy, neuritis and steroid therapy: the impact of the human immunodeficiency virus. Trans R Soc Trop Med Hyg *88*, 315-316.

Cem Mat, M., Yazici, H., Ozbakir, F., and Tuzun, Y. (1988). The HLA association of lepromatous leprosy and borderline lepromatous leprosy in Turkey. A preliminary study. Int J Dermatol *27*, 246-247.

Chakravartti, M. R., and Vogel, F. (1973). A twin study on leprosy. Top Hum Genet *1* 1–123.

Chinen, K., Nakazato, I., Iwamasa, T., Hosokawa, A., and Sawada, S. (1997). [Pathological examination of the lung in leprosy patients]. Nihon Hansenbyo Gakkai Zasshi *66*, 85-89.

Chopra, N. K., Agarawal, J. S., and Pandya, P. G. (1990). A study of relapse in paucibacillary leprosy in a multidrug therapy project, Baroda District, India. Lepr Rev *61*, 157-162.

Ciechanover, A. (2006). The ubiquitin proteolytic system: from a vague idea, through basic mechanisms, and onto human diseases and drug targeting. Neurology *66*, S7-19.

Closs, O., Reitan, L. J., Negassi, K., Harboe, M., and Belehu, A. (1982). In vitro stimulation of lymphocytes in leprosy patients, healthy contacts of leprosy patients, and subjects not exposed to leprosy. Comparison of an antigen fraction prepared from Mycobacterium leprae and tuberculin-purified protein derivative. Scand J Immunol *16*, 103-115.

Coeytaux, R. R., Kaufman, J. S., Chao, R., Mann, J. D., and Devellis, R. F. (2006). Four methods of estimating the minimal important difference score were compared to establish a clinically significant change in Headache Impact Test. J Clin Epidemiol *59*, 374-380.

Cole, S. T., Eiglmeier, K., Parkhill, J., James, K. D., Thomson, N. R., Wheeler, P. R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R. M., Devlin, K., Duthoy, S., Feltwell, T., Fraser, A., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Lacroix, C., Maclean, J., Moule, S., Murphy, L., Oliver, K., Quail, M. A., Rajandream, M. A., Rutherford, K. M., Rutter, S., Seeger, K., Simon, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Taylor, K., Whitehead, S., Woodward, J. R., and Barrell, B. G. (2001). Massive gene decay in the leprosy bacillus. Nature *409*, 1007-1011.

Conn, H. O., and Poynard, T. (1994). Corticosteroids and peptic ulcer: meta-analysis of adverse events during steroid therapy. J Intern Med *236*, 619-632.

Croft, R. P., Nicholls, P. G., Richardus, J. H., and Smith, W. C. (2000a). Incidence rates of acute nerve function impairment in leprosy: a prospective cohort analysis after 24 months (The Bangladesh Acute Nerve Damage Study). Lepr Rev *71*, 18-33.

Croft, R. P., Nicholls, P. G., Richardus, J. H., and Smith, W. C. (2000b). The treatment of acute nerve function impairment in leprosy: results from a prospective cohort study in Bangladesh. Lepr Rev *71*, 154-168.

Croft, R. P., Richardus, J. H., Nicholls, P. G., and Smith, W. C. (1999). Nerve function impairment in leprosy: design, methodology, and intake status of a prospective cohort study of 2664 new leprosy cases in Bangladesh (The Bangladesh Acute Nerve Damage Study). Lepr Rev *70*, 140-159.

Cronbach, L. J., and Meehl, P. E. (1955). Construct validity in psychological tests. Psychol Bull *52*, 281-302.

Cross, H., and Newcombe, L. (2001). An intensive self care training programme reduces admissions for the treatment of plantar ulcers. Lepr Rev *72*, 276-284.

Curry, J. L., Qin, J. Z., Bonish, B., Carrick, R., Bacon, P., Panella, J., Robinson, J., and Nickoloff, B. J. (2003). Innate immune-related receptors in normal and psoriatic skin. Arch Pathol Lab Med *127*, 178-186.

Curtis, J. R., Westfall, A. O., Allison, J., Bijlsma, J. W., Freeman, A., George, V., Kovac, S. H., Spettell, C. M., and Saag, K. G. (2006). Population-based assessment of adverse events associated with long-term glucocorticoid use. Arthritis Rheum *55*, 420-426.

Daniel, E., and Sundar Rao, P. S. (2007). Evolution of vision reducing cataract in skin smear positive lepromatous patients: does it have an inflammatory basis? Br J Ophthalmol *91*, 1011-1013.

Davey, T. F., and Rees, R. J. (1974). The nasal dicharge in leprosy: clinical and bacteriological aspects. Lepr Rev *45*, 121-134.

Degowin, R. L., Eppes, R. B., Powell, R. D., and Carson, P. E. (1966). The haemolytic effects of diaphenylsulfone (DDS) in normal subjects and in those with glucose-6-phosphate-dehydrogenase deficiency. Bull World Health Organ *35*, 165-179.

Demangel, C., and Britton, W. J. (2000). Interaction of dendritic cells with mycobacteria: where the action starts. Immunol Cell Biol *78*, 318-324.

Deps, P. D., and Lockwood, D. N. (2008). Leprosy occurring as immune reconstitution syndrome. Trans R Soc Trop Med Hyg *102*, 966-968.

Deps, P. D., Nasser, S., Guerra, P., Simon, M., Birshner Rde, C., and Rodrigues, L. C. (2007). Adverse effects from multi-drug therapy in leprosy: a Brazilian study. Lepr Rev *78*, 216-222.

Desikan, K. V., and Sreevatsa (1995). Extended studies on the viability of Mycobacterium leprae outside the human body. Lepr Rev *66*, 287-295.

Dissanayeke, S. R., Levin, S., Pienaar, S., Wood, K., Eley, B., Beatty, D., Henderson, H., Anderson, S., and Levin, M. (2009). Polymorphic variation in TIRAP is not associated with susceptibility to childhood TB but may determine susceptibility to TBM in some ethnic groups. PLoS One *4*, e6698.

Doz, E., Rose, S., Nigou, J., Gilleron, M., Puzo, G., Erard, F., Ryffel, B., and Quesniaux, V. F. (2007). Acylation determines the toll-like receptor (TLR)-dependent positive versus TLR2-, mannose receptor-, and SIGNR1-independent negative regulation of pro-inflammatory cytokines by mycobacterial lipomannan. J Biol Chem *282*, 26014-26025.

Duncan, M. E., and Pearson, J. M. (1982). Neuritis in pregnancy and lactation. Int J Lepr Other Mycobact Dis *50*, 31-38.

Duncan, M. E., and Pearson, J. M. (1984). The association of pregnancy and leprosy--III. Erythema nodosum leprosum in pregnancy and lactation. Lepr Rev *55*, 129-142.

Faber, W. R., Jensema, A. J., and Goldschmidt, W. F. (2006). Treatment of recurrent erythema nodosum leprosum with infliximab. N Engl J Med *355*, 739.

Fajardo, T. T., Villahermosa, L., Pardillo, F. E., Abalos, R. M., Burgos, J., Dela Cruz, E., and Gelber, R. H. (2009). A comparative clinical trial in multibacillary leprosy with long-term relapse rates of four different multidrug regimens. Am J Trop Med Hyg *81*, 330-334.

Fardet, L., Cabane, J., Kettaneh, A., Lebbe, C., and Flahault, A. (2007a). Corticosteroid-induced lipodystrophy is associated with features of the metabolic syndrome. Rheumatology (Oxford) *46*, 1102-1106.

Fardet, L., Cabane, J., Lebbe, C., Morel, P., and Flahault, A. (2007b). Incidence and risk factors for corticosteroid-induced lipodystrophy: a prospective study. J Am Acad Dermatol *57*, 604-609.

Feenstra, W., Van de Vijver, S., Benbow, C., Amenu, A., and Saunderson, P. (2001). Can people affected by leprosy at risk of developing plantar ulcers be identified? A field study from central Ethiopia. Lepr Rev *72*, 151-157.

Feldman, S. R., Fleischer, A. B., Jr., Reboussin, D. M., Rapp, S. R., Exum, M. L., Clark, A. R., and Nurre, L. (1996). The self-administered psoriasis area and severity index is valid and reliable. J Invest Dermatol *106*, 183-186.

Fenhalls, G., Squires, G. R., Stevens-Muller, L., Bezuidenhout, J., Amphlett, G., Duncan, K., and Lukey, P. T. (2003). Associations between toll-like receptors and interleukin-4 in the lungs of patients with tuberculosis. Am J Respir Cell Mol Biol *29*, 28-38.

Ferwerda, B., Kibiki, G. S., Netea, M. G., Dolmans, W. M., and van der Ven, A. J. (2007). The toll-like receptor 4 Asp299Gly variant and tuberculosis susceptibility in HIV-infected patients in Tanzania. Aids *21*, 1375-1377.

Ffytche, T. J. (1998). The prevalence of disabling ocular complications of leprosy: a global study. Indian J Lepr *70*, 49-59.

Filippini, G., Brusaferri, F., Sibley, W. A., Citterio, A., Ciucci, G., Midgard, R., and Candelise, L. (2000). Corticosteroids or ACTH for acute exacerbations in multiple sclerosis. Cochrane Database Syst Rev, CD001331.

Fine, P. E. (2008). Leprosy's global statistics--still room for improvement. Lepr Rev *79*, 235-238.

Fine, P. E., Sterne, J. A., Ponnighaus, J. M., Bliss, L., Saui, J., Chihana, A., Munthali, M., and Warndorff, D. K. (1997). Household and dwelling contact as risk factors for leprosy in northern Malawi. Am J Epidemiol *146*, 91-102.

Fitness, J., Floyd, S., Warndorff, D. K., Sichali, L., Mwaungulu, L., Crampin, A. C., Fine, P. E., and Hill, A. V. (2004). Large-scale candidate gene study of leprosy susceptibility in the Karonga district of northern Malawi. Am J Trop Med Hyg *71*, 330-340.

Fitness, J., Tosh, K., and Hill, A. V. (2002). Genetics of susceptibility to leprosy. Genes Immun *3*, 441-453.

Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Visintin, A., Latz, E., Monks, B., Pitha, P. M., and Golenbock, D. T. (2003). LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med *198*, 1043-1055.

Fredriksson, T., and Pettersson, U. (1978). Severe psoriasis--oral therapy with a new retinoid. Dermatologica *157*, 238-244.

Galon, J., Franchimont, D., Hiroi, N., Frey, G., Boettner, A., Ehrhart-Bornstein, M., O'Shea, J. J., Chrousos, G. P., and Bornstein, S. R. (2002). Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. Faseb J *16*, 61-71.

Garbino, J. A., Virmond Mda, C., Ura, S., Salgado, M. H., and Naafs, B. (2008). A randomized clinical trial of oral steroids for ulnar neuropathy in type 1 and type 2 leprosy reactions. Arq Neuropsiquiatr *66*, 861-867.

Gay, N. J., and Gangloff, M. (2007). Structure and function of Toll receptors and their ligands. Annu Rev Biochem *76*, 141-165.

Gay, N. J., and Keith, F. J. (1991). Drosophila Toll and IL-1 receptor. Nature *351*, 355-356.

Girdhar, B. K., Girdhar, A., and Kumar, A. (2000). Relapses in multibacillary leprosy patients: effect of length of therapy. Lepr Rev *71*, 144-153.

Godel, V., Feiler-Ofry, V., and Stein, R. (1972). Systemic steroids and ocular fluid dynamics. I. Analysis of the sample as a whole. Influence of dosage and duration of therapy. Acta Ophthalmol (Copenh) *50*, 655-663.

Grad, I., and Picard, D. (2007). The glucocorticoid responses are shaped by molecular chaperones. Mol Cell Endocrinol *275*, 2-12.

Green, J. A., Tran, C. T., Farrar, J. J., Nguyen, M. T., Nguyen, P. H., Dinh, S. X., Ho, N. D., Ly, C. V., Tran, H. T., Friedland, J. S., and Thwaites, G. E. (2009). Dexamethasone, cerebrospinal fluid matrix metalloproteinase concentrations and clinical outcomes in tuberculous meningitis. PLoS One *4*, e7277.

Gummer, C. L., Starley, J. N., Dawber, R. P., and Pearson, J. M. (1983). The distribution of Mycobacterium leprae in the hair follicle of the eyebrow. Int J Lepr Other Mycobact Dis *51*, 205-210.

Gupta, S. K., Nigam, S., Mandal, A. K., and Kumar, V. (2006). S-100 as a useful auxiliary diagnostic aid in tuberculoid leprosy. J Cutan Pathol *33*, 482-486.

Gupte, M. D., Vallishayee, R. S., Nagaraju, B., Ramalingam, A., Lourdusamy, G., and Kannan, S. (1990). Inter-observer agreement and clinical diagnosis of leprosy for prophylaxis studies. Indian J Lepr *62*, 281-295.

Gurwitz, J. H., Bohn, R. L., Glynn, R. J., Monane, M., Mogun, H., and Avorn, J. (1994). Glucocorticoids and the risk for initiation of hypoglycemic therapy. Arch Intern Med *154*, 97-101.

Hansen, G. H. A. (1874). Undersogelser angaende spedalskhedens aasager. Norsk Magazin for Laegervidenskaben *4 (Suppl.)*, 1-88.

Hao, H. N., Peduzzi-Nelson, J. D., VandeVord, P. J., Barami, K., DeSilva, S. P., Pelinkovic, D., and Morawa, L. G. (2009). Lipopolysaccharide-induced inflammatory cytokine production by Schwann's cells dependent upon TLR4 expression. J Neuroimmunol *212*, 26-34.

Hara, Y., Shiraishi, A., Kobayashi, T., Kadota, Y., Shirakata, Y., Hashimoto, K., and Ohashi, Y. (2009). Alteration of TLR3 pathways by glucocorticoids may be responsible for immunosusceptibility of human corneal epithelial cells to viral infections. Mol Vis *15*, 937-948.

Harboe, M. (1994). Overview of host-parasite relations. In Leprosy, R. C. Hastings, ed. (Edinburgh, Churchill Livingstone), pp. 87-112.

Hashimoto, K., Maeda, Y., Kimura, H., Suzuki, K., Masuda, A., Matsuoka, M., and Makino, M. (2002). Mycobacterium leprae infection in monocyte-derived dendritic cells and its influence on antigen-presenting function. Infect Immun *70*, 5167-5176.

Haslett, P. A., Roche, P., Butlin, C. R., Macdonald, M., Shrestha, N., Manandhar, R., Lemaster, J., Hawksworth, R., Shah, M., Lubinsky, A. S., Albert, M., Worley, J., and Kaplan, G. (2005). Effective treatment of erythema nodosum leprosum with thalidomide is associated with immune stimulation. J Infect Dis *192*, 2045-2053.

Hatta, M., van Beers, S. M., Madjid, B., Djumadi, A., de Wit, M. Y., and Klatser, P. R. (1995). Distribution and persistence of Mycobacterium leprae nasal carriage among a population in which leprosy is endemic in Indonesia. Trans R Soc Trop Med Hyg *89*, 381-385.

Hawn, T. R., Dunstan, S. J., Thwaites, G. E., Simmons, C. P., Thuong, N. T., Lan, N. T., Quy, H. T., Chau, T. T., Hieu, N. T., Rodrigues, S., Janer, M., Zhao, L. P., Hien, T. T., Farrar, J. J., and Aderem, A. (2006). A polymorphism in Toll-interleukin 1 receptor domain containing adaptor protein is associated with susceptibility to meningeal tuberculosis. J Infect Dis *194*, 1127-1134.

Hietaharju, A., Croft, R., Alam, R., Birch, P., Mong, A., and Haanpaa, M. (2000). Chronic neuropathic pain in treated leprosy. Lancet *356*, 1080-1081.

Hirano, T., Tsuboi, N., Homma, M., Oka, K., Takekoshi, T., Tahara, K., Takanashi, H., Abe, H., Urata, Y., and Hayashi, T. (2000). Comparative study of lymphocyte-suppressive potency between prednisolone and methylprednisolone in rheumatoid arthritis. Immunopharmacology *49*, 411-417.

Hodge, S., Hodge, G., Flower, R., and Han, P. (1999). Methyl-prednisolone up-regulates monocyte interleukin-10 production in stimulated whole blood. Scand J Immunol *49*, 548-553.

Hogerzeil, L. M., and Rees, R. J. (1975). The effect of a single dose of rifampicin on the infectivity of the nasal discharge in leprosy (preliminary communication). Lepr Rev *46*, 147.

Hogeweg, M., and Keunen, J. E. (2005). Prevention of blindness in leprosy and the role of the Vision 2020 Programme. Eye *19*, 1099-1105.

Hogeweg, M., Kiran, K. U., and Suneetha, S. (1991). The significance of facial patches and type I reaction for the development of facial nerve damage in leprosy. A retrospective study among 1226 paucibacillary leprosy patients. Lepr Rev *62*, 143-149.

Homma, T., Kato, A., Hashimoto, N., Batchelor, J., Yoshikawa, M., Imai, S., Wakiguchi, H., Saito, H., and Matsumoto, K. (2004). Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells. Am J Respir Cell Mol Biol *31*, 463-469.

Hunger, R. E., Surovy, A. M., Hassan, A. S., Braathen, L. R., and Yawalkar, N. (2008). Toll-like receptor 2 is highly expressed in lesions of acne inversa and colocalizes with C-type lectin receptor. Br J Dermatol *158*, 691-697.

Hussain, R., Lucas, S. B., Kifayet, A., Jamil, S., Raynes, J., Uqaili, Z., Dockrell, H. M., Chiang, T. J., and McAdam, K. P. (1995). Clinical and histological discrepancies in diagnosis of ENL reactions classified by assessment of acute phase proteins SAA and CRP. Int J Lepr Other Mycobact Dis *63*, 222-230.

Ishii, K. J., Suzuki, K., Coban, C., Takeshita, F., Itoh, Y., Matoba, H., Kohn, L. D., and Klinman, D. M. (2001). Genomic DNA released by dying cells induces the maturation of APCs. J Immunol *167*, 2602-2607.

Ishikawa, A., Ishikawa, S., and Hirakawa, M. (2001). Osteoporosis, bone turnover and hypogonadism in elderly men with treated leprosy. Lepr Rev *72*, 322-329.

Ishikawa, S., Ishikawa, A., Yoh, K., Tanaka, H., and Fujiwara, M. (1999). Osteoporosis in male and female leprosy patients. Calcif Tissue Int *64*, 144-147.

Ito, K., Barnes, P. J., and Adcock, I. M. (2000). Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. Mol Cell Biol *20*, 6891-6903.

Ito, T., Schaller, M., Hogaboam, C. M., Standiford, T. J., Chensue, S. W., and Kunkel, S. L. (2007). TLR9 activation is a key event for the maintenance of a mycobacterial antigen-elicited pulmonary granulomatous response. Eur J Immunol *37*, 2847-2855.

Iyer, C. G., and Ramu, G. (1976). An open trial with clofazimine in the management of recurrent lepra reaction using thalidomide as a control drug. Lepr India *48*, 690-694.

Jain, M. C. (2008). Leprosy scenario in Nepal. JNMA J Nepal Med Assoc *47*, 259-263.

Jarrousse, V., Castex-Rizzi, N., Khammari, A., Charveron, M., and Dreno, B. (2007). Zinc salts inhibit in vitro Toll-like receptor 2 surface expression by keratinocytes. Eur J Dermatol *17*, 492-496.

Jarrousse, V., Quereux, G., Marques-Briand, S., Knol, A. C., Khammari, A., and Dreno, B. (2006). Toll-like receptors 2, 4 and 9 expression in cutaneous T-cell lymphoma (mycosis fungoides and Sezary syndrome). Eur J Dermatol *16*, 636-641.

Job, C. K. (1994). Pathology of leprosy. In Leprosy, R. C. Hastings, ed. (Edinburgh, Churchill Livingstone), pp. 193-234.

Job, C. K., Jayakumar, J., Kearney, M., and Gillis, T. P. (2008). Transmission of leprosy: a study of skin and nasal secretions of household contacts of leprosy patients using PCR. Am J Trop Med Hyg *78*, 518-521.

Johnson, C. M., Lyle, E. A., Omueti, K. O., Stepensky, V. A., Yegin, O., Alpsoy, E., Hamann, L., Schumann, R. R., and Tapping, R. I. (2007). Cutting edge: A common polymorphism impairs cell surface trafficking and functional responses of TLR1 but protects against leprosy. J Immunol *178*, 7520-7524.

Jones, I. W., and Westmacott, A. (2007). Multiple immunochemical staining. In Immunohistochemistry, S. Renshaw, ed. (Bloxham, Scion Publishing Limited).

Jopling, W. H. (1976). Complications of treatment with clofazimine (Lamprene: B663). Lepr Rev *47*, 1-3.

Jugeau, S., Tenaud, I., Knol, A. C., Jarrousse, V., Quereux, G., Khammari, A., and Dreno, B. (2005). Induction of toll-like receptors by Propionibacterium acnes. Br J Dermatol *153*, 1105-1113.

Kahawita, I. P., and Lockwood, D. N. (2008). Towards understanding the pathology of erythema nodosum leprosum. Trans R Soc Trop Med Hyg *102*, 329-337.

Kanzler, H., Barrat, F. J., Hessel, E. M., and Coffman, R. L. (2007). Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med *13*, 552-559.

Kaplan, G., Mathur, N. K., Job, C. K., Nath, I., and Cohn, Z. A. (1989). Effect of multiple interferon gamma injections on the disposal of Mycobacterium leprae. Proc Natl Acad Sci U S A *86*, 8073-8077.

Karanth, S., Yang, G., Yeh, J., and Richardson, P. M. (2006). Nature of signals that initiate the immune response during Wallerian degeneration of peripheral nerves. Exp Neurol *202*, 161-166.

Kaur, C., Thami, G. P., and Mohan, H. (2005). Lucio phenomenon and Lucio leprosy. Clin Exp Dermatol *30*, 525-527.

Kawuma, H. J., Bwire, R., and Adatu-Engwau, F. (1994). Leprosy and infection with the human immunodeficiency virus in Uganda; a case-control study. Int J Lepr Other Mycobact Dis *62*, 521-526.

Kets, C. M., Van Leerdam, M. E., Van Brakel, W. H., Deville, W., and Bertelsmann, F. W. (1996). Reference values for touch sensibility thresholds in healthy Nepalese volunteers. Lepr Rev *67*, 28-38.

Khambati, F. A., Shetty, V. P., Ghate, S. D., and Capadia, G. D. (2009). Sensitivity and specificity of nerve palpation, monofilament testing and voluntary muscle testing in detecting peripheral nerve abnormality, using nerve conduction studies as gold standard; a study in 357 patients. Lepr Rev *80*, 34-50.

Khambati, F. A., Shetty, V. P., Ghate, S. D., Capadia, G. D., Pai, V. V., and Ganapati, R. (2008). The effect of corticosteroid usage on the bacterial killing, clearance and nerve damage in leprosy: a prospective cohort study: part 1--study design and baseline findings of 400 untreated multibacillary patients. Lepr Rev *79*, 134-153.

Khanolkar-Young, S., Rayment, N., Brickell, P. M., Katz, D. R., Vinayakumar, S., Colston, M. J., and Lockwood, D. N. (1995). Tumour necrosis factor-alpha (TNF-alpha) synthesis is associated with the skin and peripheral nerve pathology of leprosy reversal reactions. Clin Exp Immunol *99*, 196-202.

Khor, C. C., Chapman, S. J., Vannberg, F. O., Dunne, A., Murphy, C., Ling, E. Y., Frodsham, A. J., Walley, A. J., Kyrieleis, O., Khan, A., Aucan, C., Segal, S., Moore, C. E., Knox, K., Campbell, S. J., Lienhardt, C., Scott, A., Aaby, P., Sow, O. Y., Grignani, R. T., Sillah, J., Sirugo, G., Peshu, N., Williams, T. N., Maitland, K., Davies, R. J., Kwiatkowski, D. P., Day, N. P., Yala, D., Crook, D. W., Marsh, K., Berkley, J. A., O'Neill, L. A., and Hill, A. V. (2007). A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. Nat Genet *39*, 523-528.

Kiekens, R. C., Thepen, T., Oosting, A. J., Bihari, I. C., Van De Winkel, J. G., Bruijnzeel-Koomen, C. A., and Knol, E. F. (2001). Heterogeneity within tissue-specific macrophage and dendritic cell populations during cutaneous inflammation in atopic dermatitis. Br J Dermatol *145*, 957-965.

Kim, D., Kim, M. A., Cho, I. H., Kim, M. S., Lee, S., Jo, E. K., Choi, S. Y., Park, K., Kim, J. S., Akira, S., Na, H. S., Oh, S. B., and Lee, S. J. (2007). A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. J Biol Chem *282*, 14975-14983.

Kim, D., Lee, S., and Lee, S. J. (2009). Toll-like receptors in peripheral nerve injury and neuropathic pa. Curr Top Microbiol Immunol *336*, 169-186.

Kim, J., Ochoa, M. T., Krutzik, S. R., Takeuchi, O., Uematsu, S., Legaspi, A. J., Brightbill, H. D., Holland, D., Cunliffe, W. J., Akira, S., Sieling, P. A., Godowski, P. J., and Modlin, R. L. (2002). Activation of toll-like receptor 2 in acne triggers inflammatory cytokine responses. J Immunol *169*, 1535-1541.

Kim, M. H., Choi, Y. W., Choi, H. Y., Myung, K. B., and Cho, S. N. (2006). The expression of RAGE and EN-RAGE in leprosy. Br J Dermatol *154*, 594-601.

Kiran, K. U., Hogeweg, M., and Suneetha, S. (1991). Treatment of recent facial nerve damage with lagophthalmos, using a semistandardized steroid regimen. Lepr Rev *62*, 150-154.

Kiran, K. U., Stanley, J. N., and Pearson, J. M. (1985). The outpatient treatment of nerve damage in patients with borderline leprosy using a semi-standardized steroid regimen. Lepr Rev *56*, 127-134.

Kirkaldy, A. A., Musonda, A. C., Khanolkhar-Young, S., Suneetha, S., and Lockwood, D. N. (2003). Expression of CC and CXC chemokines and chemokine receptors in human leprosy skin lesions. Clin Exp Immunol *134*, 447-453.

Kis, K., Bodai, L., Polyanka, H., Eder, K., Pivarcsi, A., Duda, E., Soos, G., Bata-Csorgo, Z., and Kemeny, L. (2006). Budesonide, but not tacrolimus, affects the immune functions of normal human keratinocytes. Int Immunopharmacol *6*, 358-368.

Koelewijn, L. F., Meima, A., Broekhuis, S. M., Richardus, J. H., Mitchell, P. D., Benbow, C., and Saunderson, P. R. (2003). Sensory testing in leprosy: comparison of ballpoint pen and monofilaments. Lepr Rev *74*, 42-52.

Kollisch, G., Kalali, B. N., Voelcker, V., Wallich, R., Behrendt, H., Ring, J., Bauer, S., Jakob, T., Mempel, M., and Ollert, M. (2005). Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. Immunology *114*, 531-541.

Kontoyiannis, D., Kotlyarov, A., Carballo, E., Alexopoulou, L., Blackshear, P. J., Gaestel, M., Davis, R., Flavell, R., and Kollias, G. (2001). Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. Embo J *20*, 3760-3770.

Krutzik, S. R., Ochoa, M. T., Sieling, P. A., Uematsu, S., Ng, Y. W., Legaspi, A., Liu, P. T., Cole, S. T., Godowski, P. J., Maeda, Y., Sarno, E. N., Norgard, M. V., Brennan, P. J., Akira, S., Rea, T. H., and Modlin, R. L. (2003). Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. Nat Med *9*, 525-532.

Krutzik, S. R., Tan, B., Li, H., Ochoa, M. T., Liu, P. T., Sharfstein, S. E., Graeber, T. G., Sieling, P. A., Liu, Y. J., Rea, T. H., Bloom, B. R., and Modlin, R. L. (2005). TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells. Nat Med *11*, 653-660.

Ku, J. K., Kwon, H. J., Kim, M. Y., Kang, H., Song, P. I., Armstrong, C. A., Ansel, J. C., Kim, H. O., and Park, Y. M. (2008). Expression of Toll-like receptors in verruca and molluscum contagiosum. J Korean Med Sci *23*, 307-314.

Kumar, B., Dogra, S., and Kaur, I. (2004). Epidemiological characteristics of leprosy reactions: 15 years experience from north India. Int J Lepr Other Mycobact Dis *72*, 125-133.

Landis, J. R., and Koch, G. G. (1977). The measurement of observer agreement for categorical data. Biometrics *33*, 159-174.

Lawn, S. D., Wood, C., and Lockwood, D. N. (2003). Borderline tuberculoid leprosy: an immune reconstitution phenomenon in a human immunodeficiency virus-infected person. Clin Infect Dis *36*, e5-6.

Le Grand, A. (1997). Women and leprosy: a review. Lepr Rev *68*, 203-211.

Leang, B., Lynen, L., Schrooten, W., and Hines, J. (2005). Comparison of albendazole regimen for prophylaxis of strongyloides hyperinfection in nephrotic syndrome patients on long-term steroids in Cambodia. Trop Doct *35*, 212-213.

Leang, B., Lynen, L., Tootill, R., Griffiths, S., and Monchy, D. (2004). Death caused by strongyloides hyperinfection in a leprosy patient on treatment for a type II leprosy reaction. Lepr Rev *75*, 398-403.

Lee, H., Jo, E. K., Choi, S. Y., Oh, S. B., Park, K., Kim, J. S., and Lee, S. J. (2006). Necrotic neuronal cells induce inflammatory Schwann cell activation via TLR2 and TLR3: implication in Wallerian degeneration. Biochem Biophys Res Commun *350*, 742-747.

Lee, H. M., Shin, D. M., Choi, D. K., Lee, Z. W., Kim, K. H., Yuk, J. M., Kim, C. D., Lee, J. H., and Jo, E. K. (2009a). Innate immune responses to Mycobacterium ulcerans via toll-like receptors and dectin-1 in human keratinocytes. Cell Microbiol *11*, 678-692.

Lee, W. I., Liang, F. C., Huang, J. L., Jaing, T. H., Wang, C. H., Lin, T. Y., Huang, Y. C., Huang, W. L., Jou, R., Hsieh, M. Y., Chia, J. H., and Wu, T. L. (2009b). Immunologic analysis of HIV-uninfected Taiwanese children with BCG-induced disease. J Clin Immunol *29*, 319-329.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973-983.

Levy, L. (1976). Studies of the mouse foot pad technique for cultivation of Mycobacterium leprae. 3. Doubling time during logarithmic multiplication. Lepr Rev *47*, 103-106.

Li, J., Chen, J., Tan, Z., Liu, H., and Liu, Z. (2007). Expression of TLR9 and its mRNA in the lesions of lichen planus. J Huazhong Univ Sci Technolog Med Sci *27*, 203-205.

Libraty, D. H., Airan, L. E., Uyemura, K., Jullien, D., Spellberg, B., Rea, T. H., and Modlin, R. L. (1997). Interferon-gamma differentially regulates interleukin-12 and interleukin-10 production in leprosy. J Clin Invest *99*, 336-341.

Little, D., Khanolkar-Young, S., Coulthart, A., Suneetha, S., and Lockwood, D. N. (2001). Immunohistochemical analysis of cellular infiltrate and gamma interferon, interleukin-12, and inducible nitric oxide synthase expression in leprosy type 1 (reversal) reactions before and during prednisolone treatment. Infect Immun *69*, 3413-3417.

Liu, J. H., Shi, Y. F., Kong, Q. Y., Yang, L. H., Li, W. Z., and Ye, G. Y. (1984). Observations of Langerhans' cells in leprosy using monoclonal antibody OKT6. Int J Lepr Other Mycobact Dis *52*, 524-526.

Liu, P. T., Stenger, S., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., Ochoa, M. T., Schauber, J., Wu, K., Meinken, C., Kamen, D. L., Wagner, M., Bals, R., Steinmeyer, A., Zugel, U., Gallo, R. L., Eisenberg, D., Hewison, M., Hollis, B. W., Adams, J. S., Bloom, B. R., and Modlin, R. L. (2006). Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science *311*, 1770-1773.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Lockwood, D. N., Colston, M. J., and Khanolkar-Young, S. R. (2002). The detection of Mycobacterium leprae protein and carbohydrate antigens in skin and nerve from leprosy patients with type 1 (reversal) reactions. Am J Trop Med Hyg *66*, 409-415.

Lockwood, D. N., Lucas, S. B., Desikan, K., Ebenezer, G., Suneetha, S., and Nicholls, P. (2008). The histological diagnosis of leprosy Type 1 reactions: identification of key variables and an analysis of the process of histological diagnosis. J Clin Pathol.

Lockwood, D. N., and Reid, A. J. (2001). The diagnosis of leprosy is delayed in the United Kingdom. QJM *94*, 207-212.

Lockwood, D. N., Sarno, E., and Smith, W. C. (2007). Classifying leprosy patients--searching for the perfect solution? Lepr Rev *78*, 317-320.

Lockwood, D. N., and Sinha, H. H. (1999). Pregnancy and leprosy: a comprehensive literature review. Int J Lepr Other Mycobact Dis *67*, 6-12.

Lockwood, D. N., Vinayakumar, S., Stanley, J. N., McAdam, K. P., and Colston, M. J. (1993). Clinical features and outcome of reversal (type 1) reactions in Hyderabad, India. Int J Lepr Other Mycobact Dis *61*, 8-15.

Lockwood, D. N. J. (2004). Leprosy. In Rook's Textbook of Dermatology D. A. Burns, Breathnach, S.M., Cox, N.H., Griffiths, C.E.M. , ed. (Oxford, Blackwell Publishing), pp. 29.21-29.21.

Lowe, J., and Smith, M. (1949). The chemotherapy of leprosy in Nigeria; with an appendix on glandular fever and exfoliative dermatitis precipitated by sulfones. Int J Lepr *17*, 181-195.

Mahaisavariya, P., Kulthanan, K., Khemngern, S., and Pinkaew, S. (1999). Lesional T-cell subset in leprosy and leprosy reaction. Int J Dermatol *38*, 345-347.

Malhotra, D., Darvishi, K., Lohra, M., Kumar, H., Grover, C., Sood, S., Reddy, B. S., and Bamezai, R. N. (2006). Association study of major risk single nucleotide polymorphisms in the common regulatory region of PARK2 and PACRG genes with leprosy in an Indian population. Eur J Hum Genet *14*, 438-442.

Malhotra, D., Darvishi, K., Sood, S., Sharma, S., Grover, C., Relhan, V., Reddy, B. S., and Bamezai, R. N. (2005). IL-10 promoter single nucleotide polymorphisms are significantly associated with resistance to leprosy. Hum Genet, 1-6.

Manandhar, R., LeMaster, J. W., and Roche, P. W. (1999). Risk factors for erythema nodosum leprosum. Int J Lepr Other Mycobact Dis *67*, 270-278.

Marlowe, S. N., Hawksworth, R. A., Butlin, C. R., Nicholls, P. G., and Lockwood, D. N. (2004). Clinical outcomes in a randomized controlled study comparing azathioprine and

prednisolone versus prednisolone alone in the treatment of severe leprosy type 1 reactions in Nepal. Trans R Soc Trop Med Hyg *98*, 602-609.

Marlowe, S. N., Leekassa, R., Bizuneh, E., Knuutilla, J., Ale, P., Bhattarai, B., Sigdel, H., Anderson, A., Nicholls, P. G., Johnston, A., Holt, D., and Lockwood, D. N. (2007). Response to ciclosporin treatment in Ethiopian and Nepali patients with severe leprosy Type 1 reactions. Trans R Soc Trop Med Hyg *101*, 1004-1012.

Marshall, M., Lockwood, A., Bradley, C., Adams, C., Joy, C., and Fenton, M. (2000). Unpublished rating scales: a major source of bias in randomised controlled trials of treatments for schizophrenia. Br J Psychiatry *176*, 249-252.

Martens, U., and Klingmuller, G. (1984). Free subepidermal grenz zone (band of Unna) in lepromatous leprosy. Histological and ultrastructural findings. Int J Lepr Other Mycobact Dis *52*, 55-60.

Martinon, F., Petrilli, V., Mayor, A., Tardivel, A., and Tschopp, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature *440*, 237-241.

Matsushima, N., Tanaka, T., Enkhbayar, P., Mikami, T., Taga, M., Yamada, K., and Kuroki, Y. (2007). Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. BMC Genomics *8*, 124.

Matzinger, P. (1998). An innate sense of danger. Semin Immunol *10*, 399-415.

McArthur, J. H. (1998). The reliability and validity of the subjective peripheral neuropathy screen. J Assoc Nurses AIDS Care *9*, 84-94.

Mehra, V., Brennan, P. J., Rada, E., Convit, J., and Bloom, B. R. (1984). Lymphocyte suppression in leprosy induced by unique M. leprae glycolipid. Nature *308*, 194-196.

Melsom, R., Harboe, M., Myrvang, B., Godal, T., and Belehu, A. (1982). Immunoglobulin class specific antibodies to M. leprae in leprosy patients, including the indeterminate group and healthy contacts as a step in the development of methods for sero-diagnosis of leprosy. Clin Exp Immunol *47*, 225-233.

Menzies, B. E., and Kenoyer, A. (2006). Signal transduction and nuclear responses in Staphylococcus aureus-induced expression of human beta-defensin 3 in skin keratinocytes. Infect Immun *74*, 6847-6854.

Miller, R. A. (1991). Leprosy and AIDS: a review of the literature and speculations on the impact of CD4+ lymphocyte depletion on immunity to Mycobacterium leprae. Int J Lepr Other Mycobact Dis *59*, 639-644.

Mira, M. T., Alcais, A., Nguyen, V. T., Moraes, M. O., Di Flumeri, C., Vu, H. T., Mai, C. P., Nguyen, T. H., Nguyen, N. B., Pham, X. K., Sarno, E. N., Alter, A., Montpetit, A., Moraes, M. E., Moraes, J. R., Dore, C., Gallant, C. J., Lepage, P., Verner, A., Van De Vosse, E., Hudson, T. J., Abel, L., and Schurr, E. (2004). Susceptibility to leprosy is associated with PARK2 and PACRG. Nature *427*, 636-640.

Mira, M. T., Alcais, A., Van Thuc, N., Thai, V. H., Huong, N. T., Ba, N. N., Verner, A., Hudson, T. J., Abel, L., and Schurr, E. (2003). Chromosome 6q25 is linked to susceptibility to leprosy in a Vietnamese population. Nat Genet *33*, 412-415.

Misch, E. A., Macdonald, M., Ranjit, C., Sapkota, B. R., Wells, R. D., Siddiqui, M. R., Kaplan, G., and Hawn, T. R. (2008). Human TLR1 Deficiency Is Associated with Impaired Mycobacterial Signaling and Protection from Leprosy Reversal Reaction. PLoS Negl Trop Dis *2*, e231.

Modlin, R. L. (1994). Th1-Th2 paradigm: insights from leprosy. J Invest Dermatol *102*, 828-832.

Modlin, R. L., Hofman, F. M., Horwitz, D. A., Husmann, L. A., Gillis, S., Taylor, C. R., and Rea, T. H. (1984). In situ identification of cells in human leprosy granulomas with monoclonal antibodies to interleukin 2 and its receptor. J Immunol *132*, 3085-3090.

Modlin, R. L., Melancon-Kaplan, J., Young, S. M., Pirmez, C., Kino, H., Convit, J., Rea, T. H., and Bloom, B. R. (1988). Learning from lesions: patterns of tissue inflammation in leprosy. Proc Natl Acad Sci U S A *85*, 1213-1217.

Moher, D., Schulz, K. F., and Altman, D. G. (2001). The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomised trials. Lancet *357*, 1191-1194.

Monk, B., Cunliffe, W. J., Layton, A. M., and Rhodes, D. J. (1993). Acne induced by inhaled corticosteroids. Clin Exp Dermatol *18*, 148-150.

Moraes, M. O., Pacheco, A. G., Schonkeren, J. J., Vanderborght, P. R., Nery, J. A., Santos, A. R., Moraes, M. E., Moraes, J. R., Ottenhoff, T. H., Sampaio, E. P., Huizinga, T. W., and Sarno, E. N. (2004). Interleukin-10 promoter single-nucleotide polymorphisms as markers for disease susceptibility and disease severity in leprosy. Genes Immun *5*, 592-595.

Moraes, M. O., Sarno, E. N., Almeida, A. S., Saraiva, B. C., Nery, J. A., Martins, R. C., and Sampaio, E. P. (1999). Cytokine mRNA expression in leprosy: a possible role for interferon-gamma and interleukin-12 in reactions (RR and ENL). Scand J Immunol *50*, 541-549.

Moreira, A. L., Kaplan, G., Villahermosa, L. G., Fajardo, T. J., Abalos, R. M., Cellona, R. V., Balagon, M. V., Tan, E. V., and Walsh, G. P. (1998). Comparison of pentoxifylline, thalidomide and prednisone in the treatment of ENL. Int J Lepr Other Mycobact Dis *66*, 61-65.

Moschella, S. L. (1968). Primary diffuse lepromatous leprosy with erythema necrotisans (lucio phenomenon). Arch Dermatol *97*, 593-594.

Murphy, P. J., Morishima, Y., Kovacs, J. J., Yao, T. P., and Pratt, W. B. (2005). Regulation of the dynamics of hsp90 action on the glucocorticoid receptor by acetylation/deacetylation of the chaperone. J Biol Chem *280*, 33792-33799.

Naafs, B. (2003). Treatment duration of reversal reaction: a reappraisal. Back to the past. Lepr Rev *74*, 328-336.

Naafs, B., and Dagne, T. (1977). Sensory testing: a sensitive method in the follow-up of nerve involvement. Int J Lepr Other Mycobact Dis *45*, 364-368.

Naafs, B., Pearson, J. M., and Wheate, H. W. (1979). Reversal reaction: the prevention of permanent nerve damage. Comparison of short and long-term steroid treatment. Int J Lepr Other Mycobact Dis *47*, 7-12.

Narayanan, R. B., Laal, S., Sharma, A. K., Bhutani, L. K., and Nath, I. (1984). Differences in predominant T cell phenotypes and distribution pattern in reactional lesions of tuberculoid and lepromatous leprosy. Clin Exp Immunol *55*, 623-628.

Nejentsev, S., Thye, T., Szeszko, J. S., Stevens, H., Balabanova, Y., Chinbuah, A. M., Hibberd, M., van de Vosse, E., Alisjahbana, B., van Crevel, R., Ottenhoff, T. H., Png, E., Drobniewski, F., Todd, J. A., Seielstad, M., and Horstmann, R. D. (2008). Analysis of association of the TIRAP (MAL) S180L variant and tuberculosis in three populations. Nat Genet *40*, 261-262; author reply 262-263.

Nery, J. A., Vieira, L. M., de Matos, H. J., Gallo, M. E., and Sarno, E. N. (1998). Reactional states in multibacillary Hansen disease patients during multidrug therapy. Rev Inst Med Trop Sao Paulo *40*, 363-370.

Newton, R., Seybold, J., Kuitert, L. M., Bergmann, M., and Barnes, P. J. (1998). Repression of cyclooxygenase-2 and prostaglandin E2 release by dexamethasone occurs by transcriptional and post-transcriptional mechanisms involving loss of polyadenylated mRNA. J Biol Chem *273*, 32312-32321.

Nishimura, M., Koeda, A., Suzuki, E., Shimizu, T., Kawano, Y., Nakayama, M., Satoh, T., Narimatsu, S., and Naito, S. (2006). Effects of prototypical drug-metabolizing enzyme inducers on mRNA expression of housekeeping genes in primary cultures of human and rat hepatocytes. Biochem Biophys Res Commun *346*, 1033-1039.

Noordeen, S. K. (1994). The epidemiology of leprosy. In Leprosy, R. C. Hastings, ed. (Edinburgh, Churchill Livingstone), pp. 29-45.

Ochoa, M. T., Loncaric, A., Krutzik, S. R., Becker, T. C., and Modlin, R. L. (2008). "Dermal dendritic cells" comprise two distinct populations: CD1+ dendritic cells and CD209+ macrophages. J Invest Dermatol *128*, 2225-2231.

Ochoa, M. T., Stenger, S., Sieling, P. A., Thoma-Uszynski, S., Sabet, S., Cho, S., Krensky, A. M., Rollinghoff, M., Nunes Sarno, E., Burdick, A. E., Rea, T. H., and Modlin, R. L. (2001). T-cell release of granulysin contributes to host defense in leprosy. Nat Med *7*, 174-179.

Ogus, A. C., Yoldas, B., Ozdemir, T., Uguz, A., Olcen, S., Keser, I., Coskun, M., Cilli, A., and Yegin, O. (2004). The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. Eur Respir J *23*, 219-223.

Okun, E., Griffioen, K. J., Lathia, J. D., Tang, S. C., Mattson, M. P., and Arumugam, T. V. (2009). Toll-like receptors in neurodegeneration. Brain Res Rev *59*, 278-292.

Oliveira, R. B., Ochoa, M. T., Sieling, P. A., Rea, T. H., Rambukkana, A., Sarno, E. N., and Modlin, R. L. (2003). Expression of Toll-like receptor 2 on human Schwann cells: a mechanism of nerve damage in leprosy. Infect Immun *71*, 1427-1433.

Orihara, K., Nagata, K., Hamasaki, S., Oba, R., Hirai, H., Ishida, S., Kataoka, T., Oketani, N., Ogawa, M., Mizoguchi, E., Ichiki, H., and Tei, C. (2007). Time-course of Toll-like receptor 2 expression, as a predictor of recurrence in patients with bacterial infectious diseases. Clin Exp Immunol *148*, 260-270.

Oskam, L., Slim, E., and Buhrer-Sekula, S. (2003). Serology: recent developments, strengths, limitations and prospects: a state of the art overview. Lepr Rev *74*, 196-205.

Oswald-Richter, K. A., Culver, D. A., Hawkins, C., Hajizadeh, R., Abraham, S., Shepherd, B. E., Jenkins, C. A., Judson, M. A., and Drake, W. P. (2009). Cellular responses to mycobacterial antigens are present in bronchoalveolar lavage fluid used in the diagnosis of sarcoidosis. Infect Immun *77*, 3740-3748.

Pandey, B., Shrestha, K., Lewis, J., Hawksworth, R. A., and Walker, S. L. (2007). Mortality due to dapsone hypersensitivity syndrome complicating multi-drug therapy for leprosy in Nepal. Trop Doct *37*, 162-163.

Pannikar, V. K., Arunthathi, S., Chacko, C. J., and Fritschi, E. P. (1983). A clinico-pathological study of primary neuritic leprosy. Lepr India *55*, 212-221.

Pannikar, V. K., Ramprasad, S., Reddy, N. R., Andrews, P., Ravi, K., and Fritschi, E. P. (1984). Effect of epicondylectomy in early ulnar neuritis treated with steroids. Int J Lepr Other Mycobact Dis *52*, 501-505.

Patki, A. H., and Baran, R. (1991). Significance of nail changes in leprosy: a clinical review of 357 cases. Semin Dermatol *10*, 77-81.

Peduzzi, E., Groeper, C., Schutte, D., Zajac, P., Rondini, S., Mensah-Quainoo, E., Spagnoli, G. C., Pluschke, G., and Daubenberger, C. A. (2007). Local activation of the innate immune system in Buruli ulcer lesions. J Invest Dermatol *127*, 638-645.

Peiser, M., Koeck, J., Kirschning, C. J., Wittig, B., and Wanner, R. (2008). Human Langerhans cells selectively activated via Toll-like receptor 2 agonists acquire migratory and CD4+T cell stimulatory capacity. J Leukoc Biol *83*, 1118-1127.

Penna, M. L., and Penna, G. O. (2007). Trend of case detection and leprosy elimination in Brazil. Trop Med Int Health *12*, 647-650.

Pereira, G. A., Stefani, M. M., Araujo Filho, J. A., Souza, L. C., Stefani, G. P., and Martelli, C. M. (2004). Human immunodeficiency virus type 1 (HIV-1) and Mycobacterium leprae co-infection: HIV-1 subtypes and clinical, immunologic, and histopathologic profiles in a Brazilian cohort. Am J Trop Med Hyg *71*, 679-684.

Perretti, M., and D'Acquisto, F. (2006). Novel aspects of annexin 1 and glucocorticoid biology: intersection with nitric oxide and the lipoxin receptor. Inflamm Allergy Drug Targets *5*, 107-114.

Pfalzgraff, R. E., and Ramu, G. (1994). Clinical leprosy. In Leprosy, R. C. Hastings, ed. (Edinburgh, Churchill Livingstone), pp. 237-290.

Pivarcsi, A., Bodai, L., Rethi, B., Kenderessy-Szabo, A., Koreck, A., Szell, M., Beer, Z., Bata-Csorgoo, Z., Magocsi, M., Rajnavolgyi, E., Dobozy, A., and Kemeny, L. (2003). Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. Int Immunol *15*, 721-730.

Pocaterra, L., Jain, S., Reddy, R., Muzaffarullah, S., Torres, O., Suneetha, S., and Lockwood, D. N. (2006). Clinical course of erythema nodosum leprosum: an 11-year cohort study in Hyderabad, India. Am J Trop Med Hyg *74*, 868-879.

Ponnighaus, J. M., and Boerrigter, G. (1995). Are 18 doses of WHO/MDT sufficient for multibacillary leprosy; results of a trial in Malawi. Int J Lepr Other Mycobact Dis *63*, 1-7.

Ponnighaus, J. M., and Fine, P. E. (1988). Leprosy in Malawi. 1. Sensitivity and specificity of the diagnosis and the search for risk factors for leprosy. Trans R Soc Trop Med Hyg *82*, 803-809.

Prabha, C., Rajashree, P., and Sulochana, D. D. (2008). TLR2 and TLR4 expression on the immune cells of tuberculous pleural fluid. Immunol Lett *117*, 26-34.

Prabhakaran, K., Harris, E. B., and Randhawa, B. (2000). Regulation by protein kinase of phagocytosis of Mycobacterium leprae by macrophages. J Med Microbiol *49*, 339-342.

Pratt, W. B., Galigniana, M. D., Harrell, J. M., and DeFranco, D. B. (2004). Role of hsp90 and the hsp90-binding immunophilins in signalling protein movement. Cell Signal *16*, 857-872.

Rajalingam, R., Singal, D. P., and Mehra, N. K. (1997). Transporter associated with antigen-processing (TAP) genes and susceptibility to tuberculoid leprosy and pulmonary tuberculosis. Tissue Antigens *49*, 168-172.

Rambukkana, A., Das, P. K., Krieg, S., and Faber, W. R. (1992). Association of the mycobacterial 30-kDa region proteins with the cutaneous infiltrates of leprosy lesions. Evidence for the involvement of the major mycobacterial secreted proteins in the local immune response of leprosy. Scand J Immunol *36*, 35-48.

Rani, R., Fernandez-Vina, M. A., Zaheer, S. A., Beena, K. R., and Stastny, P. (1993). Study of HLA class II alleles by PCR oligotyping in leprosy patients from north India. Tissue Antigens *42*, 133-137.

Ranque, B., Nguyen, V. T., Vu, H. T., Nguyen, T. H., Nguyen, N. B., Pham, X. K., Schurr, E., Abel, L., and Alcais, A. (2007). Age is an important risk factor for onset and sequelae of reversal reactions in Vietnamese patients with leprosy. Clin Infect Dis *44*, 33-40.

Rao, P. S., Sugamaran, D. S., Richard, J., and Smith, W. C. (2006). Multi-centre, double blind, randomized trial of three steroid regimens in the treatment of type-1 reactions in leprosy. Lepr Rev *77*, 25-33.

Rastogi, N., and Rastogi, R. C. (1984). Leprosy in ancient India. Int J Lepr Other Mycobact Dis *52*, 541-543.

Rea, T. H., and Jerskey, R. S. (2005). Clinical and histologic variations among thirty patients with Lucio's phenomenon and pure and primitive diffuse lepromatosis (Latapi's lepromatosis). Int J Lepr Other Mycobact Dis *73*, 169-188.

Rea, T. H., and Ridley, D. S. (1979). Lucio's phenomenon: a comparative histological study. Int J Lepr Other Mycobact Dis *47*, 161-166.

Rees, R. J., and McDougall, A. C. (1977). Airborne infection with Mycobacterium leprae in mice. J Med Microbiol *10*, 63-68.

Renn, C. N., Sanchez, D. J., Ochoa, M. T., Legaspi, A. J., Oh, C. K., Liu, P. T., Krutzik, S. R., Sieling, P. A., Cheng, G., and Modlin, R. L. (2006). TLR activation of Langerhans cell-like dendritic cells triggers an antiviral immune response. J Immunol *177*, 298-305.

Richardus, J. H., Finlay, K. M., Croft, R. P., and Smith, W. C. (1996). Nerve function impairment in leprosy at diagnosis and at completion of MDT: a retrospective cohort study of 786 patients in Bangladesh. Lepr Rev *67*, 297-305.

Richardus, J. H., Nicholls, P. G., Croft, R. P., Withington, S. G., and Smith, W. C. (2004). Incidence of acute nerve function impairment and reactions in leprosy: a prospective cohort analysis after 5 years of follow-up. Int J Epidemiol *33*, 337-343.

Richardus, J. H., Withington, S. G., Anderson, A. M., Croft, R. P., Nicholls, P. G., Van Brakel, W. H., and Smith, W. C. (2003a). Adverse events of standardized regimens of corticosteroids for prophylaxis and treatment of nerve function impairment in leprosy: results from the 'TRIPOD' trials. Lepr Rev *74*, 319-327.

Richardus, J. H., Withington, S. G., Anderson, A. M., Croft, R. P., Nicholls, P. G., Van Brakel, W. H., and Smith, W. C. (2003b). Treatment with corticosteroids of long-standing nerve function impairment in leprosy: a randomized controlled trial (TRIPOD 3). Lepr Rev *74*, 311-318.

Ridley, D. S., and Jopling, W. H. (1966). Classification of Leprosy according to immunity. Int J Lepr Other Mycobact Dis
*34*, 255-273.

Rijal, A., Agrawal, S., Agarwalla, A., and Lakhey, M. (2004). Bullous erythema nodosum leprosum: a case report from Nepal. Lepr Rev *75*, 177-180.

Roberts, A. E., Nicholls, P. G., Maddali, P., and Van Brakel, W. H. (2007). Ensuring inter-tester reliability of voluntary muscle and monofilament sensory testing in the INFIR Cohort Study. Lepr Rev *78*, 122-130.

Rocha-Ramirez, L. M., Estrada-Garcia, I., Lopez-Marin, L. M., Segura-Salinas, E., Mendez-Aragon, P., Van Soolingen, D., Torres-Gonzalez, R., Chacon-Salinas, R., Estrada-Parra, S., Maldonado-Bernal, C., Lopez-Macias, C., and Isibasi, A. (2008). Mycobacterium tuberculosis lipids regulate cytokines, TLR-2/4 and MHC class II expression in human macrophages. Tuberculosis (Edinb) *88*, 212-220.

Roche, M., Convit, J., Medina, J. A., and Blomenfeld, E. (1951). The effects of adrenocorticotropic hormone (ACTH) in lepromatous lepra reaction. Int J Lepr *19*, 137-145.

Roche, P. W., Theuvenet, W. J., and Britton, W. J. (1991). Risk factors for type-1 reactions in borderline leprosy patients. Lancet *338*, 654-657.

Roura-Mir, C., Wang, L., Cheng, T. Y., Matsunaga, I., Dascher, C. C., Peng, S. L., Fenton, M. J., Kirschning, C., and Moody, D. B. (2005). Mycobacterium tuberculosis regulates CD1 antigen presentation pathways through TLR-2. J Immunol *175*, 1758-1766.

Roy, S., Frodsham, A., Saha, B., Hazra, S. K., Mascie-Taylor, C. G., and Hill, A. V. (1999). Association of vitamin D receptor genotype with leprosy type. J Infect Dis *179*, 187-191.

Roy, S., McGuire, W., Mascie-Taylor, C. G., Saha, B., Hazra, S. K., Hill, A. V., and Kwiatkowski, D. (1997). Tumor necrosis factor promoter polymorphism and susceptibility to lepromatous leprosy. J Infect Dis *176*, 530-532.

Rozkova, D., Horvath, R., Bartunkova, J., and Spisek, R. (2006). Glucocorticoids severely impair differentiation and antigen presenting function of dendritic cells despite upregulation of Toll-like receptors. Clin Immunol *120*, 260-271.

Rudofsky, G., Jr., Reismann, P., Witte, S., Humpert, P. M., Isermann, B., Chavakis, T., Tafel, J., Nosikov, V. V., Hamann, A., Nawroth, P., and Bierhaus, A. (2004). Asp299Gly

and Thr399Ile genotypes of the TLR4 gene are associated with a reduced prevalence of diabetic neuropathy in patients with type 2 diabetes. Diabetes Care *27*, 179-183.

Russell, D. G. (2007). Who puts the tubercle in tuberculosis? Nat Rev Microbiol *5*, 39-47.

Sakai, A., Han, J., Cato, A. C., Akira, S., and Li, J. D. (2004). Glucocorticoids synergize with IL-1beta to induce TLR2 expression via MAP Kinase Phosphatase-1-dependent dual Inhibition of MAPK JNK and p38 in epithelial cells. BMC Mol Biol *5*, 2.

Sakuntabhai, A., Turbpaiboon, C., Casademont, I., Chuansumrit, A., Lowhnoo, T., Kajaste-Rudnitski, A., Kalayanarooj, S. M., Tangnararatchakit, K., Tangthawornchaikul, N., Vasanawathana, S., Chaiyaratana, W., Yenchitsomanus, P. T., Suriyaphol, P., Avirutnan, P., Chokephaibulkit, K., Matsuda, F., Yoksan, S., Jacob, Y., Lathrop, G. M., Malasit, P., Despres, P., and Julier, C. (2005). A variant in the CD209 promoter is associated with severity of dengue disease. Nat Genet *37*, 507-513.

Sampaio, E. P., Caneshi, J. R., Nery, J. A., Duppre, N. C., Pereira, G. M., Vieira, L. M., Moreira, A. L., Kaplan, G., and Sarno, E. N. (1995). Cellular immune response to Mycobacterium leprae infection in human immunodeficiency virus-infected individuals. Infect Immun *63*, 1848-1854.

Sampaio, E. P., Moraes, M. O., Nery, J. A., Santos, A. R., Matos, H. C., and Sarno, E. N. (1998). Pentoxifylline decreases in vivo and in vitro tumour necrosis factor-alpha (TNF-alpha) production in lepromatous leprosy patients with erythema nodosum leprosum (ENL). Clin Exp Immunol *111*, 300-308.

Santaram, V., and Porichha, D. (2004). Reaction cases treated at the Regional Leprosy Training and Research Institute, Aska, Orissa: a retrospective analysis. Indian J Lepr *76*, 310-320.

Santos, A. R., Suffys, P. N., Vanderborght, P. R., Moraes, M. O., Vieira, L. M., Cabello, P. H., Bakker, A. M., Matos, H. J., Huizinga, T. W., Ottenhoff, T. H., Sampaio, E. P., and Sarno, E. N. (2002). Role of tumor necrosis factor-alpha and interleukin-10 promoter gene polymorphisms in leprosy. J Infect Dis *186*, 1687-1691.

Santos, D. O., Castro, H. C., Bourguignon, S. C., Bastos, O. M., Rodrigues, C. R., Van Heuverswyn, H., Nery, J. A., and Miranda, A. (2007). Expression of B7-1 costimulatory molecules in patients with multibacillary leprosy and reactional states. Clin Exp Dermatol *32*, 75-80.

Sarno, E. N., Grau, G. E., Vieira, L. M., and Nery, J. A. (1991). Serum levels of tumour necrosis factor-alpha and interleukin-1 beta during leprosy reactional states. Clin Exp Immunol *84*, 103-108.

Sarno, E. N., Illarramendi, X., Nery, J. A., Sales, A. M., Gutierrez-Galhardo, M. C., Penna, M. L., Sampaio, E. P., and Kaplan, G. (2008). HIV-M. leprae interaction: can HAART modify the course of leprosy? Public Health Rep *123*, 206-212.

Saunderson, P., Bizuneh, E., and Leekassa, R. (2008). Neuropathic pain in people treated for multibacillary leprosy more than ten years previously. Lepr Rev *79*, 270-276.

Saunderson, P., Gebre, S., and Byass, P. (2000a). Reversal reactions in the skin lesions of AMFES patients: incidence and risk factors. Lepr Rev *71*, 309-317.

Saunderson, P., Gebre, S., Desta, K., and Byass, P. (2000b). The ALERT MDT Field Evaluation Study (AMFES): a descriptive study of leprosy in Ethiopia. Patients, methods and baseline characteristics. Lepr Rev *71*, 273-284.

Saunderson, P., Gebre, S., Desta, K., Byass, P., and Lockwood, D. N. (2000c). The pattern of leprosy-related neuropathy in the AMFES patients in Ethiopia: definitions, incidence, risk factors and outcome. Lepr Rev *71*, 285-308.

Saunderson, P., and Groenen, G. (2000). Which physical signs help most in the diagnosis of leprosy? A proposal based on experience in the AMFES project, ALERT, Ethiopia. Lepr Rev *71*, 34-42.

Schauber, J., Oda, Y., Buchau, A. S., Yun, Q. C., Steinmeyer, A., Zugel, U., Bikle, D. D., and Gallo, R. L. (2008). Histone acetylation in keratinocytes enables control of the expression of cathelicidin and CD14 by 1,25-dihydroxyvitamin D3. J Invest Dermatol *128*, 816-824.

Schauf, V., Ryan, S., Scollard, D., Jonasson, O., Brown, A., Nelson, K., Smith, T., and Vithayasai, V. (1985). Leprosy associated with HLA-DR2 and DQw1 in the population of northern Thailand. Tissue Antigens *26*, 243-247.

Schlesinger, L. S., and Horwitz, M. A. (1991). Phenolic glycolipid-1 of Mycobacterium leprae binds complement component C3 in serum and mediates phagocytosis by human monocytes. J Exp Med *174*, 1031-1038.

Schmitt, J., Langan, S., and Williams, H. C. (2007). What are the best outcome measurements for atopic eczema? A systematic review. J Allergy Clin Immunol *120*, 1389-1398.

Schreuder, P. A. (1998a). The occurrence of reactions and impairments in leprosy: experience in the leprosy control program of three provinces in northeastern Thailand, 1987-1995 [correction of 1978-1995]. II. Reactions. Int J Lepr Other Mycobact Dis *66*, 159-169.

Schreuder, P. A. (1998b). The occurrence of reactions and impairments in leprosy: experience in the leprosy control program of three provinces in northeastern Thailand, 1987-1995 [correction of 1978-1995]. III. Neural and other impairments. Int J Lepr Other Mycobact Dis *66*, 170-181.

Schuring, R. P., Hamann, L., Faber, W. R., Pahan, D., Richardus, J. H., Schumann, R. R., and Oskam, L. (2009). Polymorphism N248S in the human Toll-like receptor 1 gene is related to leprosy and leprosy reactions. J Infect Dis *199*, 1816-1819.

Schuring, R. P., Richardus, J. H., Steyerberg, E. W., Pahan, D., Faber, W. R., and Oskam, L. (2008). Preventing nerve function impairment in leprosy: validation and updating of a prediction rule. PLoS Negl Trop Dis *2*, e283.

Schwarz, R. J., and Macdonald, M. (2004). A rational approach to nasal reconstruction in leprosy. Plast Reconstr Surg *114*, 876-882; discussion 883-874.

Scollard, D. M. (2008). The biology of nerve injury in leprosy. Lepr Rev *79*, 242-253.

Scollard, D. M., Smith, T., Bhoopat, L., Theetranont, C., Rangdaeng, S., and Morens, D. M. (1994). Epidemiologic characteristics of leprosy reactions. Int J Lepr Other Mycobact Dis *62*, 559-567.

Sehgal, V. N., Srivastava, G., and Sharma, V. K. (1987). Contemplative immune mechanism of Lucio phenomenon and its global status. J Dermatol *14*, 580-585.

Shepard, C. C., Levy, L., and Fasal, P. (1974). Further experience with the rapid bactericidal effect of rifampin on Mycobacterium leprae. Am J Trop Med Hyg *23*, 1120-1124.

Shetty, V. P., and Antia, N. H. (1997). Pathology of nerve damage in leprosy. In The peripheral nerve in leprosy and other neuropathies, N. H. Antia, and V. P. Shetty, eds. (Oxford, Oxford University Press), pp. 79-137.

Shetty, V. P., Thakar, U. H., D'Souza, E., Ghate, S. D., Arora, S., Doshi, R. P., Wakade, A. V., and Thakur, D. V. (2009). Detection of previously undetected leprosy cases in a defined rural and urban area of Maharashtra, Western India. Lepr Rev *80*, 22-33.

Shibata, M., Katsuyama, M., Onodera, T., Ehama, R., Hosoi, J., and Tagami, H. (2009). Glucocorticoids enhance Toll-like receptor 2 expression in human keratinocytes stimulated with Propionibacterium acnes or proinflammatory cytokines. J Invest Dermatol *129*, 375-382.

Shubin, H. (1965). Long term (five or more years) administration of corticosteroids in pulmonary diseases. Dis Chest *48*, 287-290.

Sibley, L. D., and Krahenbuhl, J. L. (1987). Mycobacterium leprae-burdened macrophages are refractory to activation by gamma interferon. Infect Immun *55*, 446-450.

Sieling, P. A., Jullien, D., Dahlem, M., Tedder, T. F., Rea, T. H., Modlin, R. L., and Porcelli, S. A. (1999). CD1 expression by dendritic cells in human leprosy lesions: correlation with effective host immunity. J Immunol *162*, 1851-1858.

Simmons, C. P., Thwaites, G. E., Quyen, N. T., Chau, T. T., Mai, P. P., Dung, N. T., Stepniewska, K., White, N. J., Hien, T. T., and Farrar, J. (2005). The clinical benefit of adjunctive dexamethasone in tuberculous meningitis is not associated with measurable attenuation of peripheral or local immune responses. J Immunol *175*, 579-590.

Simoes Quaresma, J. A., de Oliveira, M. F., Ribeiro Guimaraes, A. C., de Brito, E. B., de Brito, R. B., Pagliari, C., de Brito, A. C., Xavier, M. B., and Seixas Duarte, M. I. (2009). CD1a and factor XIIIa immunohistochemistry in leprosy: a possible role of dendritic cells in the pathogenesis of Mycobacterium leprae infection. Am J Dermatopathol *31*, 527-531.

Simons, J. J., and Morales, A. (1980). Minocycline and generalized cutaneous pigmentation. J Am Acad Dermatol *3*, 244-247.

Smith, C. H., Anstey, A. V., Barker, J. N., Burden, A. D., Chalmers, R. J., Chandler, D., Finlay, A. Y., Griffiths, C. E., Jackson, K., McHugh, N. J., McKenna, K. E., Reynolds, N. J., and Ormerod, A. D. (2005). British Association of Dermatologists guidelines for use of biological interventions in psoriasis 2005. Br J Dermatol *153*, 486-497.

Smith, W. C., Anderson, A. M., Withington, S. G., van Brakel, W. H., Croft, R. P., Nicholls, P. G., and Richardus, J. H. (2004). Steroid prophylaxis for prevention of nerve function impairment in leprosy: randomised placebo controlled trial (TRIPOD 1). BMJ *328*, 1459.

Smith, W. C., Nicholls, P. G., Das, L., Barkataki, P., Suneetha, S., Suneetha, L., Jadhav, R., Sundar Rao, P. S., Wilder-Smith, E. P., Lockwood, D. N., and van Brakel, W. H. (2009). Predicting Neuropathy and Reactions in Leprosy at Diagnosis and Before Incident Events-Results from the INFIR Cohort Study. PLoS Negl Trop Dis *3*, e500.

Soilleux, E. J., Sarno, E. N., Hernandez, M. O., Moseley, E., Horsley, J., Lopes, U. G., Goddard, M. J., Vowler, S. L., Coleman, N., Shattock, R. J., and Sampaio, E. P. (2006). DC-SIGN association with the Th2 environment of lepromatous lesions: cause or effect? J Pathol *209*, 182-189.

Sousa, A. L., Stefani, M. M., Pereira, G. A., Costa, M. B., Rebello, P. F., Gomes, M. K., Narahashi, K., Gillis, T. P., Krahenbuhl, J. L., and Martelli, C. M. (2007). Mycobacterium leprae DNA Associated with Type 1 Reactions in Single Lesion Paucibacillary Leprosy Treated with Single Dose Rifampin, Ofloxacin, and Minocycline. Am J Trop Med Hyg *77*, 829-833.

Sparvero, L. J., Asafu-Adjei, D., Kang, R., Tang, D., Amin, N., Im, J., Rutledge, R., Lin, B., Amoscato, A. A., Zeh, H. J., and Lotze, M. T. (2009). RAGE (Receptor for Advanced Glycation Endproducts), RAGE ligands, and their role in cancer and inflammation. J Transl Med *7*, 17.

Spierings, E., De Boer, T., Zulianello, L., and Ottenhoff, T. H. (2000). Novel mechanisms in the immunopathogenesis of leprosy nerve damage: the role of Schwann cells, T cells and Mycobacterium leprae. Immunol Cell Biol *78*, 349-355.

Srinivasan, H., Rao, K. S., and Shanmugam, N. (1982). Steroid therapy in recent "quiet nerve paralysis" in leprosy. Report of a study of twenty-five patients. Lepr India *54*, 412-419.

Stefani, M. M., Guerra, J. G., Sousa, A. L., Costa, M. B., Oliveira, M. L., Martelli, C. T., and Scollard, D. M. (2009). Potential plasma markers of Type 1 and Type 2 leprosy reactions: a preliminary report. BMC Infect Dis *9*, 75.

Stevenson, J., Hymas, W., and Hillyard, D. (2005). Effect of sequence polymorphisms on performance of two real-time PCR assays for detection of herpes simplex virus. J Clin Microbiol *43*, 2391-2398.

Streiner, D. L., and Norman, G. R. (2003). Health Measurement Scales:A practical guide to their development and use., 3rd edn (Oxford, Oxford University Press).

Stump, P. R., Baccarelli, R., Marciano, L. H., Lauris, J. R., Teixeira, M. J., Ura, S., and Virmond, M. C. (2004). Neuropathic pain in leprosy patients. Int J Lepr Other Mycobact Dis *72*, 134-138.

Sugumaran, D. S. (1998). Leprosy reactions--complications of steroid therapy. Int J Lepr Other Mycobact Dis *66*, 10-15.

Sumegi, A., Antal-Szalmas, P., Aleksza, M., Kovacs, I., Sipka, S., Zeher, M., Kiss, E., and Szegedi, G. (2005). Glucocorticosteroid therapy decreases CD14-expression and CD14-mediated LPS-binding and activation of monocytes in patients suffering from systemic lupus erythematosus. Clin Immunol *117*, 271-279.

Sumikawa, Y., Asada, H., Hoshino, K., Azukizawa, H., Katayama, I., Akira, S., and Itami, S. (2006). Induction of beta-defensin 3 in keratinocytes stimulated by bacterial lipopeptides through toll-like receptor 2. Microbes Infect *8*, 1513-1521.

Tanga, F. Y., Raghavendra, V., and DeLeo, J. A. (2004). Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. Neurochem Int *45*, 397-407.

Taris, N., Lang, R. P., and Camara, M. D. (2008). Sequence polymorphism can produce serious artefacts in real-time PCR assays: hard lessons from Pacific oysters. BMC Genomics *9*, 234.

Tauszig-Delamasure, S., Bilak, H., Capovilla, M., Hoffmann, J. A., and Imler, J. L. (2002). Drosophila MyD88 is required for the response to fungal and Gram-positive bacterial infections. Nat Immunol *3*, 91-97.

Texereau, J., Chiche, J. D., Taylor, W., Choukroun, G., Comba, B., and Mira, J. P. (2005). The importance of Toll-like receptor 2 polymorphisms in severe infections. Clin Infect Dis *41 Suppl 7*, S408-415.

Thwaites, G. E., Nguyen, D. B., Nguyen, H. D., Hoang, T. Q., Do, T. T., Nguyen, T. C., Nguyen, Q. H., Nguyen, T. T., Nguyen, N. H., Nguyen, T. N., Nguyen, N. L., Nguyen, H. D., Vu, N. T., Cao, H. H., Tran, T. H., Pham, P. M., Nguyen, T. D., Stepniewska, K., White, N. J., Tran, T. H., and Farrar, J. J. (2004). Dexamethasone for the treatment of tuberculous meningitis in adolescents and adults. N Engl J Med *351*, 1741-1751.

Touw-Langendijk, E. M., Brandsma, J. W., and Andersen, J. G. (1984). Treatment of ulnar and median nerve function loss in borderline leprosy. Lepr Rev *55*, 41-46.

Uplekar, M. W., and Antia, N. H. (1986). Clinical and histopathological observations on pure neuritic leprosy. Indian J Lepr *58*, 513-521.

van Brakel, W. H., Anderson, A. M., Withington, S. G., Croft, R. P., Nicholls, P. G., Richardus, J. H., and Smith, W. C. (2003). The prognostic importance of detecting mild sensory impairment in leprosy: a randomized controlled trial (TRIPOD 2). Lepr Rev *74*, 300-310.

van Brakel, W. H., Kets, C. M., van Leerdam, M. E., Khawas, I. B., and Gurung, K. S. (1997). Functional sensibility of the hand in leprosy patients. Lepr Rev *68*, 25-37.

van Brakel, W. H., and Khawas, I. B. (1994). Silent neuropathy in leprosy: an epidemiological description. Lepr Rev *65*, 350-360.

van Brakel, W. H., and Khawas, I. B. (1996). Nerve function impairment in leprosy: an epidemiological and clinical study--Part 2: Results of steroid treatment. Lepr Rev *67*, 104-118.

van Brakel, W. H., Khawas, I. B., Gurung, K. S., Kets, C. M., van Leerdam, M. E., and Drever, W. (1996). Intra- and inter-tester reliability of sensibility testing in leprosy. Int J Lepr Other Mycobact Dis *64*, 287-298.

van Brakel, W. H., Khawas, I. B., and Lucas, S. B. (1994). Reactions in leprosy: an epidemiological study of 386 patients in west Nepal. Lepr Rev *65*, 190-203.

van Brakel, W. H., Nicholls, P. G., Das, L., Barkataki, P., Maddali, P., Lockwood, D. N., and Wilder-Smith, E. (2005a). The INFIR Cohort Study: assessment of sensory and motor neuropathy in leprosy at baseline. Lepr Rev *76*, 277-295.

van Brakel, W. H., Nicholls, P. G., Das, L., Barkataki, P., Suneetha, S. K., Jadhav, R. S., Maddali, P., Lockwood, D. N., Wilder-Smith, E., and Desikan, K. V. (2005b). The INFIR Cohort Study: investigating prediction, detection and pathogenesis of neuropathy and reactions in leprosy. Methods and baseline results of a cohort of multibacillary leprosy patients in north India. Lepr Rev *76*, 14-34.

van Brakel, W. H., Nicholls, P. G., Lockwood, D. N., Rao, P. S., and Smith, W. C. (2007a). A scale to assess the severity of leprosy reactions. Lepr Rev *78*, 161-164.

van Brakel, W. H., Nicholls, P. G., Wilder-Smith, E. P., Das, L., Barkataki, P., and Lockwood, D. N. (2008). Early Diagnosis of Neuropathy in Leprosy-Comparing Diagnostic Tests in a Large Prospective Study (the INFIR Cohort Study). PLoS Negl Trop Dis *2*, e212.

van Brakel, W. H., Saunderson, P., Shetty, V., Brandsma, J. W., Post, E., Jellema, R., and McKnight, J. (2007b). International workshop on neuropathology in leprosy--consensus report. Lepr Rev *78*, 416-433.

van Veen, N. H., Nicholls, P. G., Smith, W. C., and Richardus, J. H. (2007). Corticosteroids for treating nerve damage in leprosy. Cochrane Database Syst Rev, CD005491.

Van Voorhis, W. C., Kaplan, G., Sarno, E. N., Horwitz, M. A., Steinman, R. M., Levis, W. R., Nogueira, N., Hair, L. S., Gattass, C. R., Arrick, B. A., and Cohn, Z. A. (1982). The cutaneous infiltrates of leprosy: cellular characteristics and the predominant T-cell phenotypes. N Engl J Med *307*, 1593-1597.

Vannberg, F. O., Chapman, S. J., Khor, C. C., Tosh, K., Floyd, S., Jackson-Sillah, D., Crampin, A., Sichali, L., Bah, B., Gustafson, P., Aaby, P., McAdam, K. P., Bah-Sow, O., Lienhardt, C., Sirugo, G., Fine, P., and Hill, A. V. (2008). CD209 genetic polymorphism and tuberculosis disease. PLoS One *3*, e1388.

Vargas-Inchaustegui, D. A., Tai, W., Xin, L., Hogg, A. E., Corry, D. B., and Soong, L. (2009). Distinct roles for MyD88 and Toll-like receptor 2 during Leishmania braziliensis infection in mice. Infect Immun *77*, 2948-2956.

Varkevisser, C. M., Lever, P., Alubo, O., Burathoki, K., Idawani, C., Moreira, T. M., Patrobas, P., and Yulizar, M. (2009). Gender and leprosy: case studies in Indonesia, Nigeria, Nepal and Brazil. Lepr Rev *80*, 65-76.

Vinay, K., Smita, J., Nikhil, G., and Neeta, G. (2009). Human immunodeficiency virus and leprosy coinfection in Pune, India. J Clin Microbiol *47*, 2998-2999.

Visentainer, J. E., Tsuneto, L. T., Serra, M. F., Peixoto, P. R., and Petzl-Erler, M. L. (1997). Association of leprosy with HLA-DR2 in a Southern Brazilian population. Braz J Med Biol Res *30*, 51-59.

von Meyenn, F., Schaefer, M., Weighardt, H., Bauer, S., Kirschning, C. J., Wagner, H., and Sparwasser, T. (2006). Toll-like receptor 9 contributes to recognition of Mycobacterium bovis Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. Immunobiology *211*, 557-565.

Walker, S. L., and Lockwood, D. N. (2008). Leprosy type 1 (reversal) reactions and their management. Lepr Rev *79*, 372-386.

Walker, S. L., Shah, M., Hubbard, V. G., Pradhan, H. M., and Ghimire, M. (2008). Skin disease is common in rural Nepal: results of a point prevalence study. Br J Dermatol *158*, 334-338.

Walker, S. L., Waters, M. F., and Lockwood, D. N. (2007). The role of thalidomide in the management of erythema nodosum leprosum. Lepr Rev *78*, 197-215.

Wandinger, K. P., Wessel, K., Trillenberg, P., Heindl, N., and Kirchner, H. (1998). Effect of high-dose methylprednisolone administration on immune functions in multiple sclerosis patients. Acta Neurol Scand *97*, 359-365.

Wang, L. M., Kimura, A., Satoh, M., and Mineshita, S. (1999). HLA linked with leprosy in southern China: HLA-linked resistance alleles to leprosy. Int J Lepr Other Mycobact Dis *67*, 403-408.

Waters, M. F., Rees, R. J., Pearson, J. M., Laing, A. B., Helmy, H. S., and Gelber, R. H. (1978). Rifampicin for lepromatous leprosy: nine years' experience. Br Med J *1*, 133-136.

Weber, A. N., Tauszig-Delamasure, S., Hoffmann, J. A., Lelievre, E., Gascan, H., Ray, K. P., Morse, M. A., Imler, J. L., and Gay, N. J. (2003). Binding of the Drosophila cytokine Spatzle to Toll is direct and establishes signaling. Nat Immunol *4*, 794-800.

Wemambu, S. N., Turk, J. L., Waters, M. F., and Rees, R. J. (1969). Erythema nodosum leprosum: a clinical manifestation of the arthus phenomenon. Lancet *2*, 933-935.

Weusten, B. L., Jacobs, J. W., and Bijlsma, J. W. (1993). Corticosteroid pulse therapy in active rheumatoid arthritis. Semin Arthritis Rheum *23*, 183-192.

WHO (1982). Chemotherapy of leprosy for control programmes. Tech Rep Ser 675 (Geneva, WHO).

WHO (1988). WHO Expert Committee on Leprosy. World Health Organ Tech Rep Ser *768*, 1-51.

WHO (1994). Chemotherapy of leprosy. Report of a WHO Study Group. World Health Organ Tech Rep Ser *847*, 1-24.

WHO (1998). WHO Expert Committee on Leprosy. World Health Organ Tech Rep Ser *874*, 1-43.

WHO (2002). Leprosy. Global situation. Wkly Epidemiol Rec *77*, 1-8.

WHO (2005). Global leprosy situation, 2005. Wkly Epidemiol Rec *80*, 289-295.

WHO (2006). Global strategy for further reducing the leprosy burden and sustaining leprosy control activities 2006-2010. Operational guidelines. Lepr Rev *77*, IX, X, 1-50.

WHO (2009). Global leprosy situation, 2009. Wkly Epidemiol Rec *84*, 333-340.

Wijdicks, E. F., Bamlet, W. R., Maramattom, B. V., Manno, E. M., and McClelland, R. L. (2005). Validation of a new coma scale: The FOUR score. Ann Neurol *58*, 585-593.

Wiken, M., Grunewald, J., Eklund, A., and Wahlstrom, J. (2009). Higher monocyte expression of TLR2 and TLR4, and enhanced pro-inflammatory synergy of TLR2 with NOD2 stimulation in sarcoidosis. J Clin Immunol *29*, 78-89.

Wilder-Smith, A., and Wilder-Smith, E. (1997). Effect of steroid therapy on parameters of peripheral autonomic dysfunction in leprosy patients with acute neuritis. Int J Lepr Other Mycobact Dis *65*, 20-27.

Wolkenstein, P., Latarjet, J., Roujeau, J. C., Duguet, C., Boudeau, S., Vaillant, L., Maignan, M., Schuhmacher, M. H., Milpied, B., Pilorget, A., Bocquet, H., Brun-Buisson, C., and

Revuz, J. (1998). Randomised comparison of thalidomide versus placebo in toxic epidermal necrolysis. Lancet *352*, 1586-1589.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640-643.

Yamamura, M., Uyemura, K., Deans, R. J., Weinberg, K., Rea, T. H., Bloom, B. R., and Modlin, R. L. (1991). Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science *254*, 277-279.

Yamashita, S., and Okada, Y. (2005). Application of heat-induced antigen retrieval to aldehyde-fixed fresh frozen sections. J Histochem Cytochem *53*, 1421-1432.

Yim, J. J., Kim, H. J., Kwon, O. J., and Koh, W. J. (2008). Association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and nontuberculous mycobacterial lung disease in a Korean population. Hum Immunol *69*, 572-576.

Yim, J. J., Lee, H. W., Lee, H. S., Kim, Y. W., Han, S. K., Shim, Y. S., and Holland, S. M. (2006). The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. Genes Immun *7*, 150-155.

Youssef, P. P., Haynes, D. R., Triantafillou, S., Parker, A., Gamble, J. R., Roberts-Thomson, P. J., Ahern, M. J., and Smith, M. D. (1997). Effects of pulse methylprednisolone on inflammatory mediators in peripheral blood, synovial fluid, and synovial membrane in rheumatoid arthritis. Arthritis Rheum *40*, 1400-1408.

Zhang, Z. Y., Zhang, Z., and Schluesener, H. J. (2009). Toll-like receptor-2, CD14 and heat-shock protein 70 in inflammatory lesions of rat experimental autoimmune neuritis. Neuroscience *159*, 136-142.

# APPENDICES

# APPENDIX 1. Ethical Approval

# Appendix 1.1

**LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE**

**ETHICS COMMITTEE**

**APPROVAL FORM**
**Application number:** 4021

Name of Principal Investigator    **Dr Diana Lockwood**

Department    **Infectious and Tropical Diseases**

Head of Department    **Professor Hazel Dockrell**

Title    **A multicentre study to critically evaluate a clinical severity score for measuring the severity of skin and nerve signs in nerve function impairment and type 1 (T1R) reactions in leprosy.**

Approval of this study is granted by the Committee.
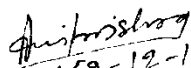
Chair
**Professor Tom Meade** .......... *T.-W. Meade* ..............................

Date .......... 29 Nov. 2005 ..............................

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the consent form must be re-submitted to the Committee.

# Appendix 1.2

## Nepal Health Research Council
Estd. 1991

————NHRC————

Date :

Ref. ? ? ?

**Executive Committee**

**Chairman**
Prof. Gopal Prasad Acharya

**Vice-Chairman**
Prof. Ramesh Kant Adhikari

**Member-Secretary Cum
Administrative Chief**
Dr. Anil Kumar Mishra

**Members**
Dr. B. D. Chataut
Dr. Laxmi Raj Pathak
Dr. Saraswati M. Padhye
Dr. Ugra Narayan Pathak
Dr. Rishi Ram Koirala

**Representative**
Ministry of Finance
National Planning Commission
Ministry of Health
Chief, Research Committee, IOM
Chairman, Nepal Medical Council

### Letter of Approval for Research Proposal

Date: 27th March, 2003

PI: Dr. Murdo Macdonald

Title: Studies To Improve The Treatment Of Acute Leprosy Reversal Reactions: A Proposal To Use Short Course High Dose Methyal Prednisolone In The Initial Treatment Of Acute Reversal Reaction In Leprosy.

Dear Dr Macdonald,

We are pleased to inform you that above mentioned proposal submitted by you has been approved by NHRC Executive Board after recommendation of Ethical Review Board (ERB) on 19th March,2003. This also certifies that there is no ethical objection

As per NHRC law you have to strictly follow the protocol stipulated in your proposal. Any changes in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit to NHRC the details of such changes intended or desired with justification prior to instituting actual change.

Moreover, you are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of your research proposal

Lastly, you are obliged to submit periodic progress reports every 3 months and three copies of the final research report with brief presentation of the findings and the financial statement of expenditure if funded by NHRC. If an article based upon that research is likely to be published, you must take prior permission of NHRC if funded for the same.

If you have any question, please contact our research officers.

Thank You,

Yours Truly,
Dr. Anil K Mishra
Member-Secretary

Tel. (977-1) 4254220, 4227460. Fax: 977-1-4262469, 4268284, Email: nhrc@nhrc.org.np, Ramshah Path, P. O. Box 7626, Kathmandu, Nepal.

# Appendix 1.3

**Nepal Health Research Council**

— NHRC —

Reference: 337·

**Executive Committee**

**Chairman**
Dr. Mahesh Kumar Maskey

**Vice-Chairman**
Dr. Buddha Basnyat

**Member-Secretary**
Dr. Sharad Raj Onta

**Members**
Dr. Rishi Ram Koirala
Dr. Basant Raj Pant
Dr. Nilambar Jha
Dr. Achala Vaidya
Dr. Kedar Prasad Baral

**Representative**
Ministry of Finance
National Planning Commission
Ministry of Health & Population
Chief, Research Committee, IOM
Chairman, Nepal Medical Council

Date: 27th November, 2006

Dr. Murdo Macdonald
Principal Investigator
Anandaban Hospital
PO Box151
Lalitpur,Nepal

Subject: Approval of the amendment

Dear Dr. Macdonald,
It is my pleasure to inform that the 48th Ethical Review Board Meeting of Nepal Health Research Council held on 19th November 2006, has decided to accept the amendment made in the research proposal entitled " Studies to Improve the Treatment of Acute Leprosy Reversal Reactions: A Proposal to Use Short Course High Dose Methylprednisolone in the Initial Treatment of Acute Reversal Reaction in Leprosy." The approval allows the researcher to proceed the research.

Thanking you for your kind cooperation.

Sincerely yours,

Dr. Sharad Raj Onta
Member-Secretary

# Appendix 1.4

**LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE**

**ETHICS COMMITTEE**

**APPROVAL FORM**
**Application number:** 4022

Name of Principal Investigator — **Diana Lockwood**

Department — **Infectious and Tropical Diseases**

Head of Department — **Professor Hazel Dockrell**

Title: **A phase 2 trial to investigate the safety of early intravenous high dose methylprednisolone in leprosy acute neuritis and leprosy type 1 reactions (T1R) with neuritis.**

Approval of this study is granted by the Committee.

Chair ....*T. W. Meade*....................................
**Professor Tom Meade**

Date ....*28 Nov 2005*.........................................

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the consent form must be re-submitted to the Committee.

## APPENDIX 2. Patient information leaflets and consent forms

# Appendix 2.1

**Information for prospective participants in the clinical scale study**

**A multicentre study to critically evaluate a clinical severity score for measuring the severity of skin and nerve signs in nerve function impairment and type 1 reactions in leprosy.**
**Dr Ajit Barkataki**
**Dr Ruth Butlin**
**Dr Diana Lockwood**
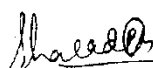**Dr José Nery**
**Dr Peter Nicholls**
**Dr Samba Sow**
**Dr Stephen Walker**


**Clinical Research Unit, 2nd Floor**
**Department of Infectious and Tropical Diseases**
**London School of Hygiene and Tropical Medicine**
**Keppel St**
**London**
**UK**
**WC1E 7HT**

Email: **steve.walker@lshtm.ac.uk**
        drstevewalker@hotmail.com

We would like to ask for your cooperation in a study that we are conducting. The study aims to improve our ability to measure skin and nerve damage that can be part of leprosy.

Leprosy is a disease affecting the skin and nerves caused by a bacterium called *Mycobacterium Leprae.* Leprosy can be treated and completely cured with antibiotics, the so-called 'multidrug therapy' or MDT. One of the complications of leprosy is that the nerves in the face, the arms and the legs are at risk of getting damaged.

The risk of nerve damage is highest during so-called 'reactions', which are like special attacks the body itself launches on the bacilli. These reactions can occur at any time. You may have experienced one already before and the doctor has diagnosed a reaction today. At the time of a reaction the skin patches on your body may become red and swollen. You may also get pain in your nerves, which may be like shooting pain in your arms or legs. At such times, you may feel that parts of the palms of your hands or the soles of your feet loose their feeling and become numb. There may also be a 'pins and needles' sensation in your face, hands or feet. These symptoms are caused by nerve damage. Another sign of nerve damage may be that muscles get weak. In that case you might experience weakness in your hands, have difficulty walking or have problems closing your eyes. Sometimes nerve damage also occurs without a reaction.

People with leprosy *may* get reactions and nerve damage but we cannot predict who will get it and who won't. As with many diseases, the earlier you treat reactions and nerve damage, the more chance you have to be completely healed. Despite this two fifths of people do not fully recover with the standard treatment of steroid tablets.

In order to improve the diagnosis and treatment of reactions it is important to be able to measure them as accurately as possible.

**The Clinical Scale study**

To achieve these aims, we have set up a study. It is called the Clinical Scale study. Over the years researchers in leprosy have developed a scale to measure reactions. We now wish to test the reliability of this scale.

If you are diagnosed as having a reaction you will be invited to take part in this study. You will be examined in the usual way. The testing of your sensation and strength will be measured and recorded on the scale. Once this has been done a doctor will also examine you and decide without using the scale the severity of the reaction affecting you. We will then use the results to compare the results from the scale with doctor to see if it is reliable.

If you take part in the study there will be no more visits to the clinic than would have been required had you not taken part. No blood or skin tests are required for the study. The study will mean that your clinic visit will last thirty minutes longer than it would do if you do not take part.

The information collected as part of this study will be kept secure and is entirely confidential. The results will be analysed in an anonymous fashion. This means that you can not be identified from the data we collect as part of the study or from the published results.

This study has received approval from the Ethics Committee of the London School of Hygiene and Tropical Medicine and the committees of the collaborating centres.

Your decision whether to take part or not will *not* affect the routine leprosy treatment you will receive. We would very much like you to take part and help us with this study, but you will *not* be penalised in any way if you refuse to take part. If, at any time after deciding to take part, you feel unhappy about continuing with the study, you have the freedom to come out of the study and do not have to give a reason for doing so. This will not negatively affect your leprosy treatment.

Thank you for taking the time to consider participating in the Clinical Scale study.

# Appendix 2.2

<div style="border:1px solid black; width:40%;">**Clinical Scale Study**</div>

Informed Consent

*You should read the statement describing the project and explain the study to the patient so that s/he understands what is involved. The patient may choose to take part or not.*

*If the patient does NOT consent to take part, s/he must NOT be entered into the Clinical Scale Study. DO NOT fill in any more Clinical Scale Study forms. Treat the patient according to the standard procedures in the clinic.*

*If the patient agrees to participate s/he must sign the statement below. This form and a copy of the information leaflet about the study must be stored with Clinic Notes. The form must also be signed by the enrolling researcher.*

**A multicentre study to critically evaluate a clinical severity score for measuring the severity of skin and nerve signs in nerve function impairment and type 1 reactions in leprosy.**

**Dr Ajit Barkataki**
**Dr Ruth Butlin**
**Dr Loretta Das**
**Prof JA Nery**
**Dr Samba Sow**

**Dr Diana Lockwood**
**Dr Peter Nicholls**
**Dr Stephen Walker**

---

**Are you willing to take part in this study?**

Yes, I have understood the purpose of the Clinical Scale study and I am willing to take part.  The accompanying description of the project has been read to me. I understand what will be required of me and what will happen to me if I take part in it.

My questions concerning this study have been answered by ……………………………………

I understand that at any time I may withdraw from this study without giving a reason and without affecting my normal care and management.

I agree to take part in this study

 Name:                                    Signature:                              Date:

---

Consent obtained and witnessed by:


Name:                                     Signature:                              Date:
(Enrolling researcher)

---

# Appendix 2.3

**Information for prospective participants in the Methylprednisolone study**

**A Phase 2 trial to investigate the safety of early intravenous high dose methylprednisolone in acute neuritis and type 1 reactions (T1R) with neuritis.**

**Dr Rachel Hawksworth**
**Dr Diana Lockwood**
**Dr Murdo Macdonald**
**Dr Peter Nicholls**
**Dr Stephen Walker**

**Anandaban Hospital**
**PO Box 151**
**Kathmandu**
**Nepal**

**Clinical Research Unit**
**Department of Infectious and Tropical Diseases**
**London School of Hygiene and Tropical Medicine**
**Keppel St**
**London**
**UK**
**WC1E 7HT**

**Email: steve.walker@lshtm.ac.uk**

We would like to ask for your cooperation in a study that we are conducting. The study has two main aims:
1. To improve our ability to treat the nerve damage that can be part of leprosy.
2. To improve our understanding of the factors causing that nerve damage.

Leprosy is a disease affecting the skin and nerves caused by a bacteria called *Mycobacterium Leprae.* Leprosy can be treated and completely cured with antibiotics, the so-called 'multidrug therapy' or MDT. One of the complications of leprosy is that the nerves in the face, the arms and the legs are at risk of getting damaged.

The risk of nerve damage is highest during so-called 'reactions', which are like special attacks the body itself launches on the bacteria. These reactions can occur at any time. You may have experienced one already before and the doctor has diagnosed a reaction today. At the time of a reaction the skin patches on your body may become red and swollen. You may also get pain in your nerves, which may be like shooting pain in your arms or legs. At such times, you may feel that parts of the palms of your hands or the soles of your feet loose their feeling and become numb. There may also be a 'pins and needles' sensation in your face, hands or feet. These symptoms are caused by nerve damage. Another sign of nerve damage may be that muscles get weak. In that case you might experience weakness in your hands, have difficulty walking or have problems closing your eyes. Sometimes nerve damage also occurs without a reaction.

People with leprosy *may* get reactions and nerve damage but we cannot predict who will get it and who won't. As with many diseases, the earlier you treat reactions and nerve damage, the more chance you have to be completely healed. Despite this two fifths of people do not fully recover with the standard treatment of steroid tablets. We would therefore like to try and improve the treatment of reactions and our understanding of what causes them.

**The Methylprednisolone study**

To achieve these aims, we have set up a study. It is called the Methylprednisolone study. Methylprednisolone is another steroid medicine which we hope will improve reactions by using it at a high dose and by giving it intravenously rather than in tablet form for the first three days of reaction treatment. Participants in the study will then take the standard steroid tablet regime. Not everyone who takes part will receive the methylprednisolone, this is because people are assigned to the treatment using a process of random allocation. This helps to minimise bias which can result in erroneous conclusions. Half of the people in the study will receive a placebo (a simple salt water solution – still given into the vein) instead but they will still be given the standard steroid tablet treatment for

reactions. This means that neither you nor your doctors will know whether you have received methylprednisolone or the placebo.

As the methylprednisolone or the placebo has to be given into a vein you will be admitted to hospital for a period of approximately four to seven days. You will also be taking steroid tablets afterwards which would have happened normally. You will have to have some blood tests, a chest x ray and provide a stool specimen to rule out certain health problems which the steroids could make worse. In addition to these as part of the study looking at the causes of the reaction we will want to take a small amount of blood (10ml) on **three** occasions during the 48 weeks which people take part in the trial. On **three** occasions during the trial we would take a small piece of skin, to be able to study the changes under the microscope. This is called a 'skin biopsy'. Before such a biopsy is taken, we would make the skin numb, so that you would have as little discomfort as possible. The removal of this small piece of skin will leave a wound which should heal in 7- 10 days. In order to record changes in the skin during the study we would also like to take photographs. These would be used to monitor progress. They would also be used to teach health care professionals about leprosy and when the results of the study are published. If you do not wish to have photographs taken you may still participate in the study.

In addition to spending at least four days in Anandaban Hospital you will have to attend the clinic on 14 occasions to be examined for changes in the skin and nerves and have the tests performed. This is approximately three more than if you did not participate in the study. All the treatment you will need as part of this study is free.

Methylprednisolone is a safe drug which has been available for many years. It has been used in high doses over a three day period in various diseases and is well tolerated. Like all steroids it can make certain conditions such as high blood pressure and diabetes worse but you will be tested for these and others. It can also cause weight gain, acne and mood disturbances just as other steroids can.

If you do experience any problems as a result of the study the doctors at Anandaban Hospital will see and treat you for free.

The information collected as part of this study will be entirely confidential and the results will be analysed in an anonymous fashion. This means that you can not be identified from the data we collect as part of the study or from the published results. The information and specimens will be kept secure. Some analysis of the information and specimens will take place in Nepal but the majority will be done in London, UK.

This study has received approval from the Medical Research Council of Nepal and the Ethics Committee of the London School of Hygiene and Tropical Medicine.

Your decision whether to take part or not will **not** affect the routine leprosy treatment you will receive. We would very much like you to take part and help us with this study, but you will **not** be penalised in any way if you refuse to take part. If, at any time after deciding to take part, you feel unhappy about continuing with the study, you have the freedom to come out of the study and do not have to give a reason for doing so. This will not negatively affect your leprosy treatment.

Thank you for taking the time to consider participating in the Methylprednisolone study.

# Appendix 2.4

<table>
<tr><td>**MP Study – Registration**</td><td>**Form 2**</td></tr>
</table>

| Informed Consent |
| --- |

*Completion of the consent procedure by PMW*

**You should read the Nepali statement describing the project and explain the study to the patient so that s/he understands what is involved. The patient may choose to take part or not.**

**If the patient does NOT consent to take part, s/he must NOT be entered into the MP Study. DO NOT fill in any more MP Study forms. Treat the patient according to the standard procedures in the clinic.**

**If the patient agrees to participate s/he must sign the statement below. This form and a copy of the Nepali statement must be stored with Clinic Notes. The form must also be signed by the enrolling researcher.**

**A Phase 2 trial to investigate the safety of early intravenous high dose methylprednisolone in acute neuritis and type 1 reactions with neuritis.**
**Dr Rachel Hawksworth**
**Dr Diana Lockwood**
**Dr Murdo Macdonald**
**Dr Peter Nicholls**
**Dr Stephen Walker**

**Anandaban Hospital**
**PO Box 151**
**Kathmandu**
**Nepal**

---

**Are you willing to take part in this study?**

Yes, I have understood the purpose of the Methylprednisolone study and I am willing to take part. The accompanying description of the project has been read to me in Nepali. I understand what will be required of me and what will happen to me if I take part in it. I am happy to attend the regular clinic appointments.
My questions concerning this study have been answered by …………………………………
I understand that at any time I may withdraw from this study without giving a
reason and without affecting my normal care and management.
I agree to take part in this study
 Name:                          Signature:                          Date:

I hereby confirm that I give consent for the photographs to be taken of me. I understand the material has educational value. I consent to the material being shown to appropriate professional staff and used in educational publications, journals, textbooks and used in any other form or medium including all forms of electronic publication or distribution anywhere in the world. As a result, I understand that the material may be seen by the general public. All or part of the material may be used in conjunction with other photographs, drawings, videotape images, sound recordings or other forms of illustration. Efforts will be made to conceal my identity but full confidentiality is not guaranteed.
Name:                          Signature:                          Date:

---

Consent obtained and witnessed by:


Name:                          Signature:                          Date:
(Enrolling researcher)

---

# Appendix 2.5

## प्रतिक्रियामा भएका सहभागीहरुको लागि मेथाइल प्रेड्नीसोलोन अध्ययनको जानकारी

### आनन्दबन अस्पताल, काठमाण्डौं र लण्डन स्कूल अफ ट्रपीकल मेडीसिन एण्ड हाईजीन, लण्डन

### परीक्षण भाग २

हामीहरुले आयोजना गरिरहेको अध्ययनमा तपाँईको सहयोगको अपेक्षा गर्दछौं । यस अध्ययनका प्रमुख दुई उद्देश्यहरु :

१  कुष्ठरोगको कारणले हुने नसाको क्षतिको उपचारमा हाम्रो कार्य क्षमतामा सुधार ल्याउनु
२  नसाको क्षति गर्ने कारक तत्वहरुका बारेमा अरु बढी जानकारी हासिल गर्नु

कुष्ठरोग माइकोब्याक्टेरीयम लेप्रे भन्ने सूक्ष्म कीटाणु बाट लाग्ने रोग हो र यसले विशेष गरि छाला र स्नायू नसामा असर गर्दछ । कुष्ठरोग बहु औषधी पद्धती बाट उपचार गरेमा पूर्ण रुपमा निको हुन्छ । कुष्ठरोगमा आउन सक्ने एउटा प्रमुख समस्या र जोखिम भनेको मुख, हात र खुट्टाको नसाहरु बिग्रने हुन सक्छ ।

प्रतिक्रिया आएको बेला नसाहरुको क्षति हुने ठुलो खतरा रहन्छ, यो एक विशेष अबस्था हुने छ जुन बेला शरीरले किटाणुको विरुद्ध प्रतिकार गर्दछ । यी प्रतिक्रियाहरु कुनै पनि बेला आउन सक्छन् । तपाईंले यस किसीमको प्रतिक्रिया पहिल्यै अनुभव गरी सक्नु भयो होला र यसलाई डाक्टरले आज पहिचान गरेका छन् । प्रतिक्रिया हुँदाको बेला शरीरका दागहरु रातो भई सुन्निन सक्छ । तपाँईको नसाहरु पनि दुख्न सक्छन् साथै तपाँईको हात र गोडाको नसाहरुमा घोचेको जस्तो गरेर दुख्न सक्छ । त्यस बेला तपाईंको हत्केला र पैताला निदाउन र लाटो हुन सक्छ साथै तपाँईको अनुहार, हात र गोडामा सियोले घोचेको जस्तो अनुभव पनि हुन्छ । यी लक्षणहरु नसा बिग्रेको कारण हुन्छ । नसा बिग्रेको अर्को लक्षण माँशपेशी कमजोर हुनु पनि हो । त्यस्तो अवस्थामा तपाईंले हातका औलाहरु कमजोर भएको, हिंड्डुल गर्न असजीलो र आँखा चिम्लन गाह्रो भएको महसूस गर्नु हुने छ । कहिलेकाहीं नसाको क्षति प्रतिक्रिया नआइकन पनि हुन सक्छ ।

कुष्ठरोग लागेका ब्यक्तिहरुलाई प्रतिक्रिया र नसाको क्षति हुन्छ, तर कुन ब्यक्तिमा यस्तो हुन्छ वा हुँदैन भनेर किटान गर्न सकिंदैन । अरु रोगहरुमा जस्तै यसमा पनि जति छिटो प्रतिक्रिया र नसाको क्षति हुनबाट जोगिने उपचार गर्न सक्यो, त्यतिनै पूरै निको हुने संभावना बढि हुन्छ । यस्तो भएतापनि पाँच भागको दुई भाग मानिसहरुमा चलन चल्तीको स्टेरोइड उपचारले पनि पूरै निको हुँदैन । त्यसैले यसको उपचारको स्तर बढाउन र कारणहरु जान्नको लागि हामी कोशिश गरिरहेका छौं ।

### मेथाइल प्रेड्नीसोलोनको अध्ययन :

यी उद्देश्यहरु प्राप्त गर्न हामीले एक अध्ययनको शुरुवात गरेको छौं । यसलाई मेथाइल प्रेड्नीसोलोन अध्ययन भनिन्छ । मेथाइल प्रेड्नीसोलोन एक स्टेराइड औषधी हो जसलाई ठुलो मात्रामा नसामा इन्जेक्शनको रुपमा प्रतिक्रिया आएको पहिलो तीन दिन भित्र दिंदा हामीलाई आशा छ कि यसैको चक्कीले भन्दा प्रतिक्रियाको उपचारमा सुधार ल्याउन सक्छ । यस अध्ययनका सहभागीहरुलाई चलन चल्तीको प्रेड्नीसोनको चक्की नियमित रुपमा दिइने छ । यस अध्ययनमा संलग्न सबै सहभागीहरुले यो मेथाइल प्रेड्नीसोलोन औषधीको सुइ पाउँदैनन् किनभने सहभागीहरुलाई टिपन टापन तरीकाले उपचार तोकिनेछ । यसबाट तथ्याङ्कलाई एकतर्फी भ्रूकाव हुनबाट कमी गर्छ र गलत निष्कर्ष निकाल्ने सम्भावनालाई न्यून पार्छ । यस अध्ययनमा संलग्न आधा ब्यक्तिहरुलाई मेथाइल प्रेड्नीसोलोन जस्तै सुई (सलाइन पानीको घोल) नसामा दिइने छ र प्रतिक्रियाको उपचारको लागी चलन चल्तीको स्टेरोइडको चक्की दिइन् छ । यसको मतलब न तपाँईले नत तपाँईको डाक्टरले तपाँईले मेथाइल प्रेड्नीसोलोन वा जस्तै कुन औषधी पाउनु भयो भनेर थाहा पाउने छन् ।

237

यी दुवै औषधीका सुईहरु नसामा दिइने हुँदा तपाई अस्पतालमा चार दिन देखि सात दिन सम्म भर्ना भएर बस्नु पर्नेछ । त्यसपछि तपाईलाई साथमा स्टेरोइडको चक्की दिइने छ । स्टेरोइडको प्रयोगले तपाईको स्वास्थ्यमा गर्न सक्ने हानिको बारेमा थाहा पाउनको लागि केही रगतका जाँचहरु, छातीको एक्सरे र दिसाको जाँच गरिने छ । यसको अलावा प्रतिक्रियाको कारण पत्ता लगाउन ४८ हप्ताको अवधीमा ६ पटक १० मिली (करीब ठुलो एक चम्चा) रगत लिने छौं । अध्ययनको अवधिमा तीन पटक छालाको सानो टुक्रा लिइने छ, जसमा सूक्ष्मदर्शक यन्त्रको सहायताले छालामा आएका परिवर्तनहरुलाई अध्ययन गरिने छ । यसलाई स्किन बायोप्सी भनिन्छ छालाको टुक्रा निकाल्नु भन्दा अघि तपाईलाई असजिलो नहोस् भनेर छालालाई लाटो पारिन्छ । छालाको टुक्रा निकालेको ठाउमा एक सानो घाउ हुन्छ, जुन निको हुन ७ देखी १० दिन लाग्न सक्छ । अध्ययन अवधिमा छालामा आएको परिवर्तनहरुलाई थाह पाउन हामी फाटो पनि खिच्ने छौं । यी फोटोहरुलाई प्रगतीको अनुगमन गर्न प्रयोग गरिने छ । यस अध्ययनको नतिजा पत्र पत्रिकाहरुमा प्रकाशित भए पछि यी फोटोहरुलाई स्वास्थ्य क्षेत्रमा काम गर्ने ब्यक्तिहरुलाई कुष्ठरोगको बारेमा सिकाउन प्रयोग गरिने छ। यदि तपाई फोटो खिचाउन अनइच्छुक हुनु भए पनि अध्ययनमा सहभागी हुन सक्नु हुने छ।

आनन्दबन अस्पतालमा चार दिन भर्ना हुनुको अलावा तपाई पाटन क्लीनिकमा १४ पटक आउनु पर्ने छ र उक्त समयमा तपाईको छाला र नसामा आएको परिवर्तनहरुलाई र अन्य जाँचहरु गरिने छ । यो भनेको यदि तपाई अध्ययनमा सहभागी नहुनु भएको भन्दा करीब ४ पटक बढी आउनु पर्ने हो । यस अध्ययनमा आवश्यक सबै औषधी उपचार निशुल्क हुनेछ ।

मेथाइल प्रेड्नीसोलोनको एक सुरक्षित औषधी हो र यो वर्षौं देखि उपलब्ध छ । यो औषधी तीन दिन सम्म अधिक मात्रामा अन्य धेरै रोगहरुको उपचारमा प्रयोग भएको छ र यसलाई मानीसहरुले सजिलै पचाई रहेका छन् । अन्य स्टेरोइड जस्तै यस औषधीको प्रयोगले पनि उच्च रक्तचाप र मधुमेहलाई खराब गर्न सक्ने संभावना भएको हुँदा यी रोगहरु र अन्य रोगहरुका बारेमा जाँच गरिने छ । अन्य स्टेरोइड जस्तै यसको प्रयोगले पनि मुखमा डण्डीफोर आउने, शरीरको तौल बढ्ने र स्वभावमा परिवर्तनहरु आउन सक्छ ।

यस अध्ययनको कारणले तपाईले कुनै समस्या महसुस गर्नु भएमा आनन्दबन अस्पतालका डाक्टरहरुले तपाईलाई निशुल्क उपचार गर्ने छन् ।

यस अध्ययनबाट प्राप्त जानकारीका अंशहरु पूरै गोप्य राखिने छ र नाम रहित तरीकाले प्रशोधन गरिने छ । यसको मतलव हामीले तपाईबाट तथ्याङ्ग लिदा र त्यसलाई प्रकाशित गर्दा कही पनि तपाईको परिचय खुलाउने छैनौ । तथ्याङ्गका जानकारीहरु र परीक्षण गरिएका नमूनाहरुलाई सुरक्षित तवरले राखिने छ । केही नमूनाहरु नेपालमा र धेरै नमूनाहरु लण्डन, बेलायतमा जाँच हने छन् ।

यस अध्ययनलाई नेपालमा संचालन गर्न नेपाल स्वास्थ्य अनुसन्धान परिषद र लण्डन स्कूल अफ हाइजीन एण्ड ट्रोपिकल मेडीसिनको आचार संहिता हेर्ने समितिले स्वीकृती दिएको छ

य यस अध्ययनमा तपाईले सहभागी हुने नहुने जुनसुकै निर्णय लिएता पनि तपाईले लिइरहनु भएको कुष्ठरोगको औषधी उपचारमा कुनै असर पर्ने छैन । हामी चाहन्छौं तपाई यस अध्ययनमा सहभागी हुनु हुनेछ र सहयोग गर्नु हुने छ तर तपाईले सहभागी हुन अस्वीकार गर्नु भए पनि तपाईलाई कुनै जरिवाना गरिने छैन । यदि अध्ययनमा सहभागी हुने निर्णय लिइसकेपछि पनि तपाईले कुनै पनि बेला यस अध्ययनलाई निरन्तरता दिन अनइच्छुक हुनु भएमा तपाई स्वतन्त्र रुपमा यस अध्ययनबाट आफ्नो सहभागिता परित्याग गर्न सक्नु हुनेछ, यसको लागी कुनै कारण दिइरहनु पर्ने छैन । यसबाट तपाईको कुष्ठरोगको उपचारमा कुनै नकारात्मक असर पर्ने छैन।

यस मेथाइल प्रेड्नीसोलोन अध्ययनमा सहभागी हुन राजी हुनु भएकोमा धेरै धन्यबाद दिन चाहन्छौं।

238

# Appendix 2.6

## प्रतिक्रियामा भएका सहभागीहरुको लागि मेथाइल प्रेड्नीसोलोन अध्ययनको सहमती पत्र

### आनन्दबन अस्पताल काठमाण्डौं र लण्डन स्कूल अफ ट्रपीकल मेडीसिन एण्ड हाइजीन लण्डन
### परीक्षण भाग २

**के तपाँई यस अध्ययनमा भाग लिन चाहनु हुन्छ ?**

हो, मैले मेथाइलप्रेड्नीसोलोन अध्ययनको उद्देश्यको बारेमा राम्रो संग बुझें र यसमा भाग लिनलाई राजी छु । सहायकले विस्तृत रुपमा यस योजनाका बारेमा नेपालीमा पढेर सुनाउनु भयो । यदि मैले यस अध्ययनमा भाग लिए भने म बाट के आवश्यक पर्ने छ र म मा कुन कुन प्रकारका जाँचहरु हुने छन् भन्ने कुरा मैले बुझेको छ । म तोकिएको समयमा नियमित रुपमा क्लीनिकमा उपस्थित हुन राजी छु ।

यस अध्ययन संग सम्वन्धित मेरा सवै जिज्ञासाहरुलाई . . . . . . . . . . . . . . . . . . . . . . . . . . . . ले जवाफ दिनु भयो ।

यस अध्ययनबाट मेरो सहभागिता कुनै पनि बेला बिना जरिवाना कुनै कारण नदिइकन परित्याग गरेमा मैले पाइरहेको नियमित उपचारमा कुनै असर नपर्ने कुरा मैले बुझेको छु ।

म यस अध्ययनमा भाग लिन राजी छु ।

नाम : . . . . . . . . . . . . . . . . . . . .    सही : . . . . . . . . . . . . . .    मिति : . . . . . . . . . . .

म निश्चित रुपमा यसै साथ जानकारी गराउन चाहन्छु कि मेरो फोटो खिच्नलाई मलाई मन्जुरी छ । यी फोटोहरुका शैक्षिक महत्व छ भन्ने कुरा मलाई थाहा छ । मेरा फोटोहरु स्वास्थ्य क्षेत्रमा कार्य गर्ने कर्मचारी र चिकित्सा क्षेत्रको शैक्षिक सामाग्रीमा प्रकाशित गर्न, पत्र पत्रिकामा प्रकाशित गर्न, पाठ्यपुस्तकमा समावेश गर्न र अन्य कुनै माध्यमहरु अथवा विद्युतिय माध्यमहरुमा प्रकाशित गर्न अथवा संसारका कुनै पनि भागमा वितरण गर्न र देखाउन पनि मेरो मन्जुरी छ । यसको नतीजा जन समुदायमा देखीन सक्छ । समाग्रीका सबै वा केही भाग अन्य फोटोमा, रेखा चित्र, श्रब्य दृश्यका सामग्री तथा अन्य प्रकारका सामाग्रीहरुमा समावेश गर्न सकिने छन् । मेरो ब्यक्तिगत परिचयलाई गोप्य राख्ने हर प्रयास गरीने छ तर पूर्ण निश्चय गर्न सकिने छैन ।

नाम : . . . . . . . . . . . . . . . . . . . .    सही : . . . . . . . . . . . . . .    मिति . . . . . . . . . . . .

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सहमती लिने र साक्षी बस्ने :

नाम : . . . . . . . . . . . . . . . . . . . .    सही : . . . . . . . . . . . . . .    मिति . . . . . . . . . . . .

(सहभागी गराउने अनुसन्धानकर्ता)

# Appendix 2.7

**Information for non-reactional controls in the Methylprednisolone study**

**A Phase 2 trial to investigate the safety of early intravenous high dose methylprednisolone in acute neuritis and type 1 reactions (T1R) with neuritis.**

**Dr Rachel Hawksworth**
**Dr Diana Lockwood**
**Dr Murdo Macdonald**
**Dr Peter Nicholls**
**Dr Stephen Walker**

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**Nepal**

**Clinical Research Unit**
**Department of Infectious and Tropical Diseases**
**London School of Hygiene and Tropical Medicine**
**Keppel St**
**London, WC1E 7HT United Kingdom**

**Email: steve.walker@lshtm.ac.uk**

We would like to ask for your cooperation in a study that we are conducting. The study has two main aims:
3. To improve our ability to treat the nerve damage that can be part of leprosy.
4. To improve our understanding of the factors causing that nerve damage.

Leprosy is a disease affecting the skin and nerves caused by a bacteria called *Mycobacterium Leprae.* Leprosy can be treated and completely cured with antibiotics, the so-called 'multidrug therapy' or MDT. One of the complications of leprosy is that the nerves in the face, the arms and the legs are at risk of getting damaged.

The risk of nerve damage is highest during so-called 'reactions', which are like special attacks the body itself launches on the bacteria. You are not having a reaction but the changes in your skin would be useful to compare with people in the study who are having a reaction.

Your doctor has arranged for you to have a skin biopsy to help diagnose your condition. We would like to ask for your permission to take a small amount (6mm diameter circle) of extra skin that we can compare with the people in the study. The removal of this small amount of additional skin will not affect the healing of the biopsy wound.

If you do experience any problems as a result of the biopsy the doctors at Anandaban Hospital will see and treat you for free.

The information collected as part of this study will be entirely confidential and the results will be analysed in an anonymous fashion. This means that you can not be identified from the data we collect as part of the study or from the published results. The information and specimens will be kept secure. Some analysis of the information and specimens will take place in Nepal but the majority will be done in London, UK.

This study has received approval from the Medical Research Council of Nepal and the Ethics Committee of the London School of Hygiene and Tropical Medicine.

Your decision whether to take part or not will ***not*** affect the routine treatment you will receive. We would very much like you to take part and help us with this study, but you will ***not*** be penalised in any way if you refuse to take part. If, at any time after deciding to take part, you feel unhappy about continuing, you have the freedom to stop the biopsy and do not have to give a reason for doing so. This will not negatively affect your treatment.

Thank you for taking the time to consider assisting in this study.

# Appendix 2.8

| Non reactional controls for the MP Study | Controls |
| --- | --- |

| Informed Consent |
| --- |

*Completion of the consent procedure by PMW*

**You should read the Nepali statement describing the reason for the biopsy and explain the study to the patient so that s/he understands what is involved. The patient may choose to take part or not.**

**If the patient does NOT consent to take part, s/he must NOT have the additional biopsy performed. Treat the patient according to the standard procedures in the clinic.**

**If the patient agrees to participate s/he must sign the statement below. This form and a copy of the Nepali statement must be stored with Clinic Notes. The form must also be signed by the enrolling researcher.**

**A Phase 2 trial to investigate the safety of early intravenous high dose methylprednisolone in acute neuritis and type 1 reactions with neuritis.**

**Dr Rachel Hawksworth**

**Dr Diana Lockwood**          **Anandaban Hospital**
**Dr Murdo Macdonald**        **PO Box 151**
**Dr Peter Nicholls**            **Kathmandu**
**Dr Stephen Walker**           **Nepal**

---

**Are you willing to take part in this study?**

Yes, I have understood the purpose of the additional skin biopsy and I am willing to take part.  The accompanying description of the project has been read to me in Nepali. I understand what will be required of me and what will happen to me if I take part in it.
My questions concerning this procedure  have been answered by …………………………………

I understand that at any time I may withdraw my consent  without giving a
reason and without affecting my normal care and management.

I agree to have an additional skin biopsy

 Name:                              Signature:                          Date:

---

Consent obtained and witnessed by:


Name:                               Signature:                          Date:
(Enrolling researcher)

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**Appendix 2.9**

## प्रतिक्रियामा नभएका सहभागीहरुको लागि मेथाइल प्रेड्नीसोलोन अध्ययनको जानकारी

### आनन्दबन अस्पताल, काठमाण्डौं र लण्डन स्कूल अफ ट्रपीकल मेडीसिन एण्ड हाईजीन, लण्डन
### परीक्षण भाग २

हामीहरुले आयोजना गरीरहेको अध्ययनमा तपाँईको सहयोगको अपेक्षा गर्दछौं । यस अध्ययनका प्रमुख दुई उद्देश्यहरु :

१  कुष्ठरोगको कारणले हुने नसाको क्षतिको उपचारमा हाम्रो कार्य क्षमतामा सुधार ल्याउनु
२  नसाको क्षति गर्ने कारक तत्वहरुका बारेमा अरु बढी जानकारी हासील गर्नु

कुष्ठरोग माइकोब्याक्टेरीयम लेप्रे भन्ने सूक्ष्म कीटाणु बाट लाग्ने रोग हो र यसले विशेष गरि छाला र स्नायू नसामा असर गर्दछ । कुष्ठरोग बहु औषधी पद्धती बाट उपचार गरेमा पूर्ण रुपमा निको हुन्छ । कुष्ठरोगमा आउन सक्ने एउटा प्रमुख समस्या र जोखिम भनेको मुख, हात र खुट्टाको नसाहरु बिग्रने हुन सक्छ ।

प्रतिक्रिया हुंदाको अवस्थामा नसाहरुको क्षति हुने ठुलो खतरा रहन्छ, यो एक विशेष अबस्था हुने छ जुन बेला शरीरले किटाणुको विरुद्ध प्रतिकार गर्दछ । तपाँईलाई प्रतिक्रिया भएको छैन तर तपाँईको छालामा आएको परिवर्तनहरुलाई यस अध्ययनमा संलग्न अन्य प्रतिक्रिया आएका ब्यक्तिहरुको छालामा आएको परिवर्तन संग दाज्न मद्दत गर्दछ ।

तपाँईको रोगको अवस्थाको निदान गर्न डाक्टरले तपाँईबाट एउटा सानो छालाको टुक्रा निकाल्ने छन् । यसको अलावा अर्को एक टुक्रा (६ मिमी गोलो)  निकाल्न तपाँईको अनुमती चाहन्छौं र  त्यसलाई  यस अध्ययनमा संलग्न अन्य ब्यक्तिको छालाको जाँच संग दाज्न प्रयोग गर्नेछौं । अर्को एक टुक्रा बढि निकाल्नाले तपाँईको घाउ निको हुने प्रकृयामा कुनै असर पर्ने छैन।

यस अध्ययनको कारणले तपाँइले कुनै समस्या महसुस गर्नु भएमा आनन्दबन अस्पतालका डाक्टरहरुले तपाँईलाई निशुल्क उपचार गर्ने छन् ।

यस अध्ययनबाट प्राप्त जानकारीका अंशहरु पूरै गोप्य राखिने छ र नाम रहित तरीकाले प्रशोधन गरिने छ । यसको मतलब हामीले तपाँईबाट तथ्याङ्क लिंदा र त्यसलाई प्रकाशित गर्दा कहीं पनि तपाँईको परिचय खुलाउने छैनौं । तथ्याङ्कका जानकारीहरु र परीक्षण गरिएका नमूनाहरुलाई सुरक्षित तवरले राखिने छ । केही नमूनाहरु नेपालमा र धेरै नमूनाहरु लण्डन, बेलायतमा जाँच हुने छन् ।

यस अध्ययनलाई नेपालमा संचालन गर्न नेपाल स्वास्थ्य अनुसन्धान परिषद र लण्डन स्कूल अफ हाइजीन एण्ड ट्रपिकल मेडीसिनको आचार संहिता हेर्ने समितिले स्वीकृति दिएको छ ।

यस अध्ययनमा तपाँइले सहभागी हुने नहुने जुनसुकै निर्णय लिएता पनि तपाँईले लिइरहनु भएको कुष्ठरोगको औषधी उपचारमा कुनै असर पर्ने छैन । हामी चाहन्छौं की तपाँई यस अध्ययनमा सहभागी हुनु हुनेछ र यसलाई सहयोग गर्नु हुने छ, तर तपाँईले सहगागी हुन अस्वीकार गर्नु भए पनि तपाँईलाई कुनै जरिवाना गरिने छैन । यदि अध्ययनमा सहभागी हुने निर्णय लिइसके पछि पनि तपाँईले कुनै पनि बेला यस अध्ययनलाई निरन्तरता दिन अनइच्छुक हुनु भएमा तपाँई स्वतन्त्र रुपमा यस अध्ययनबाट आफ्नो सहभागिता परित्याग गर्न सक्नु हुनेछ र छालाको टुक्रा निकाल्न नदिन स्वतन्त्र हुनु हुन्छ र यसको लागी कुनै कारण दिइरहनु पर्ने छैन । यसबाट तपाँईको कुष्ठरोगको उपचारमा कुनै नकारात्मक असर पर्ने छैन ।

यस अध्ययनमा सहभागी हुन राजी हुनु भएकोमा धेरै धन्यबाद दिन चाहन्छौं।

*Consent Non Reaction Control Methyl Pred Study Nepali Date: 13th Dec 2005*
*Page 1 of 2*

242

# Appendix 2.10

## प्रतिक्रियामा नभएका सहभागीहरुको लागी मेथाइल प्रेड्नीसोलोन अध्ययनको सहमती पत्र

### आनन्दबन अस्पताल, काठमाण्डौं र लण्डन स्कूल अफ ट्रोपीकल मेडीसिन एण्ड हाईजीन, लण्डन

### परीक्षण भाग २

---

**के तपाँई यस अध्ययनमा भाग लिनु चाहनु हुन्छ ?**

हो, मैले अरु बढी छालाको टुक्रा लिनु पर्ने कारणको बारेमा राम्रो संग बुझें र यसमा भाग लिनलाई राजी छु । सहायकले विस्तृत रुपमा यस योजनाका बारेमा नेपालीमा पढेर सुनाउनु भयो । यदि मैले यस अध्ययनमा भाग लिएँ भने म बाट के आवश्यक पर्ने छ र म मा के के हुने छ भन्ने कुरा मैले बुझेको छु ।

यस प्रकृयाको बारेमा मेरा सबै जिज्ञासाहरुलाई . . . . . . . . . . . . . . . . . . . . . . . . ले जवाफ दिनु भयो ।

यस अध्ययनबाट मेरो सहभागिता कुनै पनि बेला बिना जरीवाना कुनै कारण नदिइकन परित्याग गरेमा मैले पाइरहेको नियमित उपचारको ब्यवस्थामा कुनै असर नपर्ने कुरा मैले बुझेको छु ।

म अरु बढी छालाको टुक्रा दिनको लागि राजी छु ।

नाम : . . . . . . . . . . . . . . . . . . .    सही : . . . . . . . . . . . .    मिति : . . . . . . .

---

सहमती लिने र साक्षी बस्ने :

नाम : . . . . . . . . . . . . . . . . . . .    सही : . . . . . . . . . . . .    मिति : . . . . . . .

(सहभागी गराउने अनुसन्धान कर्ता)

---

# APPENDIX 3. Protocols and data collection forms

# Appendix 3.1

**Dr A and P Barkatakis**
**Dr CR Butlin**
**Dr Diana Lockwood**
**Dr JAC Nery**
**Dr Peter Nicholls**
**Dr Samba Sow**
**Dr Stephen Walker**

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**and**

**drstevewalker@hotmail.com**

## Eligibility

### Entry criteria
- All individuals presenting with a new Type 1 (reversal) reaction who have not received corticosteroids in the previous three months. Those who have experienced previous type 1 reactions are eligible.

### Exclusion criteria

- Anyone unwilling to give consent.

## Informed consent

### Consent
- Trial carefully explained by investigator.
- Written explanatory note available in local language and English.
- Individual's signature or mark obtained as proof of consent to the take part in the trial.
- Signature of enrolling researcher.

### Confidentiality

- A trial database is to be kept at each participating centre by the lead investigator.
- The database should record the date an individual took part in the trial. The participant's unique clinic number should be recorded (but no other identifying details) alongside the generated "study number". This will prevent inadvertently enrolling someone twice.
- The database should be kept secure (ie locked in a safe) by the lead investigator
- The "study number" will be the two letter code for each centre followed by the numerical order in which the individual was enrolled starting with 1 for the first participant at any given centre.

## History and examination at presentation on Form A

### Examiner A

Record on Form A

- Sex
- Age
- Type of leprosy (Ridley-Jopling)
- Date of onset of T1R
- Current treatment for reaction (Record none if treatment has yet to be started)
- MDT start date
- Number of previous type 1 reactions (record 0 if this first)
- Time since previous type 1 reaction (record x if this is first)
- Duration of leprosy

Using Form A record examination scores

- Skin
- Oedema
- Nerves
- Fever

Using Form A record sensory testing scores

- The trigeminal nerve is tested on each side using cotton wool.
- The ulnar, median and posterior tibial nerves are tested on each side. Trigeminal sensation should be tested with cotton wool.
- The 2g and 10g Semmes-Weinstein monofilaments are used at 3 sites for the ulnar and median.
- The 10g and 300g monofilament at 3 sites for the posterior tibial nerves.
- Record on the diagram of the hands and feet the result of the monofilament testing at each test site using the following symbols

  Purple 2g felt -  ▲

  Orange 10g felt - ■

  Pink 300g felt -  #

  Orange not felt on hands, Pink not felt on feet then mark an **A** at the site in question.

- Record in the table the score for each nerve, determined by the results of the monofilament testing.

Using Form A record voluntary motor testing scores

- The modified MRC grading for muscle power is used to assess the facial, ulnar, radial, median and lateral popliteal nerves on each side.

  **Testing procedure for each movement**
  The patient should be seated in comfortably.

  *Facial nerve - Forced eye closure*
  - The patient is asked to close the eyes as tight as (s)he can.
  - The tester tries to pull down the lower lid on both sides using       his/her thumbs

  **Median nerve -Thumb abduction**

  - The wrist is held in extension and the patient is asked to lift his thumb up.
  - Pressure is applied over the lateral side of the base of the proximal phalanx.

  **Ulnar nerve - Little finger abduction**
  - Ask the patient to abduct the little finger with MCP in slight flexion.
  - Pressure is applied over the base of the proximal phalanx.

  **Radial nerve - Wrist extention**
  - Ask the patient to make a fist and lift the wrist up.
  - Pressure is applied over the dorsum of hand.

  **Lateral popliteal nerve** - **Foot dorsiflexion**
  - Ask the patient to lift the foot up.
  - Pressure is applied over the dorsum of foot.

- The score is derived for each as follows:

MRC = 5 scores 0
MRC = 4 scores 1
MRC = 3 scores 2
MRC < 3 scores 3

**THE SCORES NEED TO BE RECORDED ON FORM A ALONG WITH THE MONOFILAMENT RESULTS ON THE HANDS AND FEET DIAGRAM**

Add the three individual scores for the total severity score

**Examiner B**

Record on Form B

- The presence of signs of reaction
- The treatment including any drug dosages that would best manage the reaction
- The severity of the reaction – not present, mild, moderate or severe
- Mark an X on the line to indicate the degree of severity, the further to the right end of the line (10) the more severe.
- Free text box for any additional comments.

**PROTOCOL FOR ASSESSMENT OF TYPE 1 REACTION CLINICAL SEVERITY SCORE**

1) An individual who is eligible should be invited to take part in the study and be given the information about the trial verbally and in writing.
2) If they consent to taking part then continue with steps 3 and on.
3) Examiner A performs the initial examination including completing the assessment form A.
4) Examiner B who is unaware of A's findings then undertakes the assessment he/she would normally perform for an individual in reaction and then complete the form B.
5) The treatment of the reaction is recorded
6) The two sets of data are then placed together in an envelope without being compared.
7) In a given centre an investigator who is designated the role of Examiner A can not subsequently perform Examiner B's role and vice versa.
8) Analysis of the data will be performed by Drs Lockwood, Nicholls and Walker at LSHTM.

## Appendix 3.2

| Clinical Severity Scale Study | Form A |
|---|---|

| Study Patient Number: <br> *Enter Study code* ie BRAS1 | _____ |
|---|---|
| Today's Date: <br> *dd-mmm-yyyy* | __/____/_____ |
| Assessed by: <br> *Name* | |
| Is this a presentation of a new Type 1 reaction? | Yes/No |

| Sex: <br> *(M/F)* | |
|---|---|
| Age: <br> *(Yrs)* | |
| Classification: <br> *BT/BB/BL/LL/PN./I* | |
| MDT Start Date: <br> *dd-mmm-yyyy* | __/____/_____ |
| Duration of Type1 reaction symptoms on this occasion | |
| Current treatment for reaction symptoms (including dose) | |
| Number of previous Type 1 reactions <br> - if first record 0 | |
| Time since last Type 1 reaction ( in months) <br> **-** if first reaction then record X | |
| Duration of leprosy: <br> *Number of **months** since first sign* | |
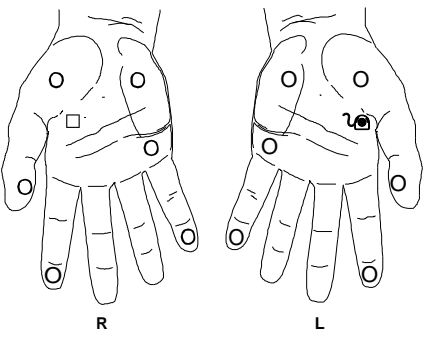
Type 1 reaction Severity Assessment

| | Criteria | 0 | 1 | 2 | 3 | Score |
|---|---|---|---|---|---|---|
| A1 | Degree of inflammation of skin lesions | **None** | Erythema | Erythema, raised | Ulceration | |
| A2 | Number of raised and/or inflamed lesions | **0** | **1-5** | **6-10** | **>10** | |
| A3 | Peripheral oedema due to reaction | **None** | **Minimal** | Visible, but not affecting function | Oedema affecting function | |
| A4 | Nerve pain and/or paraesthesia | **None** | Pain on activity | Pain at rest | Pain disturbing sleep | |
| A5 | Nerve tenderness (worst affected nerve only) | **None** | Mild tenderness | Withdrawal or wincing | Not allowing palpation | |
| A6 | Fever (°C) | **<37.5** | **37.5-38.5** | **38.6-40** | **>40** | |
| | TOTAL A SCORE | | | | | |

**Sensory Assessment by Monofilament**
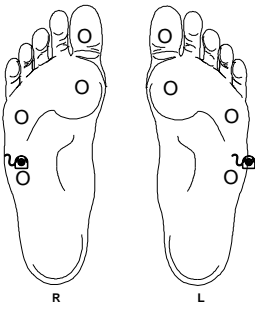


Mark the symbols clearly on the diagram above:
2g – Purple - ▲
10g – Orange - ■
Not felt at 10g **- A**
Missing/unable to test – Mark =U

Mark the symbols clearly on the diagram above:
10g – Orange ■
300g – Pink **#**
Not felt at 300g **- A**
Missing/unable to test – Mark = U

| | Hands | Purple 2g Monofilament scores | | | | Orange 10g Monofilament scores | | | Score |
|---|---|---|---|---|---|---|---|---|---|
| | Nerves | 0 | 1 | 2 | 3 | 4 | 5 | 6 | |
| B1 | RIGHT Trigeminal | | | | | | | Not felt | |
| B2 | LEFT Trigeminal | | | | | | | Not felt | |
| B3 | RIGHT ulnar | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| B4 | LEFT ulnar | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| B5 | RIGHT median | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| B6 | LEFT median | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| | Feet | Orange 10g Monofilament scores | | | | Pink 300g Monofilament scores | | | Score |
| | Nerves | 0 | 1 | 2 | 3 | 4 | 5 | 6 | |
| B7 | RIGHT posterior tibial | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| B8 | LEFT posterior tibial | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| | **TOTAL B SCORE** | | | | | | | | |

Motor Assessment by VMT

| | Nerve | 0 | 1 | 2 | 3 | Score |
|---|---|---|---|---|---|---|
| C1 | RIGHT Facial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C2 | LEFT Facial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C3 | RIGHT Ulnar | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C4 | LEFT Ulnar | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C5 | RIGHT Median | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C6 | LEFT Median | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C7 | RIGHT Radial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C8 | LEFT Radial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C9 | RIGHT Lateral Popliteal | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C10 | LEFT Lateral Popliteal | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| TOTAL C SCORE | | | | | | |

| CLINICAL SEVERITY SCORE = A + B +C | |
|---|---|

# Appendix 3.3

| Clinical Severity Scale Study | **Form B** |
|---|---|

| | |
|---|---|
| **Study Patient Number:** <br> *Enter Study code* ie BRAS1 | ____/_____ |
| **Today's Date:** <br> *dd-mmm-yyyy* | ___/_____/_____ |
| **Assessed by:** *Name* | |
| **Is this a new presentation of a Type 1 reaction?** | Yes/No |

| **Features of type 1 reaction** | |
|---|---|
| **Signs of reaction** | **Present (Y/N)** |
| Skin involvement | |
| Nerve involvement | |
| Oedema | |
| Fever | |
| **Treatment of type 1 reaction** | |
| **Treatment of reaction** | **Drug including dose** |
| No treatment | |
| Paracetamol/ Non-steroidal anti-inflammatory drug | |
| Corticosteroids | |
| Other (please indicate reason below) | |
| **Severity of type 1 reaction   (circle answer)** | |

**No evidence of reaction        Mild                Moderate            Severe**

**Please mark an X on the line below to indicate the severity of the reaction (10 = most severe)**

_____

**0**                                                                                                  **10**

| **Additional comments (if any):** |
|---|
| |

# Appendix 3.4

**PROTOCOL**

**A Phase 2 trial to investigate the safety of early intravenous high dose methylprednisolone in acute neuritis and type 1 reactions (T1R) with neuritis.**

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**Form 1- Eligibility**

**Entry criteria**

- Individuals with clinical evidence of T1R **with** new nerve function impairment (NFI). A T1R is clinically defined by the acute development of erythema and oedema of skin lesions, often accompanied by neuritis and oedema of the hands, feet and face. New NFI is defined as less than **6 months** duration of reduction in sensory, motor or autonomic function on history or examination.

**OR**

Individuals with new nerve function impairment without inflammation of skin lesions (**if** skin lesions are present)

- Aged 16-65
- Weigh more than 30Kg

**Exclusion criteria**

- Anyone unwilling to give consent.
- **T1R without new NFI**.
- Individuals with severe active infection such as tuberculosis.
- Individuals with severe intercurrent disease (cardiac, hepatic or renal disorder)
- Contraindications to high dose methylprednisolone such as peptic ulcer disease, diabetes mellitus, glaucoma and uncontrolled hypertension or known allergy to methylprednisolone.
- Pregnant women and those females of child bearing capacity without at least one month of adequate contraception.
- Individuals who have taken systemic steroids or thalidomide within 3 months.
- Anyone unwilling to be admitted or return for follow-up.

**Form 2 – Informed consent**

**Consent**

- Trial carefully explained by investigator.
- Written explanatory note available in Nepali and English.
- Individual's signature or mark obtained as proof of consent to take part in the trial.
- Individual's signature or mark obtained as proof of consent to photography (participation in the trial is not dependent on giving consent for photography).
- Signature of enrolling researcher.
- **Attach adhesive label stating MP Study and the patient's STUDY number to front of clinic notes.**

- **RECORD DATE, NAME ,CLINIC NUMBER, STUDY NUMBER AND CATEGORY (SEVERE OR MILD) IN THE METHYLPREDNISOLONE STUDY RECORD BOOK IN STUDY BOX**

**Form 3 - History and examination at registration**

**Pre-treatment assessment**

2. History
   - Date of onset of T1R
   - Symptoms of T1R  (with particular attention to date of onset)
   - Leprosy classification and date of diagnosis
   - Leprosy treatment (type, starting and completion dates)
   - Time since completion of leprosy treatment
   - Previous history of T1R

3. Clinical Examination
   - Full general clinical examination including T°, blood pressure    and weight
   - Leprosy clinical examination

     i.  Nerves  - signs and symptoms of neuritis
                  (pain, tenderness, enlargement)

     ii. Skin    - location of lesions (body chart)
                - type of lesions
                       (patches, plaques, papules, nodules)
                     - signs of inflammation in lesions
                     - oedema of the hands and/or feet

**Form 4 - Sensory testing (ST)**

- Trigeminal*, ulnar, median and posterior tibial nerves on each side. The Purple 2g and Orange 10g Semmes-Weinstein monofilaments are used at 3 sites for each nerve on the hand (median and ulnar). The Orange 10g and Pink 300g monofilament at 3 sites for the posterior tibial nerves. (* cotton wool is used)

  - Record on the diagram of the hands and feet the result of the monofilament testing at each test site using the following symbols
    Purple 2g felt -   ▲
    Orange 10g felt - ■
    Pink 300g felt -   #
    Neither monofilament felt – **A**
    (Orange not felt on hands, Pink not felt on feet then mark an **A** at the site in question).

**Form 5** - **Voluntary motor testing (VMT)**
   i.  Facial, ulnar, radial, median and lateral popliteal nerves on each side. Using the modified MRC grading for muscle power.
       **Facial nerve -** Forced eye closure (orbicularis oculi)
       **Median nerve** - Thumb abduction (abductor pollicis brevis)
       **Ulnar nerve** - Little finger abduction (abductor digiti minimi)
       **Radial nerve** - Wrist extension (extensor muscles)
       **Lateral popliteal nerve-** Foot dorsiflexion (tibialis anterior,            peroneus longus and brevis)

   **Testing procedure for each movement**

   The patient should be seated comfortably.

*Facial nerve -* *Forced eye closure*
- The patient is asked to close the eyes as tight as (s)he can.
- The tester tries to pull down the lower lid on both sides using his/her thumbs

**Median nerve** -Thumb abduction
- The wrist is held in extension and the patient is asked to lift his thumb up.
- **Pressure is applied over the lateral side of the base of the proximal phalanx.**

Ulnar nerve **- Little finger abduction**
- **Ask the patient to abduct the little finger with MCP in slight flexion.**
- **Pressure is applied over the base of the proximal phalanx.**

Radial nerve **- Wrist extention**
- **Ask the patient to make a fist and lift the wrist up.**
- **Pressure is applied over the dorsum of hand.**

Lateral popliteal nerve - **Foot dorsiflexion**
- **Ask the patient to lift the foot up.**
- **Pressure is applied over the dorsum of foot.**

ii. Score is derived for each nerve.
MRC = 5 scores 0
MRC = 4 scores 1
MRC = 3 scores 2
MRC < 3 scores 3

If there is evidence of NFI for a given nerve then confirmation of the duration of the NFI should be sought from the affected individual to determine whether or not this is new.

| MRC modified grading of muscle power | | Severity Scale Score |
|---|---|---|
| Score | Muscle response | |
| 5 | Full range of movement (FROM) | 0 |
| 4 | FROM but less than normal resistance | 1 |
| 3 | FROM but no resistance | 2 |
| 2 | Partial range of movement with no resistance | 3 |
| 1 | Perceptible contraction of the muscle not resulting in joint movement | 3 |
| 0 | Complete paralysis | 3 |

**Form 7**

**Pre-enrolment Screening Tests**
- Full clinical examination.
- Full blood count, creatinine, random blood sugar.
- Stool specimen will be examined for ova, cysts and parasites
- Chest radiograph
- Sputum examination for AFB
- Pregnancy test

**Staging and initial trial investigations**
- Skin smears from four sites including both ear lobes and two active skin lesions (the elbow or thigh should be used if there is only one skin lesion and both should be used if there are none).Smears are unnecessary if they have been done within 3 months of enrolment into the trial.
- 6mm punch biopsy of skin for Ridley Jopling classification if not already done.
- 6mm punch biopsy of skin at baseline. The site of biopsy should be clearly documented on **FORM 8** to enable subsequent biopsies to be taken from an adjacent site. Ulcerated lesions should be avoided if possible. **USE PLAIN 1 0R 2% LIGNOCAINE DO NOT USE LIGNOCAINE WITH ADRENALINE**. The skin biopsy should be halved **lengthwise**. Place half in the test vials with RNA*later*, the other half should be placed in a dry test vial. A yellow top should be placed in the cap of the vial containing the RNA*later*. These should be taken by the **doctor** to the laboratory and handed to the member of laboratory staff responsible
- 5 ml heparinised venous blood specimen for trial at baseline.

**Subsequent trial investigations**
- 5ml heparinised venous blood specimens at days 3, 14, 28 and weeks 16 and 24.
- 6mm punch biopsy of skin at day 4 and week 16 taken from same site as baseline specimen.

**Admission**
- All those enrolled in the trial will be admitted for the infusions (either methylprednisolone or placebo)
- Period of admission is 7-14 days for uncomplicated cases

**Allocation of treatment**

**Preparatory actions.**

A Randomisation Table has been prepared specifying the order in which individuals recruited to the study are to be allocated to MP and P arms. This lists allocations within two separate lists, one for individuals with severe reactions and one for individuals with mild reactions, as clinically assessed at intake. Each list has been generated using random numbers in an Excel spreadsheet and includes a sequence number. In each, the randomisation is organised so that, among each block of four individuals recruited to the study, there are two allocations to the MP arm and two the P arm. Example(not the actual allocations):

| Seq No. | Severe Reaction List | Seq No. | Mild Reaction List |
|---|---|---|---|
| 1 | MP | 1 | MP |
| 2 | MP | 2 | Placebo |
| 3 | Placebo | 3 | Placebo |
| 4 | Placebo | 4 | MP |
| 5 | Placebo | 5 | MP |
| | Etc. | | Etc. |

If this were the true allocation, the first recruited individual with a severe reaction would be allocated to the MP arm, the second also to the MP arm, the third to the Placebo arm etc.

The randomisation allows for up to 40 allocations of individuals with severe reactions and up to 40 individuals with mild reactions. The total recruitment to the study will be 60, 30 in each arm.

At this stage, the relative frequency of mild and severe reactions is unknown and no target has been set for the numbers of mild or severe reactions.

**Allocation process**

To ensure blinding of clinicians to allocation to treatment arms, the following procedure is to be followed.

Two designated individuals in the hospital pharmacy will need to provide prednisolone or placebo tablets and normal saline or methylprednisolone infusions during the first three days after enrolment to the trial. We propose this be the responsibility of just one senior pharmacist and that this individual alone have access to information about the allocation to treatment arms. It should be the responsibility of this individual to maintain a record of recruitment and to deliver appropriate tablets and infusions to individual patients in the hospital ward. This will be achieved as follows:

From the lists in the Randomisation Table we have prepared duplicate sets of 80 labelled and sealed envelopes containing the details of how each individual recruited to the study is to be allocated to Placebo and MP arms. Each envelope and its contents will be linked to the Randomisation Table by the details of reaction severity and sequence number.

The labelling on each envelope will identify the reaction severity group – Mild or Severe – and the sequence number within the group – 1 to 40. The labelling will NOT specify the treatment allocation (See Appendix One).

Inside the sealed envelopes will be placed a one page form with three sections with contents as follows (See Appendix Two):

Part One describes the allocated treatment arm, either methyl prednisolone or placebo.
Part Two provides space for the pharmacist to record the patients details.
Part Three guides the pharmacist as to the tablets and infusion to be collected from pharmacy and delivered to the patient in the ward on each of the first three days from intake.

The full set of sealed envelopes will be handed to the designated pharmacist at the start of the study. They are to be kept in a secure place. No other staff should have access to the envelopes. Only the pharmacist is permitted to open the envelopes.

When a clinician identifies an individual to be enrolled in the study he will request the pharmacist to register this individual and identify the reaction group as mild or severe. The pharmacist will then take the following actions:

Register the new case on a master list held in the pharmacy, recording details of hospital card number, name and registration date. This list will have the format following:

| Severe Reaction Group | | | | Mild Reaction Group | | | |
|---|---|---|---|---|---|---|---|
| Envelope Number | Hospital Number | Patient Name | Date | Envelope Number | Hospital Number | Patient Name | Date |
| 1 | | | | 1 | | | |
| 2 | | | | 2 | | | |
| 3 | | | | 3 | | | |
| 4 | | | | 4 | | | |
| 5 | | | | 5 | | | |
| Etc | | | | Etc | | | |

The pharmacist will then give the sequence number/envelope number to the clinician so that this information can be recorded on the appropriate data form – FORM 3 and ultimately be entered onto computer (e.g. Severe Reaction Group #1).

This is an essential action that will ensure that the treatment arm can be identified on the computerized database.

The pharmacist will then retrieve the envelope containing the form specifying the treatment arm for the designated sequence number and reaction severity group. He/she will open the envelope, note the allocation to a treatment arm and file the form in a temporary location designated for individuals under process (i.e. covering the first three days from admission when MP/saline is being given).

He/she will then proceed to pharmacy, collect the appropriate infusion and tablets and deliver them to the patient in the hospital ward. The labelling on both tablets and infusion should NOT specify contents other than "Treatments relating to MP study".

The pharmacist will repeat these actions on the second and third days.

After the third day he/she will record the delivery of tablets and infusion as completed and transfer the form to a permanent file held in a secure place.

Access to files or forms by any of the clinical staff during the course of the study is denied.

**Other points**

The pharmacists must be absolutely certain to assign the correct envelope number and to provide the specified tablets and infusion on each of the first days following registration. This is fundamental to the success of the research.

A second person from the pharmacy should be involved to try and ensure that no mistakes are made.

In the event of some clinical emergency during the first 96 hours after registration of each new patient it may be necessary to ascertain if the patient concerned received MP. To provide access to this information, a second set of sealed envelopes, identical to the first, will be provided. These will be in the safe-keeping of the project director. Should the need arise, he may open the envelope relating to the individual concerned.

**Treatment**
- All individuals will receive **albendazole 400mg** daily for three days at enrolment.
- All individuals will receive **famotidine 40mg** daily for whilst on steroids.
- If the stool sample demonstrates *Entamoeba histolytica* then Metronidazole/Diloxanide furoate (**Metrin DF**) should be prescribed for 5 days
- Steroid protocol:

**Methylprednisolone/prednisolone arm ≡ 6.15g of prednisolone**

| | |
|---|---|
| **Assessment 1** | Day 1 IV methylprednisolone 1g (in 100ml Normal Saline) |
| | + **placebo tablets** |
| | Day 2 IV methylprednisolone 1g (in 100ml Normal Saline) + **placebo tablets** |
| | Day 3 IV methylprednisolone 1g (in 100ml Normal Saline) + **placebo tablets** |
| **Assessment 2** | Day 4-7 prednisolone 40mg |
| **Assessment 3** | Week 2 prednisolone 40mg |
| **Assessment 4** | Week 3 prednisolone 35mg |
| | Week 4 prednisolone 35mg |
| **Assessment 5** | Week 5 prednisolone 30mg |
| | Week 6 prednisolone 30mg |
| | Week 7 prednisolone 25mg |
| | Week 8 prednisolone 25mg |
| **Assessment 6** | Week 9 prednisolone 20mg |
| | Week 10 prednisolone 20mg |
| | Week 11 prednisolone 15mg |
| | Week 12 prednisolone 15mg |
| **Assessment 7** | Week 13 prednisolone 10mg |
| | Week 14 prednisolone 10mg |
| | Week 15 prednisolone 5mg |
| | Week 16 prednisolone 5mg |
| **Assessment 8** | Week 17 Off steroids |

**Prednisolone alone arm = 2.52g of prednisolone**

| | |
|---|---|
| **Assessment 1** | Day 1 IV placebo (100ml Normal Saline) + prednisolone 40mg |
| | Day 2 IV placebo (100ml Normal Saline)+ prednisolone 40mg |
| | Day 3 IV placebo (100ml Normal Saline)+ prednisolone 40mg |
| **Assessment 2** | Day 4-7 prednisolone 40mg |
| **Assessment 3** | Week 2  prednisolone 40mg |
| **Assessment 4** | Week 3  prednisolone 35mg |
| | Week 4  prednisolone 35mg |
| **Assessment 5** | Week 5  prednisolone 30mg |
| | Week 6  prednisolone 30mg |
| | Week 7  prednisolone 25mg |
| | Week 8  prednisolone 25mg |
| **Assessment 6** | Week 9  prednisolone 20mg |
| | Week 10 prednisolone 20mg |
| | Week 11 prednisolone 15mg |
| | Week 12 prednisolone 15mg |
| **Assessment 7** | Week 13 prednisolone 10mg |
| | Week 14 prednisolone 10mg |
| | Week 15 prednisolone 5mg |
| | Week 16 prednisolone 5mg |
| **Assessment 8** | Week 17 Off steroids |

**Form 6 - Assessment during study and after finishing prednisolone**

- Full general clinical assessment (including temperature, blood pressure and weight) at days 4, 8, 15, 29 and 4 weekly thereafter for 48 weeks.
- Leprosy clinical examination at days 4, 8, 15, 29 and 4 weekly thereafter for 48 weeks.
- Nerve function tests (ST and VMT) at days 4, 8, 15, 29 and 4 weekly thereafter for 48 weeks.
- Severity score at days 4, 8, 15, 29 and 4 weekly thereafter for 48 weeks.
- Any clinical examinations that are not part of the trial schedule should be recorded on a separate form – **Form 8**. Additional forms are kept in the protocol file in the study box.
- The date, reason and outcome of any additional examinations should also be recorded.
- **RECORD THE DATE AND NUMBER OF PATIENT'S NEXT ASSESSMENT IN THE DIARY IN THE STUDY BOX**

**Safety monitoring**

- Specific questioning at each visit with respect to adverse events or new symptoms possibly related to trial interventions.
- Major adverse events
    - i. Gastrointestinal bleeding
    - ii. Nocturia, polyuria, polydipsia
    - iii. Diabetes mellitus
    - iv. Psychosis or other mental health problems
    - v. Weight loss >5kg
    - vi. Weight gain
    - vii. Glaucoma
    - viii. Cataract
    - ix. Hypertension >160/90 on two separate readings at least one week apart
    - x. Infections
    - xi. Infected ulcers
    - xii. Corneal ulcer
    - xiii. Tuberculosis
    - xiv. Night sweats
- Minor adverse events
    - i. Moon face
    - ii. Acne
    - iii. Cutaneous (including nails)fungal infections
    - iv. Gastric pain requiring antacids

**Criteria for unblinding**

- In the event of a major adverse event in the first 96 hours which is felt could be related to methylprednisolone then the code can be broken for that individual in order to aid management of the problem.

**Recurrence of T1R OR neuritis**

- Criteria for using additional prednisolone

    - i. Sustained deterioration for a period of at least two weeks of:
        - a. Deterioration in nerve function

b. Nerve pain unresponsive to analgesics
c. Palpable swelling of skin patches
d. New erythematous and raised skin patches

    ii. Deterioration in nerve function which the study doctors believe requires immediate additional prednisolone

- The patient must be examined by at least two of the study doctors and they should be in agreement about giving the patient additional prednisolone.
- The reasons for the additional prednisolone and the date started should be recorded.

**Regimen for additional prednisolone**

- If there is recurrence of T1R with NFI (or nerve pain unresponsive to analgesics) on treatment then add extra prednisolone to make up a total of 40mg and then taper according to the original regimen.

- If there is recurrence of T1R with skin signs but **no** NFI then:

    i. If recurrence within the first ten weeks of treatment or there is facial involvement then add extra prednisolone to make up a total of 40mg and then taper according to the original regimen.
    ii. If recurrence after ten weeks of treatment then add extra prednisolone to make up a total of 20mg and then taper according to the original regimen.

**Monitoring the trial**

- Drs KV Krishna Moorthy and PS S Sundar Rao who are independent of the conception, design and management of the trial have agreed to act as trial monitors.

**Data entry**

- Each subject enrolled into the study will have an individual case booklet for recording of all clinical and laboratory data.
- An anonymised Access database will be created for storage of trial data which will subsequently be analysed using standard statistical packages.

**Late Clinic Attendances**

If a trial subject does not attend a scheduled assessment then they will be contacted and asked to come to the next clinic for their assessment. It is essential that the date of the attendance is recorded. **The number of the Assessment should not be changed regardless of how late the assessment is carried out.**
The next assessment after this should be scheduled as though the original assessment had been performed as planned. If the assessment is so late that the following assessment has also been missed then the next assessment should be scheduled for 28 days (four weeks) later.
**If a participant has missed certain trial investigations then these should be performed when they next attend.**

**Unscheduled Clinic Attendances/examinations**

- All unscheduled examinations (if an inpatient) or clinic attendances should be recorded on Form 8.
- If the reason for the attendance/examination is related to their leprosy diagnosis then Forms 4,5 and 6 should also be completed.
- All Forms should be completed in red ink or clearly marked at the top UNSCHEDULED.
- The **next** assessment number should be used for unscheduled visits/examinations.
- It should be documented if the clinician feels the attendance is related to corticosteroids.

**Non reactional controls**

Borderline patients who are undergoing a diagnostic skin biopsy should be approached to see if they would consent to a 6mm skin punch biopsy done at the same time as their diagnostic one.
They should be guided through the specific information leaflet and if agreeable asked to sign the consent form for the additional biopsy. The aim is to recruit 10 controls.
**THESE CONTROLS MUST <u>NOT</u> BE IN REACTION.**

**CHECKLIST ON ADMISSION**

Type 1 reaction with nerve function impairment or neuritis less than 6 months duration.
Reaction mild or severe
Inform pharmacy of admission and reaction type
Physical examination
FBC, creatinine, random glucose
Stool sample
Chest Xray
Sputum
Pregnancy test females with childbearing capacity
Appropriate contraception in females with childbearing capacity
Prescribe albendazole and famotidine
Intravenous access

**REQUIREMENTS AT EACH VISIT**

Assessment 1 (Enrolment/Admission)

Form 1 Eligibility
Form 2 Consent
Form 3 History and examination
Form 4 Sensory testing
Form 5 Motor testing
Form 7 Investigations
FBC, Creatinine, random blood glucose
5ml of venous blood collected in heparin (for whole blood assay and Luminex)
Stool specimen
Chest Xray
Sputum
Pregnancy test
Contraception
Albendazole and famotidine
Skin smears if not done in previous 3 months
Skin biopsy for Ridley Jopling classification if not done at a previous attendance
Skin biopsy from the edge of an area of reactional (non-ulcerated) skin
Inform pharmacy of clinical categorisation of the reaction – severe or not severe
Intravenous access
Infusion duration 1 hour
Take oral prednisolone or placebo at same time

**Assessment 2 (Day 4- the day after the last infusion)**
Form 4
Form 5
Form 6
Form 7
5ml of venous blood collected in heparin (for whole blood assay and Luminex)
Skin biopsy from the edge of an area of reactional (non-ulcerated) skin
Assessment 3 (Day 8 after one week of steroids)
Form 4
Form 5
Form 6

**Assessment 4 (Day 15 after two weeks of steroids)**
Form 4
Form 5
Form 6
Form 7
5ml of venous blood collected in heparin (for whole blood assay and Luminex)
**REMOVE BIOPSY SUTURES**

**Assessment 5 (Day 29 after four weeks of steroids)**
Form 4
Form 5
Form 6
Form 7
5ml of venous blood collected in heparin (for whole blood assay and Luminex)

**Assessment 6 (Day 57 after eight weeks steroids)**
Form 4
Form 5
Form 6

**Assessment 7 (Day 85 after 12 weeks steroids)**
Form 4
Form 5
Form 6

**Assessment 8 (Day 113 after 16 weeks steroids)**
Form 4
Form 5
Form 6
Form 7
5ml of venous blood collected in heparin (for whole blood assay and Luminex)
Skin biopsy from an area of reactional (non-ulcerated) or previously reactional skin
STOP STEROIDS IF NO DETERIORATION

**Assessment 9 (Day 141 after 20 weeks in trial)**
Form 4
Form 5
Form 6

**Assessment 10 (Day 169 after 24 weeks in trial)**

Form 4
Form 5
Form 6
Form 7
5ml of venous blood collected in heparin (for whole blood assay and Luminex)
**Assessment 11 (Day 197 after 28 weeks in trial)**
Form 4
Form 5
Form 6
**Assessment 12 (Day 225 after 32 weeks in trial)**
Form 4
Form 5
Form 6
**Assessment 13 (Day 253 after 36 weeks in trial)**
Form 4
Form 5
Form 6
**Assessment 14 (Day 281 after 40 weeks in trial)**
Form 4
Form 5
Form 6
**Assessment 15 (Day 309 after 44 weeks in trial)**
Form 4
Form 5
Form 6
**Assessment 16 (Day 337 after 48 weeks in trial)**
Form 4
Form 5
Form 6

**USE OF THE TRIAL FORMS**
**Form 1**
Used to screen and register patients at the first visit
**Form 2**
Consent form for the MP study.
**Form 3**
Initial history and examination for admission
**Form 4**
Monofilament sensory testing
**Form 5**
Voluntary motor testing
**Form 6**
Follow up assessments including documentation of the current prednisolone dose and any additional prednisolone that may be required.
**Form 7**
Recording any investigations performed at an assessment.
**Form 8**
Information that might need to be recorded but is not covered by the other trial documentation. It should also be used to document skin biopsy procedure and site.
It should also be used if a participant withdraws and **wishes** to give a reason.
**TRIAL SPECIMENS**

**Skin biopsies**

- 6mm punch biopsies taken from the edge of an active non–ulcerated skin lesion should be bisected.
- Half is to be placed in a cryogenic vial with a WHITE cap and snap frozen in liquid nitrogen.
- The other half is to be placed together with RNA*later* in a cryogenic vial with a YELLOW cap. This should be stored overnight at 4°C to allow penetration of the RNA*later*. The excess should be removed the following day and the specimen in the vial stored at -80°C.
- Each vial should be clearly labelled with the participants unique study code and the date.

**Venous blood samples**

- These should be collected into heparin. 5ml is required.
- The stimulation is undertaken and the supernatant removed and frozen at -70°C after 24 hours with the exception of the DAY 4 (if taken on a Saturday) sample which will be stimulated for 48 hours.

**Appendix One**
**Envelope labelling**

| | Methyl Prednisolone Study<br><br>Reaction type:<br><br>Sequence Number: | |
|---|---|---|

**Appendix Two**
**Form enclosed in Envelope**
**Version One – for Methylprednisolone arm**

| | **Severity Group**: | |
|---|---|---|
| | **Sequence Number** | |
| **Part One** | **Study Arm** | **Methylprednisolone** |
| **Part Two** | **Patient details**:<br><br>Name<br>Clinic Card<br>Study Number<br>Enrolment date: | |
| **Part Three** | | |
| **Day 1** | **8 placebo tablets**<br>**1 g Methylprednisolone IV** | **Completed by**: |
| **Day 2** | **8 placebo tablets**<br>**1g Methylprednisolone IV** | **Completed by**: |
| **Day 3** | **8 placebo tablet**<br>**1 g Methylprednisolone IV** | **Completed by**: |

**Version Two – for Placebo arm**

| | **Severity Group**: | |
|---|---|---|
| | **Sequence Number** | |
| **Part One** | **Study Arm** | **Placebo** |
| **Part Two** | **Patient details**:<br><br>Name<br>Clinic Card<br>Study Number<br>Enrolment date: | |
| **Part Three** | | |
| **Day 1** | **40mg prednisolone tablets**<br>**IV Normal Saline** | **Completed by**: |
| **Day 2** | **40mg prednisolone tablets**<br>**IV Normal Saline** | **Completed by**: |
| **Day 3** | **40mg prednisolone tablets**<br>**IV Normal Saline** | **Completed by**: |

# Appendix 3.5

| MP Study – Patient Screening and registration | Form 1 |
|---|---|

*Ask questions 1-4 of each patient who has evidence of a Type 1 reaction and new nerve function impairment. Mark each answer Yes or No*

| Patient Screening – Part One – Demographics | | | |
|---|---|---|---|
| 1. | Is the patient below 16 years of age? | Yes | No |
| 2. | Is the patient above 65 years of age? | Yes | No |
| 3. | Is the patient unable to come regularly for follow up? | Yes | No |
| 4. | Is the urine sugar test positive ? | Yes | No |

***If you answered YES to any of these questions, the patient must NOT be entered into the* MP Study. *You should place this form with clinic records and treat the patient according to the standard procedures in your clinic.***

**If the answer to each question was NO you should explain to the patient why special screening is needed:**

1) **Need to enrol patients to participate in the MP research project**
2) **Need to meet MP MO to see if qualify for inclusion in the study**

**Take the patient to the MP Medical Officer with this Form**

MP  MO - Ask the patient the following questions. Mark each answer Yes or No

| Patient Screening – Part Two – History and Current Condition | | | |
|---|---|---|---|
| 5. | Is there any reason why the patient should not start reaction treatment? | Yes | No |
| 6. | Is there evidence that the patient will be irregular in attending the clinic for regular visits? | Yes | No |
| 7. | Does the patient have a history or clinical signs of non-leprosy-related  neurological conditions or of psychosis/abnormal behaviour? | Yes | No |
| 8. | Does the patient have any history of mental incapacity? | Yes | No |
| 9. | Does the patient have any evidence of TB? | Yes | No |
| 10. | Does the patient have any history of diabetes mellitus? | Yes | No |
| 11. | Does the patient have any history of alcohol abuse? | Yes | No |
| 12. | Does the patient have any current illness or taking any long-term treatment? | Yes | No |
| 13. | Does the patient have any severe infection? | Yes | No |
| 14. | Is the patient taking steroids or has taken steroids in the last three months for any reason? | Yes | No |
| 15. | Is there any other reason for exclusion? ie pregnancy | Yes | No |

**If you answered YES to any of these questions, the patient must NOT be entered into the MP Cohort Study. You should:**

1. **Place this form with clinic notes**
2. **Explain to the patient that they do not qualify for inclusion in the *MP* study**
3. **Treat the patient according to the standard procedures in your clinic.**

**Only if you answered NO to all the questions 5 to 15 should  you continue with the neurological examination.**

| | |
|---|---|
| **Patient's Name:** | |

*From the Register of patients enrolled in the study assign the next available number*

| | |
|---|---|
| **Study Patient Number:**<br>*Enter AN1,AN2 etc* | _____ |
| **Enrolment Card Number:** | |
| **Study Enrolment Date:**<br>*dd-mmm-yyyy* | ___/_____/_____ |
| **Enrolled by:** | |
| **Sex:**<br>*(M/F)* | |
| **Age:**<br>*(Yrs)* | |
| **Occupation** | *Primary* / *Secondary* |
| **Reading and writing ability** *(Y/N)* | *Reading* / *Writing* |
| **Classification:**<br>*TT/BT/BB/BL/LL/PN/I* | |
| **Type of MDT?**<br>*PB/MB* | |
| **MDT Start Date:**<br>*dd-mmm-yyyy* | ___/_____/_____ |
| **MDT CompletionDate:**<br>*dd-mmm-yyyy            (X if still taking MDT)* | |
| **Duration of leprosy:**<br>*Number of **months** since first sign       (missing value = 999)* | |

| **REACTION TYPE** (CIRCLE) | **SKIN AND NERVES** | **SKIN ONLY** | **NERVES ONLY** |
|---|---|---|---|
| **PREVIOUS STEROIDS** | | | |
| **CURRENT DOSE OF PREDNISOLONE** | | | |
| **DOSE WHEN SYMPTOMS DETERIORATED** | | | |
| **TOTAL AMOUNT OF STEROIDS IN LAST 12 MONTHS** | | | |

Longstanding Nerve Status – Record the details of the history/ examination of nerve status 6 months prior to enrolment date

**Record of sensory or motor impairment LONGER than six months  duration at the time of enrolment**

| Nerve | Facial | | Ulnar | | Median | | Radial | | Lat Pop | | Pos Tib | | Sural | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Side** | R | L | R | L | R | L | R | L | R | L | R | L | R | L |
| **Sensory (Y/N)** | | | | | | | | | | | | | | |
| **Motor (Y/N)** | | | | | | | | | | | | | | |

263

**Appendix 3.6**

| MP Study – History and Examination | **Form 3** |
|---|---|

| **Study Patient Number:** *Enter AN1,AN2 etc* | _____ |
|---|---|
| **Registration Card Number:** | |
| **Today's Date:** *dd-mmm-yyyy* | __/____/____ |
| **Assessment Number:** *(1 for newly registered cases)* | |
| **Assessed by:** *Name* | |
| **TOTAL REACTION SCORE A+B+C** | |

| Part A – Patient History |
|---|

Ask the patient if s/he has experienced any of the following symptoms <u>in the last 6 months</u>:

| *Patient's report of new symptoms (<6 months old)* | *RIGHT* | | | | *LEFT* | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *ELBOW* | *HAND* | *KNEE* | *FOOT* | *ELBOW* | *HAND* | *KNEE* | *FOOT* | *OTHER* |
| *Diminished sensation* – **eg unable to feel hot or cold, numbness (Y/N)** | | | | | | | | | |
| *New Weakness* **(Y/N)** | | | | | | | | | |
| *Paraesthesia* - **eg pins and needles, insects crawling (y/n)** | | | | | | | | | |
| *Nerve Pain* **eg burning sensation, shooting pain (Y/)** | | | | | | | | | |

Patient's report of s*kin lesions and extent of disease*

| | | | | |
|---|---|---|---|---|
| **How long have they had inflamed skin patches?** | | | | |
| **Have they developed new skin patches recently?** (Y/N) | | | | |
| **How many new skin patches have developed recently?** | | | | |
| **Facial patch?** (Y/N) | | | | |
| **Facial patch inflammation.** (Circle) | NONE | ERYTHEMA | ERYTHEMA AND RAISED | ULCER-ATED |
| **Have they had a reaction in the past?** (Y/N) | | | | |
| **Previous reaction documented in notes?** (Y/N) | | | | |
| **Previous reaction confirmed by patient?** (Y/N) | | | | |

| Study Patient Number:<br>*Enter AN1,AN2 etc* | _____ |
|---|---|
| **Registration Card Number:** | |
| **Visit Number:**<br>*(1 for newly registered cases)* | |
| **Today's Date:**<br>**dd-mmm-yyyy** | ___/____/_____ |

***Does the patient have any current illnesses or drug treatment (other than MDT)? If yes, please describe***
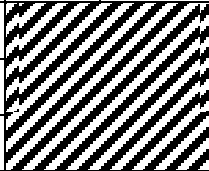
| Part B – Clinical Assessment | | | |
|---|---|---|---|
| *Weight (Kg)* | *Height (cm)* | *Temperature (°C)* | *Blood pressure (mmHg)* |
| | | | |

| Part C – Details of Reaction and Neuritis |
|---|
| Reaction Severity Assessment |

Score reaction signs and symptoms in the right hand column:

| | Criteria | 0 | 1 | 2 | 3 | Score |
|---|---|---|---|---|---|---|
| A1 | **Degree of inflammation of skin lesions** | **None** | **Erythema** | **Erythema, raised** | **Ulceration** | |
| A2 | **Number of raised and/or inflamed lesions** | **0** | **1-5** | **6-10** | **>10** | |
| A3 | **Peripheral oedema due to reaction** | **None** | **Minimal** | **Visible, but not affecting function** | **Oedema affecting function** | |
| A4 | **Nerve pain and/or paraesthesia** | **None** | **Pain on activity** | **Pain at rest** | **Pain disturbing sleep** | |
| A5 | **Nerve tenderness (worse affected nerve only)** | **None** | **Mild tenderness** | **Withdrawal or wincing** | **Not allowing palpation** | |
| | **TOTAL A SCORE** | | | | | |

| | Any | Mild | Form 3 Severe |
|---|---|---|---|
| **Reactional skin lesions?** *(Y/N)* | | | |
| **Type 1 reaction?** *(Y/N)* | | | |
| **Neuritis – New nerve function impairment according to the monofilament or voluntary muscle test?** *(Y/N)* | | ///// | ///// |
| **Neuritis – other evidence?** *(Y/N)* | | ///// | ///// |
| **Mixed-signs neuritis?** *(Y/N)* | | ///// | ///// |

| **Record here any problem with the patient affecting the completion of the assessment:** |
|---|
| |

Record details of any eye problems:

| Eye Problems | | |
|---|---|---|
| **Right** | | **Left** |
| | **Cataract** *(Y/N)* | |
| | **Visual Acuity** | |

Part D– Admission

| | |
|---|---|
| **Study Patient Number:** *Enter AN1,AN2 etc* | _____ |
| **Registration Card Number:** | |
| **Today's Date:** *dd-mmm-yyyy* | ___/_____/_____ |
| **Randomisation Envelope Number** *(to be done by pharmacist)* | |
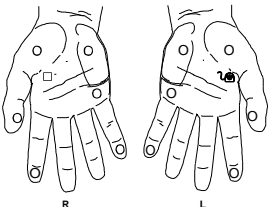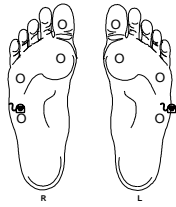
Inform ward staff of admission of trial patient
Inform pharmacy of admission of trial patient and category ie severe or not severe
Request the baseline and trial investigations
Ensure that all the necessary medication including albendazole is prescribed

| *MP  Data Entry:* |
|---|
| *Entered by:*                                     *Entered on:* |

266

# Appendix 3.7

**Sensory Assessment by Monofilament**

| | |
|---|---|
| **Study Patient Number:** *Enter AN1,AN2 etc* | _____ – |
| **Registration Card Number:** | |
| **Today's Date:** *dd-mmm-yyyy* | ___/___/_____ |
| **Assessment Number:** *(1 for newly registered cases)* | |

| **Assessed by:** *Name* | **Other Anandaban Tests points affected?    Y/N** |
|---|---|

Right   Left

**Mark the symbols clearly on the diagram above:**
2g – Purple - ▲
10g – Orange - ■
Not felt at 10g **- A**
Missing/unable to test – Mark =U

Right   Left

**Mark the symbols clearly on the diagram above:**
2g – Purple - ▲
10g – Orange - ■
Not felt at 10g **- A**
Missing/unable to test – Mark =U

| | **Hands** | **Purple 2g Monofilament scores** | | | | **Orange 10g Monofilament scores** | | | **Score** |
|---|---|---|---|---|---|---|---|---|---|
| | **Nerves** | **0** | **1** | **2** | **3** | **4** | **5** | **6** | |
| **B1** | **RIGHT Trigeminal** | Felt | | | | | | Not felt | |
| **B2** | **LEFT Trigeminal** | Felt | | | | | | Not felt | |
| **B3** | **RIGHT ulnar** | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| **B4** | **LEFT ulnar** | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| **B5** | **RIGHT median** | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| **B6** | **LEFT median** | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| | **Feet** | **Orange 10g Monofilament scores** | | | | **Pink 300g Monofilament scores** | | | **Score** |
| | | **0** | **1** | **2** | **3** | **4** | **5** | **6** | |
| **B7** | **RIGHT posterior tibial** | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| **B8** | **LEFT posterior tibial** | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| | **TOTAL B SCORE** | | | | | | | | |

**Appendix 3.8**

| MP Study – Regular Assessments | Form 5 |
|---|---|

**Motor Assessment by VMT**

| | |
|---|---|
| **Study Patient Number:**<br>*Enter AN1,AN2 etc* | |
| **Registration Card Number:** | |
| **Today's Date:**<br>*dd-mmm-yyyy* | |
| **Assessment Number:**<br>*(1  for newly registered cases)* | |
| **Assessed by:**<br>*Name* | |

| | Nerve | 0 | 1 | 2 | 3 | Score |
|---|---|---|---|---|---|---|
| C1 | RIGHT Facial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C2 | LEFT Facial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C3 | RIGHT Ulnar | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C4 | LEFT Ulnar | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C5 | RIGHT Median | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C6 | LEFT Median | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C7 | RIGHT Radial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C8 | LEFT Radial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C9 | RIGHT Lateral Popliteal | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C10 | LEFT Lateral Popliteal | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| **TOTAL C SCORE** | | | | | | |

**Comment:  Record here any problem with the patient affecting the completion of the test:**

# Appendix 3.9

| MP Study – Follow up assessments |
|---|

|  | **Form 6** |
|---|---|
| Study Patient Number:<br>*Enter AN1,AN2 etc* | _____ |
| **Registration Card Number:** |  |
| **Today's Date:**<br>*dd-mmm-yyyy* | \_\_\_/\_\_\_\_\_/_____ |
| **Assessment Number:**<br>*(1 for newly registered cases)* |  |
| **Assessed by:**<br>*Name* |  |
| **TOTAL REACTION SCORE A+B+C** |  |

| Part A – Patient History |
|---|

Ask the patient if s/he has experienced any of the following symptoms <u>since the last assessment</u>:

| *Patient's report of <u>new</u> symptoms since last assessment* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *RIGHT* | | | | *LEFT* | | | | |
| | *ELBOW* | *HAND* | *KNEE* | *FOOT* | *ELBOW* | *HAND* | *KNEE* | *FOOT* | *OTHER* |
| *Diminished sensation –* **eg unable to feel hot or cold, numbness (Y/N)** | | | | | | | | | |
| *New Weakness* **(Y/N)** | | | | | | | | | |
| *Paraesthesia -* **eg pins and needles, insects crawling (Y/N)** | | | | | | | | | |
| *Nerve Pain* **eg burning sensation, shooting pain (Y/N)** | | | | | | | | | |

| Patient's report of s*kin lesions <u>since last assessment</u>* | | | | |
|---|---|---|---|---|
| **Have the inflamed skin patches improved?** (Y/N/STABLE) | | | | |
| **How many skin patches have improved since last visit?** | | | | |
| **Have they developed new skin patches recently?** (Y/N) | | | | |
| **How many new skin patches have developed recently?** | | | | |
| **Do you feel your skin is worse, the same or better?** | | | | |
| **Facial patch?** (Y/N) | | | | |
| **Facial patch inflammation.** (Circle) | NONE | ERYTHEMA | ERYTHEMA AND RAISED | ULCER-ATED |

| Study Patient Number: | |
| :--- | :--- |
| *Enter AN1,AN2 etc* | _____ |
| **Registration Card Number:** | |
| **Today's Date:** *dd-mmm-yyyy* | |

*Has the patient had any problems with the reaction treatment or any of the following symptoms or conditions diagnosed since starting the reaction treatment?(Please tick)*

| | |
| :--- | :---: |
| Moon face | ☐ |
| Acne | ☐ |
| Cutaneous (including nails) fungal infections | ☐ |
| Gastric pain requiring antacid | ☐ |
| Gastrointestinal bleeding | ☐ |
| Nocturia, polyuria, polydipsia | ☐ |
| Diabetes mellitus | ☐ |
| Psychosis or other mental health problems | ☐ |
| Weight loss >5kg | ☐ |
| Weight gain | ☐ |
| Glaucoma | ☐ |
| Cataract | ☐ |
| Hypertension BP > 160/90 on two separate readings at least one week apart | ☐ |
| Infections | ☐ |
| Infected ulcers | ☐ |
| Corneal ulcer | ☐ |
| Tuberculosis | ☐ |
| Night sweats | ☐ |

*If yes, please describe:*

```
```

*Does the patient have any current illnesses or drug treatment (other than MDT and/or prednisolone)?  If yes, please describe:*

```
```

270

| | | | | |
|---|---|---|---|---|
| **Study Patient Number:**<br>*Enter AN1,AN2 etc* | | | | _____ |
| **Registration Card Number:** | | | | |
| **Today's Date:**<br>*dd-mmm-yyyy* | | | | \_\_\_/\_\_\_\_\_/_____ |

| **Part B – Clinical Assessment** | | |
|---|---|---|
| *Weight (kg)* | Temperature (°C) | Blood pressure (mmHg) |
| | | |

**Part C – Details of Reaction and Neuritis**

**Reaction Severity Assessment**

Score reaction signs and symptoms in the right hand column:

| | Criteria | 0 | 1 | 2 | 3 | Score |
|---|---|---|---|---|---|---|
| A1 | **Degree of inflammation of skin lesions** | **None** | **Erythema** | **Erythema, raised** | **Ulceration** | |
| A2 | **Number of raised and/or inflamed lesions** | **0** | **1-5** | **6-10** | **>10** | |
| A3 | **Peripheral oedema due to reaction** | **None** | **Minimal** | **Visible, but not affecting function** | **Oedema affecting function** | |
| A4 | **Nerve pain and/or paraesthesia** | **None** | **Pain on activity** | **Pain at rest** | **Pain disturbing sleep** | |
| A5 | **Nerve tenderness (worse affected nerve only)** | **None** | **Mild tenderness** | **Withdrawal or wincing** | **Not allowing palpation** | |
| | **TOTAL A SCORE** | | | | | |

| Study Patient Number: *Enter AN1,AN2 etc* | _____ |
|---|---|

| Registration Card Number: | |
|---|---|

| Today's Date:*dd-mmm-yyyy* | \_\_ / \_\_\_ / \_\_\_\_ |
|---|---|

| *Current dose of prednisolone?* | |
|---|---|
| *How much prednisolone is left from the last assessment?* | |
| *Is there any evidence of recurrence or deterioration? (Y/N)* | |
| *Does the patient require additional prednisolone?(Y/N)* | |

| Nature of problem | *Action* | Recurrence (Y/N) |
|---|---|---|
| **If there is recurrence of T1R with NFI (or nerve pain unresponsive to analgesics) on treatment** | add extra prednisolone to make up a total of 40mg and then taper according to the original regimen. | |
| **If there is recurrence of T1R with skin signs but no NFI then** | If recurrence is within the first ten weeks of enrolling **or** the face is affected then add **extra** prednisolone to make up a **total** of 40mg and taper according to the original regimen. | |
| | If recurrence after ten weeks of treatment then add **extra** prednisolone to make up a **total** of 20mg and then taper according to the original regimen. | |
| **Dose of prednisolone for the next week?** | | |
| **Total dose of prednisolone dispensed?** | | |

Record details of any eye problems:

| Eye Problems | | |
|---|---|---|
| **Right** | | **Left** |
| | **Cataract** (Y/N) | |
| | **Visual Acuity** (Days 113 and 337 ONLY) | |
| | Part D – Next Appointment | |

*Check the Appointments Diary and set a date for the next visit.*

| Date of next visit: *dd-mmm-yyyy* | \_\_ / \_\_ / \_\_\_\_ |
|---|---|

**Write the date on the patient's Next Visit Card**
Remaining actions:
1. **Request the prescribed tests from the laboratory and send patient to physiotherapists**
2. **According to the prescribed list, request biopsies and specimens.**
To complete the full assessment:
1. **Update the Study Register with the patient name, study number, Registration Card number and registration date**
2. **Check all tasks identified on the Checklist held by the patient have been completed.**
3. **Send the patient to the assigned PMW to take address details.**

| *MP Data Entry:* | |
|---|---|
| *Entered by:* | *Entered on:* |

# Appendix 3.10

| MP Study – Regular Assessments | |
|---|---|
| **Results of local laboratory tests and recording of trial investigations** | |
| **Study Patient Number: Enter AN1,AN2 etc** | _____ |
| **Registration Card Number:** | |
| **Today's Date:** *dd-mmm-yyyy* | ___/___/_____ |
| **Assessment Number:** *(1 for newly registered cases)* | |

*Available tests:*

| Code | Name of test | Code | Name of test |
|---|---|---|---|
| A | Smear BI | I | Skin biopsy stored |
| B | Smear MI | J | Stool specimen |
| C1 | Blood Hct | K | Chest X-ray |
| C2 | Blood Hb | L | Sputum |
| D | Blood White cell count | M | Pregnancy test |
| E | Platelets | N | Urinalysis |
| F | Blood sugar | O | |
| G | Blood creatinine | P | |
| H | Serum separated and stored | Q | |

Use the table below to record the results of tests, using as many lines as necessary. If a test is not listed above write its name in the appropriate column

| Item | Test Letter *(from table above)* | Result: | | | | | Comments/Sites: |
|---|---|---|---|---|---|---|---|
| 1 | | | | | | | |
| 2 | | | | | | | |
| 3 | | | | | | | |
| 4 | | | | | | | |
| 5 | | | | | | | |
| 6 | | | | | | | |
| 7 | | | | | | | |
| 8 | | | | | | | |
| 9 | | | | | | | |
| 10 | | | | | | | |
| 11 | | | | | | | |
| 12 | | | | | | | |
| 13 | | | | | | | |

**Comment: Record here any problem with the patient affecting the completion of the test or additional relevant information:**

| |
|---|
| |

| *MP Data Entry:* | |
|---|---|
| *Entered by:* | *Entered on:* |

# Appendix 3.11

**MP Study – Miscellaneous**

| Record any information not covered by the trial forms or protocol that is felt to be relevant | |
|---|---|
| **Study Patient Number:**<br>*Enter AN1,AN2 etc* | _____ |
| **Registration Card Number:** | |
| **Today's Date:**<br><br>*dd-mmm-yyyy* | __/__/____ |
| **Assessment Number:**<br><br>*(1  for newly registered cases)* | |

| |
|---|
| **Problem**<br><br><br><br><br><br>**Examination** |
| **Investigations** |
| **Treatment** |

*MP Data Entry:*

*Entered by:*                                      *Entered on:*

**Appendix 3.12**

**METHYLPREDNISOLONE STUDY**

**PHARMACY PROCEDURES FOR MOHAN AND RAM KUMAR**

- PATIENT ENROLLED IN STUDY

- DOCTOR INFORMS PHARMACY – MILD OR SEVERE

- IF MILD – NEXT WHITE ENVELOPE OPENED
- IF SEVERE – NEXT BROWN ENVELOPE OPENED

- INSTRUCTION SHEET IN ENVELOPE :

- RECORD ON SHEET SEVERITY GROUP IE MILD OR SEVERE
    - ENVELOPE NUMBER
    - PATIENT DETAILS: NAME,CLINIC AND STUDY NUMBERS
    - DATE

- METHYLPREDNISOLONE 1g IN 500ML NORMAL SALINE IV + PLACEBO TABLETS ORALLY

# OR

- 500ML NORMAL SALINE IV + PREDNISOLONE 40MG ORALLY

- ALL PREPARATIONS  BOTH IV AND ORAL SHOULD BE LABELLED WITH THE PATIENT NAME, STUDY NUMBER AND DATE.

- NONE OF THIS INFORMATION IS TO BE PASSED ON TO MEDICAL OR NURSING STAFF

- SIGN EACH DAY IN THE "COMPLETED" SECTION

- THIS IS FOR 3 CONSECUTIVE DAYS

- THE COMPLETED INSTRUCTION SHEET SHOULD BE FILED AND KEPT LOCKED IN PHARMACY

- AFTER THIS DOCTORS WILL PRESCRIBE ORAL PREDNISOLONE AS PER THE PROTOCOL AND THE USUSAL PREDNISOLONE CAN BE GIVEN

# APPENDIX 4. Final Clinical Severity Scale

**4.1 Final Scale**                                                  **277**

## 4.1 The Final Scale

| | Criteria | 0 | 1 | 2 | 3 | Score |
|---|---|---|---|---|---|---|
| A1 | Degree of inflammation of skin lesions | None | Erythema | Erythema and raised | Ulceration | |
| A2 | Number of raised and/or inflamed lesions | 0 | 1-5 | 6-10 | >10 | |
| A3 | Peripheral oedema due to reaction | None | Minimal | Visible, but not affecting function | Oedema affecting function | |
| | **A SCORE** | | | | | |

| | **HANDS** | **Purple 2g Monofilament scores** | | | | **Orange 10g Monofilament scores** | | | **Score** |
|---|---|---|---|---|---|---|---|---|---|
| | **Nerves** | 0 | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 | |
| B1 | RIGHT Trigeminal | | | | | | | Not felt | |
| B2 | LEFT Trigeminal | | | | | | | Not felt | |
| B3 | RIGHT ulnar | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| B4 | LEFT ulnar | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| B5 | RIGHT median | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| B6 | LEFT median | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| | **FEET** | **Orange 10g Monofilament scores** | | | | **Pink 300g Monofilament scores** | | | **Score** |
| | **Nerves** | 0 | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 | |
| B7 | RIGHT posterior tibial | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| B8 | LEFT posterior tibial | All sites felt | 1 site not felt | 2 sites not felt | 3 sites not felt | 1 site not felt | 2 sites not felt | 3 sites not felt | |
| | **B SCORE** | | | | | | | | |

| | NERVE | 0 | 1 | 2 | 3 | Score |
|---|---|---|---|---|---|---|
| C1 | RIGHT Facial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C2 | LEFT Facial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C3 | RIGHT Ulnar | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C4 | LEFT Ulnar | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C5 | RIGHT Median | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C6 | LEFT Median | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C7 | RIGHT Radial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C8 | LEFT Radial | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C9 | RIGHT Lateral Popliteal | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| C10 | LEFT Lateral Popliteal | MRC =5 | MRC=4 | MRC=3 | MRC<3 | |
| **C SCORE** | | | | | | |

| **Total score** | **Scores of A+B+C** | |
|---|---|---|

# APPENDIX 5. Real-time PCR assays

# Appendix 5.1 Melt Report

**Table A5.1 Thermal cycler conditions**

| Cycle | Cycle Point |
|---|---|
| Hold @ 95°c, 15 min 0 secs | |
| Cycling (50 repeats) | Step 1 @ 95°c, hold 10 secs |
| | Step 2 @ 60°c, hold 15 secs |
| | Step 3 @ 72°c, hold 20 secs, acquiring to Cycling A(FAM) |
| Melt (72-95°c) , hold 45 secs on the 1st step, hold 5 secs on next steps, Melt A(FAM) | |

**Fig A5.1 Representative Melt data for Melt A.FAM**
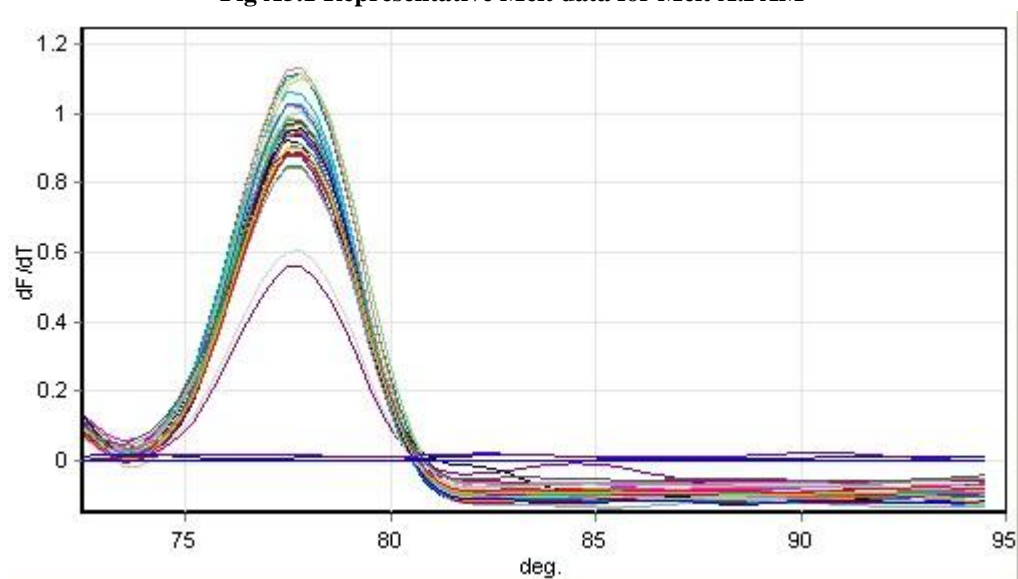
| No. | Colour | Name | Peak 1 | No. | Colour | Name | Peak 1 |
|-----|--------|------|--------|-----|--------|------|--------|
| A1 |  | TLR4 27A | 77.7 | C7 |  | TLR4 30C | 77.7 |
| A2 |  | TLR4 27A | 77.5 | C8 |  | TLR4 30C | 77.7 |
| A3 |  | TLR4 27B | 77.7 | D1 |  | TLR4 31A | 77.7 |
| A4 |  | TLR4 27B | 77.5 | D2 |  | TLR4 31A | 77.7 |
| A5 |  | TLR4 27C | 77.5 | D3 |  | TLR4 31B | 77.7 |
| A6 |  | TLR4 27C | 77.7 | D4 |  | TLR4 31B | 77.7 |
| A7 |  | TLR4 28A | 77.7 | D5 |  | TLR4 31C | 77.7 |
| A8 |  | TLR4 28A | 77.7 | D6 |  | TLR4 31C | 77.7 |
| B1 |  | TLR4 28B | 77.7 | D7 |  | TLR4 32A | 77.7 |
| B2 |  | TLR4 28B | 77.7 | D8 |  | TLR4 32A | 77.7 |
| B3 |  | TLR4 28C | 77.5 | E1 |  | TLR4 32B | 77.7 |
| B4 |  | TLR4 28C | 77.7 | E2 |  | TLR4 32B | 77.7 |
| B5 |  | TLR4 29A | 77.8 | E3 |  | TLR4 NC | 73.7 |
| B6 |  | TLR4 29A | 77.8 | E4 |  | TLR4 NTC | 76.7 |
| B7 |  | TLR4 29B | 77.7 | | | | |
| B8 |  | TLR4 29B | 77.7 | | | | |
| C1 |  | TLR4 29C | 77.7 | | | | |
| C2 |  | TLR4 29C | 77.7 | | | | |
| C3 |  | TLR430A | 77.7 | | | | |
| C4 |  | TLR4 30A | 77.7 | | | | |
| C5 |  | TLR4 30B | 77.8 | | | | |
| C6 |  | TLR4 30B | 77.7 | | | | |

**Table A5.2 Representative T$_m$ TLR4**

# Appendix 5.2 Electrophoresis of real-time PCR products



**Figure A5.2. Representative 3% Agarose gel of PCR products. EasyLadder I (Bioline, London, UK).**

# Appendix 5.3 hARP-P0 and toll-like receptor efficiency testing data

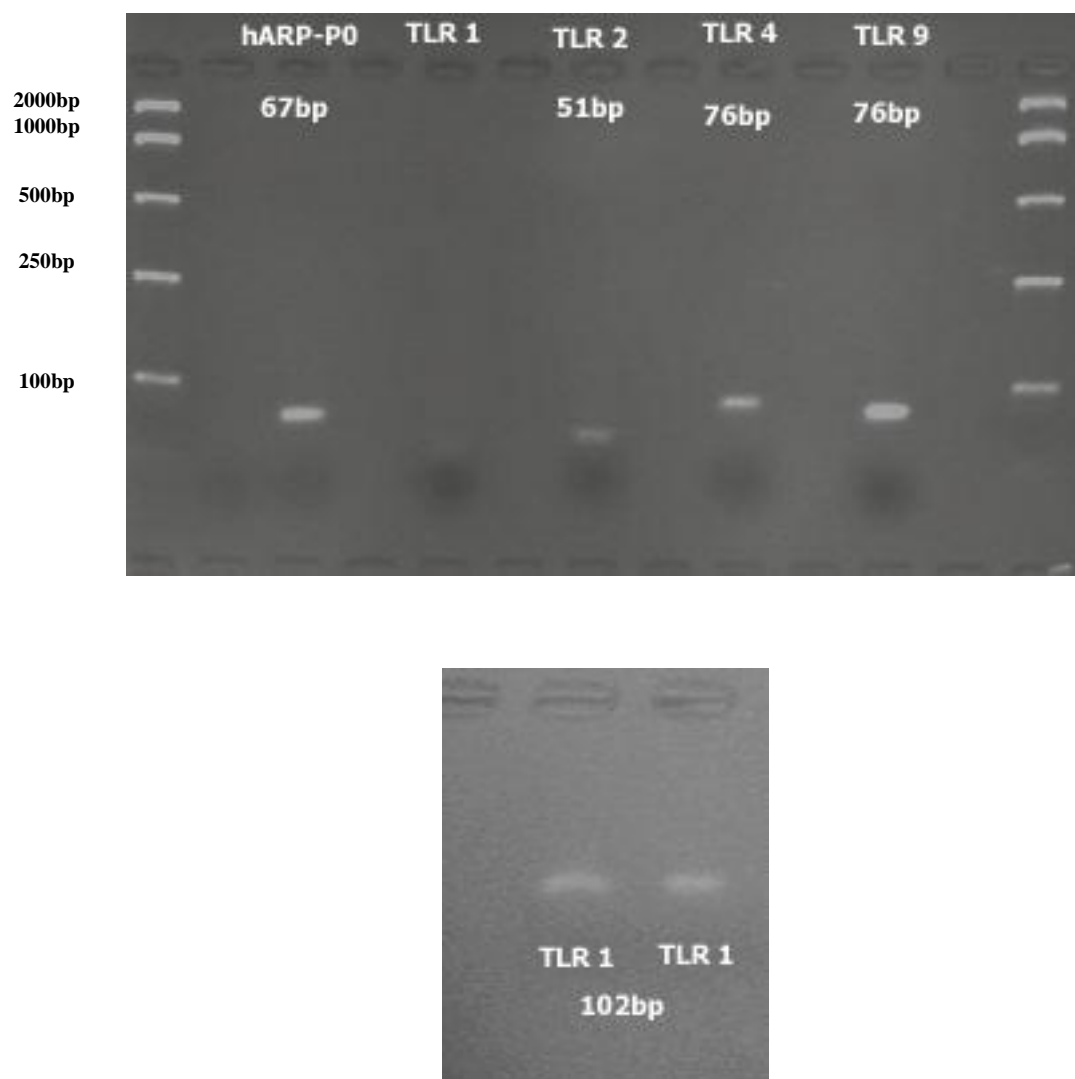| TLR1 dilution | Ct | TLR1 MEAN Ct | hARP-P0 dilution | Ct | hARP-P0 MEAN Ct | TLR1 -hARP-P0 (delta Ct) |
|---|---|---|---|---|---|---|
| tlr1_1/ 1 | 28.09 | | hARP-P0 1/1 | 18.12 | | |
| tlr1_1/ 1 | 27.84 | | hARP-P0 1/1 | 18.23 | | |
| tlr1_1/ 1 | 28 | 27.97667 | hARP-P0 1/1 | 18.18 | 18.17667 | 9.8 |
| tlr1_1/ 2 | 29.09 | | hARP-P0 1/2 | 20.05 | | |
| tlr1_1/ 2 | 29.23 | | hARP-P0 1/2 | 20.12 | | |
| tlr1_1/ 2 | 29.38 | 29.23333 | hARP-P0 1/2 | 20.21 | 20.12667 | 9.106667 |
| tlr1_1/ 4 | 29.27 | | hARP-P0 1/4 | 20.77 | | |
| tlr1_1/ 4 | 29.79 | | hARP-P0 1/4 | 20.82 | | |
| tlr1_1/ 4 | 29.63 | 29.56333 | hARP-P0 1/4 | 21.6 | 21.06333 | 8.5 |
| tlr1_1/ 8 | 30.72 | | hARP-P0 1/8 | 21.88 | | |
| tlr1_1/ 8 | 30.79 | | hARP-P0 1/8 | 22.18 | | |
| tlr1_1/ 8 | 30.44 | 30.65 | hARP-P0 1/8 | 22.06 | 22.04 | 8.61 |
| tlr1_1/ 16 | 31.84 | | hARP-P0 1/16 | 23.34 | | |
| tlr1_1/ 16 | 32.43 | | hARP-P0 1/16 | 23.27 | | |
| tlr1_1/ 16 | 31.96 | 32.07667 | hARP-P0 1/16 | 23.16 | 23.25667 | 8.82 |
| tlr1_1/ 32 | 32.62 | | hARP-P0 1/32 | 23.52 | | |
| tlr1_1/ 32 | 32.7 | | hARP-P0 1/32 | 23.55 | | |
| tlr1_1/ 32 | 33.38 | 32.9 | hARP-P0 1/32 | 23.58 | 23.55 | 9.35 |
| tlr1_1/ 64 | 33.84 | | hARP-P0 1/64 | 24.8 | | |
| tlr1_1/ 64 | 34.2 | | hARP-P0 1/64 | 24.97 | | |
| tlr1_1/ 64 | 34.59 | 34.21 | hARP-P0 1/64 | 24.64 | 24.80333 | 9.406667 |
| tlr1_1/ 128 | 34.9 | | hARP-P0 1/128 | 25.91 | | |
| tlr1_1/ 128 | 35 | 34.95 | hARP-P0 1/128 | 25.78 | 25.81667 | 9.133333 |
| | | | hARP-P0 1/128 | 25.76 | | |

**Table A5.3 Ct TLR1 and hARP-P0**

| TLR2 dilution | Ct | TLR2 MEAN Ct | hARP-P0 dilution | Ct | hARP-P0 MEAN Ct | TLR2 -hARP-P0 (delta Ct) |
|---|---|---|---|---|---|---|
| tlr2_1/1 | 30.06 | | hARP-P0 1/1 | 21.4 | | |
| tlr2_1/1 | 30.91 | | hARP-P0 1/1 | 20.04 | | |
| tlr2_1/1 | 30.39 | 30.45333 | hARP-P0 1/1 | 19.51 | 20.31667 | 10.13667 |
| tlr2_1/2 | 31.03 | | hARP-P0 1/2 | 21.09 | | |
| tlr2_1/2 | 32.65 | | hARP-P0 1/2 | 20.76 | | |
| tlr2_1/2 | 31.48 | 31.72 | hARP-P0 1/2 | 20.34 | 20.73 | 10.99 |
| tlr2_1/4 | 32.4 | | hARP-P0 1/4 | 22.06 | | |
| tlr2_1/4 | 32.11 | | hARP-P0 1/4 | 21.51 | | |
| tlr2_1/4 | 33.84 | 32.78333 | hARP-P0 1/4 | 21.14 | 21.57 | 11.21333 |
| tlr2_1/8 | 33.26 | | hARP-P0 1/8 | 23.01 | | |
| tlr2_1/8 | 33.64 | | hARP-P0 1/8 | 22.37 | | |
| tlr2_1/8 | 32.92 | 33.27333 | hARP-P0 1/8 | 22.07 | 22.48333 | 10.79 |
| tlr2_1/16 | 34.31 | | hARP-P0 1/16 | 23.86 | | |
| tlr2_1/16 | 35.39 | | hARP-P0 1/16 | 23.74 | | |
| tlr2_1/16 | 34.46 | 34.72 | hARP-P0 1/16 | 22.96 | 23.52 | 11.2 |
| tlr2_1/32 | 35.82 | | hARP-P0 1/32 | 24.99 | | |
| tlr2_1/32 | 34.68 | | hARP-P0 1/32 | 24.34 | | |
| tlr2_1/32 | 35.42 | 35.30667 | hARP-P0 1/32 | 24.46 | 24.59667 | 10.71 |
| tlr2_1/64 | 37.06 | | hARP-P0 1/64 | 25.69 | | |
| tlr2_1/64 | 35.68 | | hARP-P0 1/64 | 25.41 | | |
| tlr2_1/64 | 36.82 | 36.52 | hARP-P0 1/64 | 25.2 | 25.43333 | 11.08667 |
| tlr2_1/128 | 36.01 | | hARP-P0 1/128 | 26.96 | | |
| tlr2_1/128 | 36.46 | | hARP-P0 1/128 | 26.43 | | |
| tlr2_1/128 | | 36.235 | hARP-P0 1/128 | 26.35 | 26.58 | 9.655 |

**Table A5.4 Ct TLR2 and hARP-P0**

| TLR4 | Ct | TLR4 MEAN Ct | hARP-P0 | Ct | hARP-P0 MEAN Ct | TLR4 -hARP-P0 (delta Ct) |
|---|---|---|---|---|---|---|
| tlr4_1/ 1 | 33.99 | | hARP-P0 1/1 | 18.12 | | |
| tlr4_1/ 1 | 34.49 | | hARP-P0 1/1 | 18.23 | | |
| tlr4_1/ 1 | 34.47 | 34.31667 | hARP-P0 1/1 | 18.18 | 18.17667 | 16.14 |
| tlr4_1/ 2 | 36.64 | | hARP-P0 1/2 | 20.05 | | |
| tlr4_1/ 2 | 35.93 | | hARP-P0 1/2 | 20.12 | | |
| tlr4_1/ 2 | 36.54 | 36.37 | hARP-P0 1/2 | 20.21 | 20.12667 | 16.24333 |
| tlr4_1/ 4 | 37.58 | | hARP-P0 1/4 | 20.77 | | |
| tlr4_1/ 4 | 38.1 | | hARP-P0 1/4 | 20.82 | | |
| tlr4_1/ 4 | 37.37 | 37.68333 | hARP-P0 1/4 | 21.6 | 21.06333 | 16.62 |
| tlr4_1/ 8 | 38.3 | | hARP-P0 1/8 | 21.88 | | |
| tlr4_1/ 8 | 37.6 | | hARP-P0 1/8 | 22.18 | | |
| tlr4_1/ 8 | 37.01 | 37.63667 | hARP-P0 1/8 | 22.06 | 22.04 | 15.59667 |
| tlr4_1/ 16 | 39.24 | | hARP-P0 1/16 | 23.34 | | |
| tlr4_1/ 16 | 39.29 | | hARP-P0 1/16 | 23.27 | | |
| tlr4_1/ 16 | 38.49 | 39.00667 | hARP-P0 1/16 | 23.16 | 23.25667 | 15.75 |
| tlr4_1/ 32 | 39.25 | | hARP-P0 1/32 | 23.52 | | |
| tlr4_1/ 32 | 39.29 | | hARP-P0 1/32 | 23.55 | | |
| tlr4_1/ 32 | 39.47 | 39.33667 | hARP-P0 1/32 | 23.58 | 23.55 | 15.78667 |
| tlr4_1/ 64 | 40.91 | | hARP-P0 1/64 | 24.8 | | |
| tlr4_1/ 64 | 40.24 | | hARP-P0 1/64 | 24.97 | | |
| tlr4_1/ 64 | 40.77 | 40.64 | hARP-P0 1/64 | 24.64 | 24.80333 | 15.83667 |
| tlr4_1/ 128 | 41.48 | | hARP-P0 1/128 | 25.91 | | |
| tlr4_1/ 128 | 42.34 | 41.83333 | hARP-P0 1/128 | 25.78 | 25.81667 | 16.01667 |
| tlr4_1/ 128 | 41.68 | | hARP-P0 1/128 | 25.76 | | |

**Table A5.5 Ct TLR4 and hARP-P0**

| TLR9 dilution | Ct | TLR9 MEAN Ct | hARP-P0 dilution | Ct | hARP-P0 MEAN Ct | TLR4 -hARP-P0 (delta Ct) |
|---|---|---|---|---|---|---|
| tlr9_1/ 1 | 28.89 | | hARP-P0 1/1 | 18.12 | | |
| tlr9_1/ 1 | 29.03 | | hARP-P0 1/1 | 18.23 | | |
| tlr9_1/ 1 | 29.26 | 29.06 | hARP-P0 1/1 | 18.18 | 18.17667 | 10.88 |
| tlr9_1/ 2 | 30.55 | | hARP-P0 1/2 | 20.05 | | |
| tlr9_1/ 2 | 30.68 | | hARP-P0 1/2 | 20.12 | | |
| tlr9_1/ 2 | 30.59 | 30.60667 | hARP-P0 1/2 | 20.21 | 20.12667 | 10.48 |
| tlr9_1/ 4 | 31.3 | | hARP-P0 1/4 | 20.77 | | |
| tlr9_1/ 4 | 30.91 | | hARP-P0 1/4 | 20.82 | | |
| | | 31.105 | hARP-P0 1/4 | 21.6 | 21.06333 | 10.04167 |
| tlr9_1/ 8 | 31.85 | | hARP-P0 1/8 | 21.88 | | |
| tlr9_1/ 8 | 32.66 | | hARP-P0 1/8 | 22.18 | | |
| tlr9_1/ 8 | 31.57 | 32.02667 | hARP-P0 1/8 | 22.06 | 22.04 | 9.986667 |
| tlr9_1/ 16 | 34.19 | | hARP-P0 1/16 | 23.34 | | |
| tlr9_1/ 16 | 34.54 | | hARP-P0 1/16 | 23.27 | | |
| tlr9_1/ 16 | 33.33 | 34.02 | hARP-P0 1/16 | 23.16 | 23.25667 | 10.76333 |
| tlr9_1/ 32 | 33.83 | | hARP-P0 1/32 | 23.52 | | |
| tlr9_1/ 32 | 33.55 | | hARP-P0 1/32 | 23.55 | | |
| | | 33.69 | hARP-P0 1/32 | 23.58 | 23.55 | 10.14 |
| tlr9_1/ 64 | | | hARP-P0 1/64 | 24.8 | | |
| tlr9_1/ 64 | | | hARP-P0 1/64 | 24.97 | | |
| tlr9_1/ 64 | 36.61 | 36.61 | hARP-P0 1/64 | 24.64 | 24.80333 | 11.80667 |
| tlr9_1/ 128 | 35.5 | | hARP-P0 1/128 | 25.91 | | |
| tlr9_1/ 128 | | 35.5 | hARP-P0 1/128 | 25.78 | 25.81667 | 9.683333 |
| | | | hARP-P0 1/128 | 25.76 | | |

**Table A5.6 Ct TLR9 and hARP-P0**

# APPENDIX 6. PUBLICATIONS, POSTERS AND ABSTRACTS

**PLoS Negl Trop Dis. 2008;2(12):e351**

Development and validation of a severity scale for leprosy type 1 reactions.

Walker SL, Nicholls PG, Butlin CR, Nery JA, Roy HK, Rangel E, Sales AM, Lockwood DN.

**5th International Dermato-Epidemiology Association Congress, Nottingham, UK.**

**7th – 11th September 2008. Poster presentation.**

Abstract 46 J Invest Dermatol 2008;128:2559

**17th International Leprosy Congress, Hyderabad, India.**

**30th January – 4th February 2008**

ABS163ILC - The validation of a severity scale for leprosy type 1 reactions

**6th South Asian Regional Congress of Dermatology, Kathmandu, Nepal.**

**13th -15th November 2009**

# Development and Validation of a Severity Scale for Leprosy Type 1 Reactions

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## Abstract

**Objectives:** To develop a valid and reliable quantitative measure of leprosy Type 1 reactions.

**Methods:** A scale was developed from previous scales which had not been validated. The face and content validity were assessed following consultation with recognised experts in the field. The construct validity was determined by applying the scale to patients in Bangladesh and Brazil who had been diagnosed with leprosy Type 1 reaction. An expert categorized each patient's reaction as mild or moderate or severe. Another worker applied the scale. This was done independently. In a subsequent stage of the study the agreement between two observers was assessed.

**Results:** The scale had good internal consistency demonstrated by a Cronbach's alpha $>0.8$. Removal of three items from the original scale resulted in better discrimination between disease severity categories. Cut off points for Type 1 reaction severities were determined using Receiver Operating Characteristic curves. A mild Type 1 reaction is characterized using the final scale by a score of 4 or less. A moderate reaction is a score of between 4.5 and 8.5. A severe reaction is a score of 9 or more.

**Conclusions:** We have developed a valid and reliable tool for quantifying leprosy Type 1 reaction severity and believe this will be a useful tool in research of this condition, in observational and intervention studies, and in the comparison of clinical and laboratory parameters.

## Introduction

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae*. More than 254 000 new cases were reported to the World Health Organization in 2007 [1].

The disease predominantly affects the skin and nerves. The nerve involvement associated with the disease may lead to permanent deformity and disability. A spectrum of disease phenotypes is recognised and these are determined by the host response to the organism [2]. The tuberculoid pole of the spectrum is characterised by strong host cell mediated immunity to the organism, whereas patients with lepromatous leprosy have a predominantly humoral immune response [3]. The borderline states of the disease are immunologically unstable.

Leprosy may be exacerbated by immunological complications Type 1 (reversal) reactions and erythema nodosum leprosum (Type 2 reactions).

Type 1 reactions occur predominantly in individuals with the borderline forms of leprosy. They are characterised by inflammation of the skin, nerves or both. Type 1 reactions may occur before, during or after the successful completion of multi-drug therapy. Type 1 reactions affecting the peripheral nerves may

result in decreased sensory and motor function and lead to disability. 20–30% of individuals diagnosed with leprosy will have a Type 1 reaction [4,5].

Type 1 reactions are usually treated with oral corticosteroids but approximately 40% of individuals do not experience complete recovery of clinically detectable nerve function impairment (NFI) [6]. Clinical trials with appropriate outcome measures are needed to determine the most effective treatment regimens [7]. It has proved difficult to compare the small number of studies because of the different outcome measures used. There are also difficulties in comparing the severity of Type 1 reactions between different cohorts and even between different arms of clinical trials.

A tool which enables clinicians to accurately assess the severity of leprosy Type 1 reactions would be useful in defining outcomes for clinical trials. It would facilitate the even distribution of patients with similar disease severity between the arms of clinical trials. A measure of reaction severity could also be used in treatment guidelines to indicate the need for therapy. A quantitative measure of reaction severity may be a useful prognostic tool.

A scale devised as part of the ILEP Nerve Function Impairment and Reaction (INFIR) Cohort study examined 21 parameters for the basis of a severity scale of both Types of reactions and

288

## Author Summary

Leprosy is caused by a bacterium and is curable with a combination of antibiotics known as multi-drug therapy which patients take for six or 12 months. However, a significant proportion of leprosy patients experience inflammation in their skin and/or nerves which may occur even after successful completion of multi-drug therapy. These episodes of inflammation are called leprosy Type 1 reactions. Type 1 reactions are an important complication of leprosy because they may result in nerve damage which leads to disability and deformity. Type 1 reactions require treatment with immunosuppressive agents such as corticosteroids. The severity of Type 1 reactions varies with time, treatment and between individuals. We have developed a clinical severity scale to measure the severity of Type 1 reactions. The scale has three sections. The first measures the involvement of the skin using the number of affected skin lesions, the degree of inflammation of those lesions and the presence of swelling of the hands, feet or face. The second section is a measurement of the sensory function of the nerves supplying the eyes, hands and feet by assessing a patient's ability to feel graded nylon fibres. The third section uses a standard measure of muscle power to assess motor function of the nerves of the face, hands and feet. The clinical severity scale we have developed will facilitate the study of Type 1 reactions and enable direct comparison between different studies. This will improve the management of this disabling complication of leprosy.

retrospectively assessed the performance of this scale [8]. There was good agreement between items in the scale.

A different scale (with 24 parameters) was used by Marlowe et al in a different INFIR study of azathioprine and prednisolone in Type 1 reactions but it was not validated [9]. An "indice névritique"–a composite scale using various assessments of nerves including electrophysiological studies–was developed by Naafs and colleagues but has not been validated [10,11].

Using the INFIR scales as a starting point we decided to develop and validate a scale for Type 1 reactions and nerve function impairment in leprosy.

## Methods

### Expert opinion

A questionnaire was sent to eight leprologists who were not involved in the development of the current scale. The questionnaire used open questions to ascertain the signs they believed to be important in Type 1 reaction, which signs indicated a more severe reaction and how they categorised Type 1 reaction severity.

### Scale development

The severity scale for leprosy Type 1 reactions was developed by modifying the two previous scales used in the INFIR studies. The scale we developed and tested has 24 parameters grouped into three parts (see Appendix S1):

Section A contained six parameters which scored between 0 and 3 depending on the assessment of their severity by the examiner using the scale.

Section B is an assessment of sensory function of each of the trigeminal, ulnar, median and posterior tibial nerves. Cotton wool is used to assess the trigeminal nerve. Graded Semmes-Weinstein monofilaments (SWM) are used for the ulnar, median and posterior tibial nerves.

The ulnar and median nerves are examined using a 2 and 10g monofilament at three sites on the palmar aspect of the hand for each nerve (ulnar and median) and the posterior tibial nerves are assessed using 10 and 300g at four sites on the sole of the foot (Fig. 1). A score from 0 to 6 was assigned depending on the ability of the patient to successfully recognise the weighted monofilaments and the number of sites in which they were felt. For example, on the hand if a person could feel the 2g monofilament at the three sites innervated by the ulnar nerve then a score of zero was recorded. If the 2g was felt at two sites and the 10g at the third site a score of one was recorded. If however the 10g monofilament was not felt at one site then a score of 4 was recorded even if the patient was able to feel the 2g monofilament at the other two sites.

Section C measures motor function of ten nerves (facial, ulnar, median, radial, posterior tibial) by voluntary muscle testing (VMT) using the MRC grading system [12]. Normal muscle power (MRC Grade 5) scores zero on the scale. Grade 4 scores 1 and grade 3 scores two. An MRC grade of less than three scores three on the severity scale.
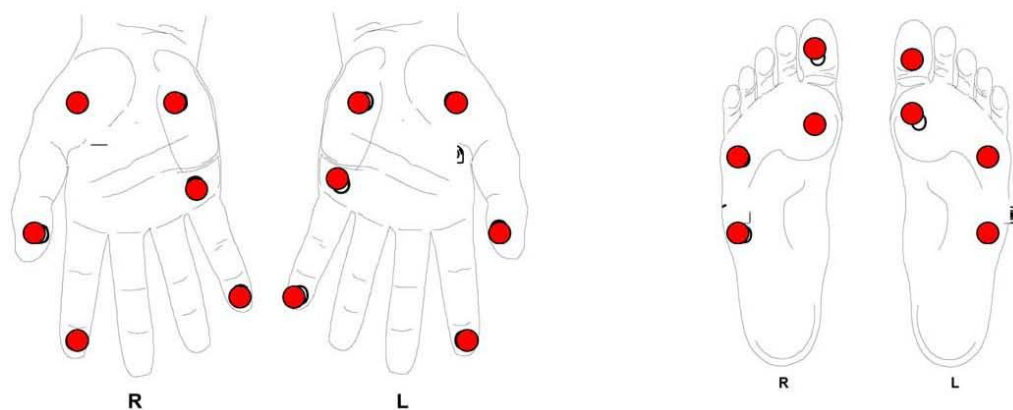


**Figure 1. Test sites on the hands (2 and 10g) and the feet (10 and 300g).**
doi:10.1371/journal.pntd.0000351.g001

289

The sum of the total for each section gives the overall severity scale score which ranges from 0 96, the lower the score the less severe the reaction.

## Scale testing

The assessment of the severity scale was performed at the specialist leprosy referral centres of DBLM Hospital, Nilphamari, Bangladesh and Oswaldo Cruz Institute, Rio de Janeiro, Brazil between June 2006 and November 2007.

Ethical approval was granted for the external validation of the scale and the assessment of inter-observer agreement by the Ethics committee of the London School of Hygiene and Tropical Medicine, the Bangladesh Medical Research Council and the Institutional Review Board of the Oswaldo Cruz Institute.

Patients attending the centres with evidence of a Type 1 reaction or nerve function impairment of less than 6 months duration were eligible. Eligible individuals were invited to participate by the attending physician.

Written informed consent was obtained from individuals who participated in the external validation of the scale and also from those enrolled in the study of inter-observer agreement.

Individuals were examined independently by a worker who was trained to use the scale and experienced leprologists (>20 years experience) who categorized the reaction as mild or moderate or severe. Neither assessor (nor the patient) was aware of the result of the others examination. All of the demographic and clinical data were recorded on a standard form. The Ridley-Jopling classification was used to classify the type of leprosy each patient had [2].

Inter-observer agreement was tested at the two centres in a subsequent stage of the study using the same eligibility criteria. Two assessors independently used the scale to assess individuals diagnosed as having Type 1 reactions. The scale was applied in the same way as in the validation part of the study. The time interval between the two assessments was kept as short as was practicable. Four pairs of assessors were used.

The results were entered into an Access database. The data were analysed using the Statistical Package for the Social Sciences (SPSS version 14. SPSS Inc, Illinois, Chicago).

## Statistical Methods

The item to total score correlation was examined using Spearman rank correlation.

The internal consistency or reliability was assessed using Cronbach's alpha. An alpha between 0.7 and 0.9 is considered acceptable [13]. The contribution of each item in the scale was assessed by calculating Cronbach's alpha for the scale if that item were removed.

The ability of the scale to discriminate between different clinical severity categories was determined using analysis of variance. The threshold for accepting statistical significance was $p < 0.05$.

Inter-observer reliability was evaluated using Intra-Class Correlation of the total score of each examiner using a two-way analysis of variation ( 5% level of significance) and the strength of agreement criteria of Landis and Koch [14]. A Bland Altman plot of the difference between pairs of observations and the mean of those pairs was used to highlight any potential systematic differences between raters.

Receiver Operating Characteristic (ROC) curves were used to determine cut off points for mild, moderate and severe reactions by calculating the sensitivity and specificity of the scale scores for mild and moderate groups and moderate and severe groups respectively.

## Results

### Expert opinion

The questionnaire sent to eight leprologists was returned by seven. The features of Type 1 reaction that were considered important indicators of severity were extent and degree of inflammation of skin lesions, the presence of peripheral oedema, nerve tenderness and nerve function impairment. These parameters are all part of the clinical severity scale we have developed and thus gives our scale face validity.

### Scale testing

81 individuals were recruited (56 from Bangladesh and 25 from Brazil). 64 (79%) were male and 17 (21%) female. The clinical features are summarised in Table 1.

**Table 1.** Characteristics of the participants in each stage of the study, validity and interobserver agreement.

| | Validity Number (%) | | Interobserver Agreement Number (%) | |
|---|---|---|---|---|
| Number | 81 | | 39 | |
| Gender | Male 64 (79) | | Male 29 (74.4) | |
| | Female 17 (21) | | Female 10 (25.6) | |
| Mean Age in years (range) | 39.5 (11–86) | | 40.9 (11–95) | |
| Type of leprosy | BT | 56(69.1) | BT | 17 (43.6) |
| | BB | 6 (7.4) | BB | 3 (7.7) |
| | BL | 18 (22.2) | BL | 15 (38.5) |
| | LL | 4 (4.9) | LL | 4 (10.3) |
| | PNL | 1 (1.2) | PNL | 0 (0) |
| First episode of Type 1 reaction | 52 (64.2) | | 19 (48.7) | |
| Type of reaction | Skin and nerves | 56 (69.1) | Skin and nerves | 28 (71.8) |
| | Skin only | 18 (22.2) | Skin only | 9 (23.1) |
| | Nerves only | 7 (8.6) | Nerves only | 7 (5.1) |

290

The range of the item to total score correlation was −0.09 to +0.73. Nerve pain and nerve tenderness appeared to show no correlation with the total score.

The internal consistency of the scale was assessed using Cronbach's alpha. The Cronbach's alpha was 0.819. Removal of the following individual items resulted in an increase in the alpha: the degree of inflammation of skin lesions, the number of raised inflamed lesions, nerve pain, nerve tenderness, fever, function of right trigeminal nerve, function of the left trigeminal nerve, motor function of the right and left radial nerves (Table 2). This indicates that removal of one or more of these items might improve the ability of the remaining items to measure the severity of Type 1 reactions.

Principal component analysis (PCA) identified a general factor to which all but nerve pain, nerve tenderness and the number of inflamed lesions contributed accounting for 23.5% of total variance. The important variables in the second factor accounting for 11.6% of the total variance were those related to the eye, namely, trigeminal nerve sensation and facial nerve motor function. The

**Table 2.** The Cronbach α for the scale when individual item indicated is removed.

| Type of Parameter | Item | Cronbach's Alpha if Item Deleted |
|---|---|---|
| Skin and oedema signs | Degree of inflammation of skin | .822 |
| | Number of raised and/or inflamed lesions | .824 |
| | Peripheral oedema due to reaction | .814 |
| Nerve symptom | Nerve pain and/or paraesthesia | .826 |
| Nerve sign | Nerve tenderness (worst affected nerve only) | .825 |
| Systemic sign | Fever (°C) | .820 |
| Sensory function of nerve | Right trigeminal | .821 |
| | Left trigeminal | .821 |
| | Right ulnar | .799 |
| | Left ulnar | .789 |
| | Right median | .795 |
| | Left median | .803 |
| | Right posterior tibial | .797 |
| | Left posterior tibial | .800 |
| Motor function of nerve | Right facial | .817 |
| | Left facial | .816 |
| | Right ulnar | .810 |
| | Left ulnar | .807 |
| | Right median | .809 |
| | Left median | .808 |
| | Right radial | .821 |
| | Left radial | .821 |
| | Right lateral popliteal | .809 |
| | Left lateral popliteal | .816 |

An increase in α indicates that removal of the item is improving agreement of the remaining scale items. (The overall α for the original 24 item scale was 0.819.)
doi:10.1371/journal.pntd.0000351.t002

third factor which accounted for 10.7% contrasted individuals with skin signs and no NFI with those who only had NFI.

The severity of the Type 1 reaction was categorized as mild in 19 (23.5%), moderate in 40 (49.4%) and severe in 12 (14.8%). The severity was not recorded in 10 cases.

The median scores for each category of reaction severity are shown in the box plots in Fig. 2 with the inter-quartile range (IQR). The median scores for each category were: mild = 5.0 (IQR = 11), moderate = 10.5 (IQR = 13) and severe = 18.0 (IQR = 29).

The differences between the mild and moderate group and the moderate and severe groups did not reach statistical significance (p = 0.053 and 0.052 respectively). The performance of the scale was not materially affected by excluding the seven individuals who did not have skin involvement.

Thirty nine individuals (27 from Bangladesh and 12 from Brazil) were recruited to the second stage of the study to assess inter-observer agreement. The details of these patients are presented in Table 1.

The Intra-Class Correlation coefficient based on a two-way analysis of variance with a random effects model is 0.994. The strength of agreement is very good [14].

A Bland and Altman plot [15] (Fig. 3) of the difference between the scores for pairs of observers plotted against the mean of the scores shows good agreement between observers with 95% of differences less than two standard deviations from the mean.

## The Final scale

The scale was adjusted and the analysis repeated in the light of the data obtained (see Appendix S2).

The items nerve pain, nerve tenderness and fever were removed. The rationale for removing these items was that nerve pain and nerve tenderness performed least well of all the items in the scale (in terms of Cronbach's alpha). Fever was removed because occurred in only four of the 120 participants in the study as a whole.

We felt it was important to retain the cutaneous signs and trigeminal and radial nerve function parameters as these are important clinical features of Type 1 reactions.

The scores for the sensory testing (using SWM and cotton wool) were reduced by 50% to make the maximum score possible for each sensory nerve three. This is the maximum score possible for each of the motor and cutaneous items.

These adjustments result in the final scale which consists of 21 items and has a range of 0 63. The maximum score possible for sections A, B and C are 9, 24 and 30 respectively.

For this adjusted version of the scale Cronbach's alpha remained satisfactory at 0.833.

The median scores for each severity group were: mild = 5.0, moderate = 7.5 and severe = 15.25. The differences between the mild and moderate groups (p = 0.038) and the moderate and severe groups (p = 0.048) reached statistical significance.

The ROC curve for the final scale scores was plotted for individuals identified as mild or moderate by the expert raters and for those categorized as moderate or severe (Fig. 4). This facilitates the determination of cut off scores for each category [13].

Using the ROC curves in conjunction with a consideration of the clinical meaning of a given score we determined the following cut off points. A mild Type 1 reaction is characterized using the final scale by a score of 4 or less. A moderate reaction is a score of between 4.5 and 8.5. A severe reaction is a score of 9 or more. The area under the curve for mild and moderate categories is 0.701 for the final scale (0.688 for the original scale). The area under the curve for the moderate and severe categories is 0.734 for the final
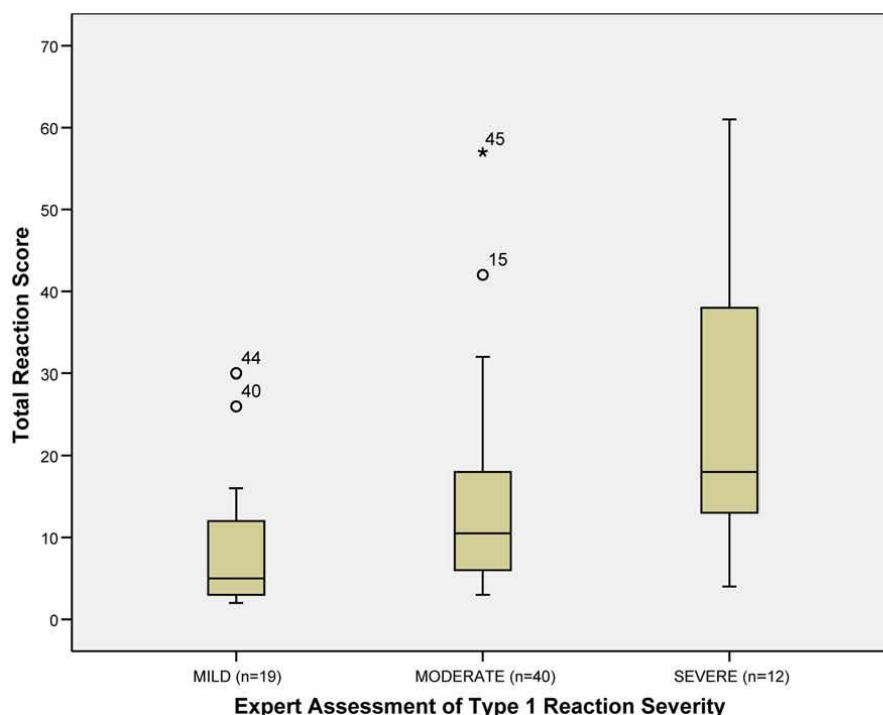
291

**Figure 2. Box plot of the Original Scale Scores by expert severity classification showing medians, interquartile ranges and minimum and maximum scores.**
doi:10.1371/journal.pntd.0000351.g002

scale (0.731 for the original scale). These values indicate that the final scale is a fair discriminator between the severity categories traditionally used by clinicians.

## Discussion

In many branches of medicine a single test or diagnostic criterion is either not available or insufficient to adequately measure or describe a clinical syndrome. This has led to difficulties in measuring the severity and prognosis of conditions. The response by researchers has been to develop composite measurement scales.

Psychologists have for many years been concerned with accurately measuring and predicting behaviour and there is a large literature on how to develop and test such measures [13,16].

The use of unpublished scales to measure outcome has been shown to be a significant source of bias in psychiatry [17]. The lack of clear descriptions of scales and familiarity with them make clinical research difficult to interpret.

We have developed and prospectively validated a reliable 21 item severity scale to measure leprosy Type 1 reactions.

This scale requires the examiner to be proficient in recognising the cutaneous signs of Type 1 reaction, the assessment of VMT and the use of SWM. These skills are not widely practised in many leprosy endemic countries and we anticipate that the main use of this tool, at least initially, will be in the context of research and referral settings.

We believe the scale is easy to use and requires little additional training or equipment for workers based in referral centres. Using a standard assessment form the additional time required to use the scale is minimal.

Type 1 reactions are a significant cause of nerve function impairment and this is the major concern of the physician managing a patient with this condition. The scale we have developed reflects the importance of NFI in the severity of Type 1 reactions.

VMT and SWM in the assessment of NFI have been shown to be reliable [18]. Monofilaments have been shown to be concordant with other sensory function tests [19]. These factors undoubtedly contribute to the robustness of the current scale but careful training and assessment of examiners is required [20].

The use of two monofilaments on the hands (2g and 10g) and feet (10g and 300g) simplifies the system used in the INFIR Cohort Study. However this also results in a higher sensory threshold before an individual's NFI impacts on their Type 1 reaction severity scale score.

The INFIR Cohort study also used a single monofilament test site for the purely sensory radial cutaneous and sural nerves [4]. These two nerves are not commonly tested in routine clinical practice and are not included in the severity scale.

The radial cutaneous and sural nerves may be assessed using various forms of quantitative sensory testing before new impairment identified by monofilaments is demonstrable. Recently published data analysing 188 individuals from the INFIR Cohort
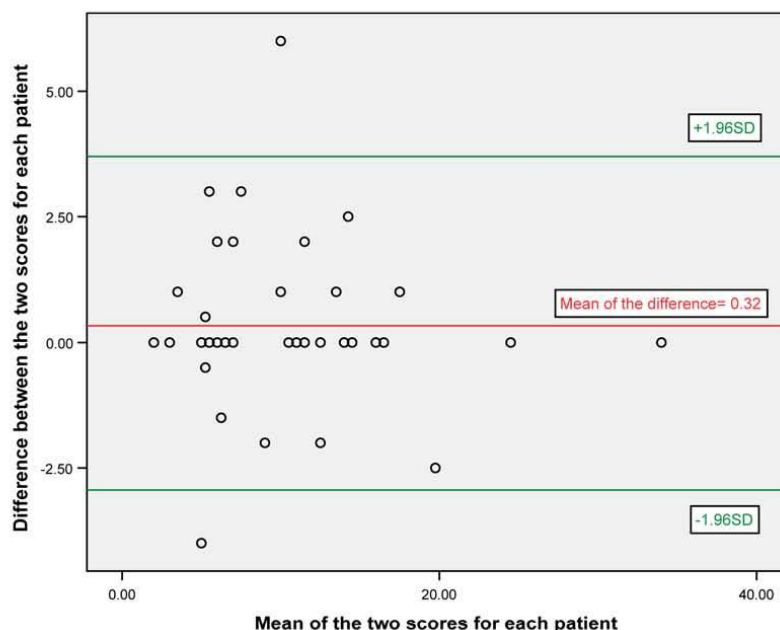
292

**Figure 3. A Bland Altman plot of the difference between the scores of the examiners and the mean of those scores (n = 39).**
doi:10.1371/journal.pntd.0000351.g003

who did not present with reaction or nerve involvement has shown that impairment identified using monofilaments occurred in the radial cutaneous nerve in 7% of individuals and in the sural nerve in 6.1% [21]. However the definition of impairment in the radial cutaneous nerve was the inability to feel monofilaments less than 10g or in the sural nerve less than 300g [4].

The lack of a gold standard measure of Type 1 reactions has resulted in us having to compare the scale with the variable and somewhat vague clinical categories of severity as mild, moderate or severe. This has undoubtedly led to a degree of heterogeneity of Type 1 reaction severity within these categories but despite this the scale has performed well.

The final scale has a high degree of inter-observer reliability. We were unable to test intra-observer reliability because of the effect of treatment on the signs of reaction. It would be unethical to withhold treatment. The assessment of intra-observer variation is desirable but not absolutely necessary in scales with a high level of inter-observer reliability [13]. The assessment of intra-observer variation has not been possible in the development of valid scales in other fields such as neurology [22].

In its present form we have found the adjusted scale to be valid and sensitive. Neurological parameters are well represented and reflect the importance of nerve function impairment. The addition of weighting of the different components of the scale would add to its complexity.

A consideration we have not addressed is the performance of the scale in individuals who have nerve damage of greater than 6 months duration. The treatment of nerve damage present for this length of time with corticosteroids is not associated with significant clinical benefit compared to placebo [23]. Nerve damage greater than six months duration should not be included in the severity

score. The issue of longstanding NFI can be problematic as patients who are presenting for the first time may be unsure as to the duration of the NFI and may have some acute NFI in a nerve which already has some pre-existing permanent impairment.

Longstanding nerve damage in an individual who experiences a Type 1 reaction would lead to a higher score than an individual with an identical reaction but who has no pre-existing nerve damage. The severity of the Type 1 reaction in the two individuals is presumably the same. However it could be argued that individuals who already have some degree of permanent nerve damage have less neurological reserve and are thus more at risk from even a mild reaction. This however needs to be formally tested.

The scale is currently being used as an additional measure in a clinical trial of methylprednisolone in Type 1 reactions. In this cohort the performance of the scale over time and its ability to reflect change will be assessed.

This is the first prospective validation of a severity scale for leprosy Type 1 reactions. We believe it will prove a useful tool in more accurately assessing Type 1 reactions particularly in clinical trials where the ability to accurately compare the severity of Type 1 reactions in different patients is vital.
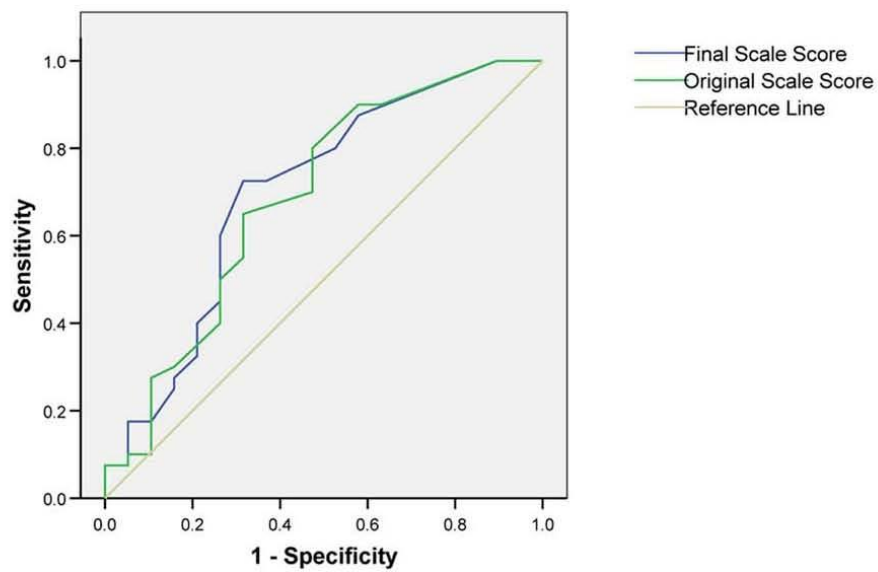
## Supporting Information

**Appendix S1**
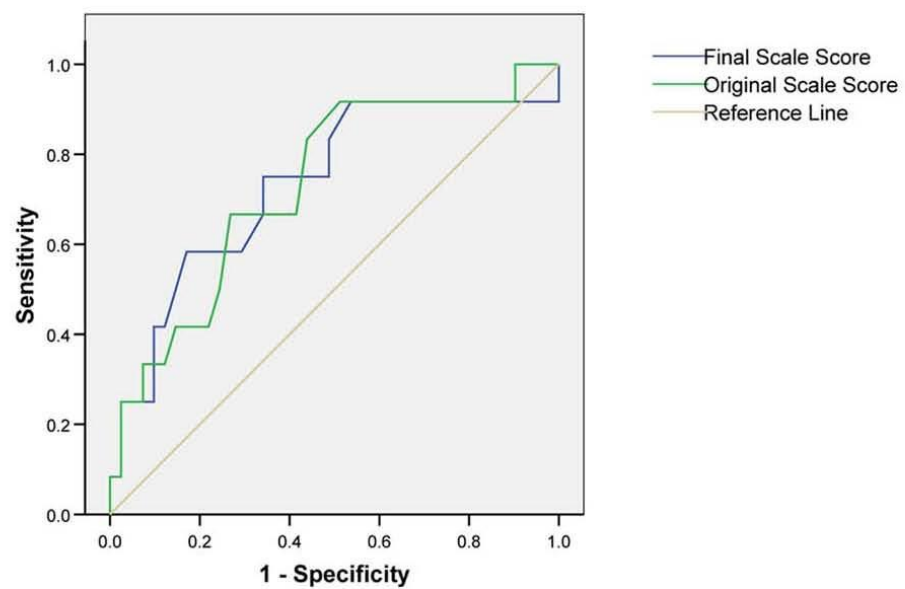Found at: doi:10.1371/journal.pntd.0000351.s001 (0.08 MB DOC)

**Appendix S2**
Found at: doi:10.1371/journal.pntd.0000351.s002 (0.07 MB DOC)

293

## ROC curve for mild and moderate cases



## ROC curve for moderate and severe cases



Diagonal segments are produced by ties.

294

**Figure 4. ROC curve for mild and moderate cases and moderate and severe cases.**
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## Author Contributions

Conceived and designed the experiments: SLW PGN DNJL. Performed the experiments: CRB JACN HKR ER AMS. Analyzed the data: SLW PGN. Contributed reagents/materials/analysis tools: SLW. Wrote the paper: SLW PGN CRB DNJL.

## References

1. (2008) Global leprosy situation, beginning of 2008. Wkly Epidemiol Rec 83: 293 300.
2. Ridley DS, Jopling WH (1966) Classification of Leprosy according to immunity. Int J Lepr Other Mycobact Dis 34: 255 273.
3. Modlin RL (1994) Th1-Th2 paradigm: insights from leprosy. J Invest Dermatol 102: 828 832.
4. van Brakel WH, Nicholls PG, Das L, Barkataki P, Suneetha SK, et al. (2005) The INFIR Cohort Study: investigating prediction, detection and pathogenesis of neuropathy and reactions in leprosy. Methods and baseline results of a cohort of multibacillary leprosy patients in north India. Lepr Rev 76: 14 34.
5. Ranque B, Nguyen VT, Vu HT, Nguyen TH, Nguyen NB, et al. (2007) Age is an important risk factor for onset and sequelae of reversal reactions in Vietnamese patients with leprosy. Clin Infect Dis 44: 33 40.
6. van Brakel WH, Khawas IB (1996) Nerve function impairment in leprosy: an epidemiological and clinical study Part 2: Results of steroid treatment. Lepr Rev 67: 104 118.
7. Van Brakel WH, Saunderson P, Shetty V, Brandsma JW, Post E, et al. (2007) International workshop on neuropathology in leprosy consensus report. Lepr Rev 78: 416 433.
8. Van Brakel WH, Nicholls PG, Lockwood DN, Rao PS, Smith WC (2007) A scale to assess the severity of leprosy reactions. Lepr Rev 78: 161 164.
9. Marlowe SN, Hawksworth RA, Butlin CR, Nicholls PG, Lockwood DN (2004) Clinical outcomes in a randomized controlled study comparing azathioprine and prednisolone versus prednisolone alone in the treatment of severe leprosy Type 1 reactions in Nepal. Trans R Soc Trop Med Hyg 98: 602 609.
10. Naafs B, Dagne T (1977) Sensory testing: a sensitive method in the follow-up of nerve involvement. Int J Lepr Other Mycobact Dis 45: 364 368.
11. Naafs B, van Drogenbroeck JBA (1977) Decompression des nevrites reaction-nelles dans la lepre: justification physiopathologique et methodes objectives pour en aprecier les resultats. Med Trop 37: 763 770.
12. Brain (2000) Aids to the examination of the peripheral nervous system. Philadelphia: W.B. Saunders.
13. Streiner DL, Norman GR (2003) Health Measurement Scales:A practical guide to their development and use. Oxford: Oxford University Press.
14. Landis JR, Koch GG (1977) The measurement of observer agreement for categorical data. Biometrics 33: 159 174.
15. Bland JM, Altman DG (1995) Comparing methods of measurement: why plotting difference against standard method is misleading. Lancet 346: 1085 1087.
16. Cronbach LJ, Meehl PE (1955) Construct validity in psychological tests. Psychol Bull 52: 281 302.
17. Marshall M, Lockwood A, Bradley C, Adams C, Joy C, et al. (2000) Unpublished rating scales: a major source of bias in randomised controlled trials of treatments for schizophrenia. Br J Psychiatry 176: 249 252.
18. Anderson AM, Croft RP (1999) Reliability of Semmes Weinstein monofilament and ballpoint sensory testing, and voluntary muscle testing in Bangladesh. Lepr Rev 70: 305 313.
19. Van Brakel WH, Nicholls PG, Das L, Barkataki P, Maddali P, et al. (2005) The INFIR Cohort Study: assessment of sensory and motor neuropathy in leprosy at baseline. Lepr Rev 76: 277 295.
20. Roberts AE, Nicholls PG, Maddali P, Van Brakel WH (2007) Ensuring inter-tester reliability of voluntary muscle and monofilament sensory testing in the INFIR Cohort Study. Lepr Rev 78: 122 130.
21. van Brakel WH, Nicholls PG, Wilder-Smith EP, Das L, Barkataki P, et al. (2008) Early Diagnosis of Neuropathy in Leprosy-Comparing Diagnostic Tests in a Large Prospective Study (the INFIR Cohort Study). PLoS Negl Trop Dis 2: e212. doi:10.1371/journal.pntd.0000212.
22. Wijdicks EF, Bamlet WR, Maramattom BV, Manno EM, McClelland RL (2005) Validation of a new coma scale: The FOUR score. Ann Neurol 58: 585 593.
23. Richardus JH, Withington SG, Anderson AM, Croft RP, Nicholls PG, et al. (2003) Treatment with corticosteroids of long-standing nerve function impairment in leprosy: a randomized controlled trial (TRIPOD 3). Lepr Rev 74: 311 318.

295

## 45
### Comparison of patients' and providers' severity evaluation of oral mucosal diseases

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The importance for providers of empathizing with patients is widely advocated. This is true also in oral health, which is an important component of general health, and has bi-directional and complex interactions with systemic health. In particular, oral mucosal lesions, such as oral ulcerations, candidiasis, burning mouth syndrome (BMS), morphology/color changes of tongue, and oral lichen planus (OLP), may have an important impact on patients' health.

The aim of this study was to compare the evaluation of the severity of different oral mucosal diseases by dermatologists and by the patients themselves. The underlying hypothesis was that, even in the global severity assessment of his/her own disease, it is plausible and understandable that a patient does not provide a simple clinical evaluation, but includes subjective aspects, which contribute to the burden of a disease. This would lead to different results compared to the providers.

For each patient, an overall clinical severity evaluation of the disease was given by the dermatologist (Physician Global Assessment, PGA) and by the patient him/herself (Patient Global Assessment, PtGA) on a 5-point scale: "very mild", "mild", "moderate", "severe", "very severe".

Data on both PGA and PtGA were complete for 145 patients (70% women; mean age 54 years). PtGA scores were concordant with PGA for 42.9-47.8% of patients, while 13.0% of patients evaluated as "mild/very mild" by physicians, were self-evaluated as "severe/very severe". However, there were also 23.8% of patients, whose clinical severity was considered as "severe/very severe" by the physician, who evaluated their disease as "mild/very mild". Cohen's kappa coefficient between PGA and PtGA was 0.140, indicating a low concordance. The highest kappa coefficient was observed for patients with BMS (0.265), while in patients with non malignant lesions the concordance PGA-PtGA was below that observed by chance (−0.234). Under-estimation by the physician was particularly high in patients with ulcerations, with bacterial or fungal diseases, and with OLP.

In conclusion, we observed that the agreement between patients' and providers' evaluation of disease severity was very low. It is important to study the possible causes of these discrepancies, in order to improve the patients' satisfaction of care.

## 46
### Development and validation of a severity scale for leprosy type 1 reactions

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Type 1 reactions occur predominantly in individuals with the borderline forms of leprosy. 20–30% of individuals diagnosed with leprosy will have a type 1 reaction.

Type 1 reactions are characterised by inflammation of the skin, nerves or both. Type 1 reactions affecting the peripheral nerves may result in decreased sensory and motor function and lead to disability. A tool which enables clinicians to accurately assess the severity of leprosy type 1 reactions would be useful in defining outcomes for clinical trials and facilitating the even distribution of patients with similar disease severity between the arms of clinical trials. A measure of reaction severity could also be used in treatment guidelines to indicate the need for therapy and may be a useful prognostic tool.

A scale was developed and the face and content validity were assessed following consultation with recognised experts in the field. The construct validity was determined by applying the scale to 81 patients in Bangladesh and Brazil who had been diagnosed with leprosy type 1 reaction. An expert categorized each patient's reaction as mild or moderate or severe. Another worker applied the scale. This was done independently. The agreement between two observers (four pairs) using the scale was assessed on 39 participants at a subsequent stage.

The scale had good internal consistency demonstrated by a Cronbach's alpha >0.8. Removal of three items from the original scale resulted in statistically significant discrimination between disease severity categories ($P=0.048$). Cut off points for reaction severities were determined using receiver operator characteristic curves. A mild reaction is characterized using the final scale by a score of 4 or less. A moderate reaction is a score of between 4.5 and 8.5. A severe reaction is a score of 9 or more. There was very good inter-observer agreement.

This is the first validated tool for quantifying leprosy type 1 reaction severity.

## 47
### Methodological quality of studies on diagnostic criteria for atopic dermatitis: the QUADAS tool

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Atopic dermatitis (AD) is a highly pruritic and chronic inflammatory disease with an increasing prevalence. Since there are no adequate (laboratory) tests to diagnose AD, several lists of diagnostic criteria have been introduced, validated and used in population and hospital based studies over the years. A systematic review (SR) was conducted to summarize all the evidence concerning the validation of diagnostic criteria. Lack of methodological quality of diagnostic validation studies may overestimate the performances of the diagnostic criteria under evaluation. To assess the quality of these studies, a checklist was developed: the Quality Assessment of Diagnostic Accuracy tool (QUADAS). The QUADAS tool uses predefined criteria based on elements of study design, conduct and analysis, which are likely to have a direct relationship to bias in test accuracy studies.

We assessed the methodological quality of studies concerning the validation of diagnostic criteria for AD.

In our SR, 27 studies concerning the validation of 6 diagnostic criteria were included of the 925 articles identified through a Pubmed and Embase search. By using the QUADAS tool for assessment of diagnostic accuracy, it was shown that the methodological quality of the assessed studies differed substantially. Selection criteria, reference standard and index test were well described in all studies, withdrawals and intermediate results were not. Approximately half of the studies showed a lack of blinding, of which there is evidence that it may cause seriously biased results. Another important point of discussion is item 7 of the QUADAS tool. Since the current diagnostic criteria of AD are based on clinical experience and the gold standard is the clinical diagnosis, the results of the index test are used in establishing the final diagnosis. With this, an incorporation bias is inevitable.

We conclude that improvement of methodological design for validation studies is needed to improve future validation studies of diagnostic criteria. The QUADAS is found to be a useful tool to assess methodological quality in diagnostic accuracy studies.

## 48
### Prevalence of inflammatory acne in Ghanaian schoolchildren

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We aimed to investigate the prevalence of inflammatory acne vulgaris in children and adolescents who are living in rural and urban areas in Ghana in relation to the age, sex, body mass index and socio-economic status of the children.

A total of 1,394 children and adolescents attending 6 rural and 5 urban schools in the Greater Accra Region were examined for inflammatory acne vulgaris. Baseline characteristics, such as age, sex, length, weight, and socio-economic status were collected by physical examination and questionnaire. Logistic regression analyses were performed to estimate the effect of potential risk factors on the development of inflammatory acne vulgaris.

Inflammatory acne vulgaris was found in 4.7% in de total group en 6% in the age group 9-20 years. In the rural schools only 1 child had inflammatory acne, compared to 65 (10.1%) out of 641 children in the urban schools ($P<0.001$). The risk with 95% confidence interval, adjusted for age and body mass index, of girls compared to boys to develop inflammatory acne was 3.0 (1.6-5.2). The risk to develop inflammatory acne adjusted for age and sex for children with a high body mass index was 3.0 (1.7-5.8) and for children with a low body mass index 0.181 (0.22-2.0) compared to children with a normal body mass index, respectively.

The prevalence of inflammatory acne vulgaris in Ghanaian schoolchildren and adolescents ranged between 0.1% in rural and 10.1% in urban areas. Age, female gender, high body mass index and living in an urban area were the main risk factors associated with an increased risk of inflammatory acne vulgaris.

296

# The development and validation of a severity scale for leprosy type 1 reactions

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## Introduction

Leprosy type 1 reactions are an important complication of borderline leprosy. They occur before, during or after multi-drug therapy. They may affect the skin, nerves or both and are a significant cause of nerve function impairment. Nerve function impairment leads to the disability and deformity associated with leprosy.
Approximately 40% of individuals with nerve function impairment will have some residual impairment despite treatment with oral corticosteroids. Studies to optimise treatment are needed.

Leprosy type 1 reaction

## Why is a Severity Scale needed?

A scale would:
• allow the combining of cutaneous and neurological signs.
• facilitate the development of treatment guidelines.
• aid the comparability of clinical trials.
• possibly help to determine prognosis.

## The Severity Scale

The current scale was developed from previously used scales which had not been validated, following consultation with leprologists independent of the scale developers.
It is a clinical tool in which fixed criteria are assigned a score. The higher the total score the worse the reaction. It is a measure of severity and not a diagnostic tool.
The score for each item is recorded on a standard form divided into sections to permit different staff members to complete the part relevant to them.
The score for each section is added together to give a total.
The scale is made up of 24 items:
• Inflammation of skin lesions
• Number of raised or inflamed skin lesions
• Peripheral oedema due to reaction
• Nerve pain and/or paraesthesia
• Nerve tenderness
• Degree of fever (°C)
• Graded monofilament sensory testing using 2 and 10g on the hands and 10 and 300g on the feet
• Voluntary Motor Testing of muscles supplied by 10 nerves.
Range of scores possible 0-96

## Methods – Step 1

• An examiner applied the scale to an individual diagnosed as having a type 1 reaction.
• The individual was then examined by an "expert" who categorised the reaction as mild or moderate or severe.
• Assessments were performed independently in a blinded manner.

## Methods – Step 2

• Two examiners independently applied the scale to patients diagnosed with type 1 reaction.
• Performed using 4 pairs of examiners on individuals with type 1 reaction.
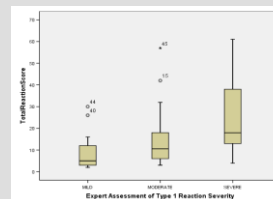
Voluntary Motor Testing

Sensory testing using graded monofilaments

## Results

Participants:
• 81 enrolled (79% male)
• Mean age 39.5 (11-86)
• Bangladesh 56, Brazil 25
• 64.2% experiencing 1st reaction
• 56 skin and nerve involvement
• 18 skin only
• 7 nerve only
Median scores:
• Mild=5.0 (IQR= 11.0)
• Moderate=10.5 (IQR = 13.0)
• Severe= 18.0  (IQR = 29)

Using one-way ANOVA did not demonstrate a statistically significant difference (p= 0.053).
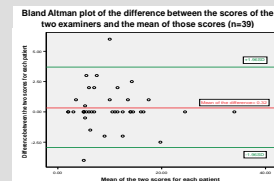
Lagophthalmos due to nerve damage following a type 1 reaction. This may lead to blindness.

Internal Consistency (Reliability)

• Cronbach's alpha = 0.828

• This measures how well a set of items (the scale components) measure a single construct, namely type 1 reaction severity. An alpha of >0.7 is considered desirable.

• Criteria that performed less well were
– Inflammation of skin lesions
– Number of skin lesions
– Nerve pain
– Nerve tenderness
– Fever

Bland Altman plot of the difference between the scores of the two examiners and the mean of those scores (n=39)

## Scale adjustments

Adjustments to the scale were made and the analysis repeated.
• The maximum sensory test score possible for each nerve was changed to 3 rather than 6.
• The components fever, nerve pain and nerve tenderness were removed.
• The reliability of the scale was maintained.
• The differences between the median scores of each severity category became significant.

## Conclusions and further work

• This is the first prospective validation of a severity scale for leprosy type 1 reactions.
• The scale is valid, reliable and has good inter-observer agreement.
• The impact of nerve function impairment greater than 6 months on severity needs to be assessed.
• Further testing of the modified scale in other settings is warranted.

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**ABS163ILC - The validation of a severity scale for leprosy type 1 reactions**

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Type 1 reactions are an important complication of borderline leprosy and are a significant cause of disability. A validated tool providing a standard measure of disease severity would aid the development of treatment guidelines and clinical trials.

Methodology: Leprosy patients in Bangladesh and Brazil diagnosed with type 1 reaction were assessed using a modified version of the INFIR reaction severity scale which incorporates signs of cutaneous involvement, neuritis and nerve function impairment. The scale was administered independently of an examination performed by an experienced leprologist who categorized the reaction as mild, moderate or severe prior to treatment.

Results: 81 patients (64 male) were recruited. 70 had complete data. 18 were diagnosed as having a mild type 1 reaction, 40 moderate and 12 severe. The median scores for reactions categorized as mild were 6.0 (Range 2-30), moderate 10.5 (3-57) and severe 18.0 (4-61). Comparing the scores (Mann-Whitney test) of the categories of severity: mild and moderate and moderate and severe showed significant differences between the mild and moderate groups (p=0.03) and the moderate and severe groups (p=0.015)

Discussion: This type 1 reaction severity scale is a valid measure of disease severity. Further work is underway to measure inter-observer reliability and to determine the weighting of individual items in the scale. The ability of the scale to reflect changes in disease severity over time and with treatment is also being assessed.

Key words: Leprosy, type 1 reactions, severity scale

**6<sup>th</sup> South Asian Regional Congress of Dermatology, Kathmandu, Nepal.**

**13<sup>th</sup> -15<sup>th</sup> November 2009**

Topic : A randomized double blind controlled phase 2 study of methylprednisolone in the management of leprosy Type 1 reactions and nerve function impairment;

**Authors : SL Walker, RA Hawksworth, S Dhakal, K Mahat, PG Nicholls, DN Lockwood;**

Introduction : Leprosy Type 1 reactions are a significant cause of nerve function impairment in people affected by leprosy. Treatment with oral prednisolone is not always effective. Leprosy Type 1 reactions are a significant cause of nerve function impairment in people affected by leprosy. Treatment with oral prednisolone is not always effective.
The optimal dose and duration of corticosteroid therapy for Type 1 reactions needs clarification. High dose intravenous methylprednisolone has not been used previously in a trial of treatment of leprosy Type 1 reactions.

Objectives : We wished to assess the safety and tolerability of high dose methylprednisolone in leprosy patients in Nepal.

Methodology : A randomized double blind controlled trial comparing intravenous high dose methylprednisolone and oral prednisolone with prednisolone alone was used.
The primary outcome measure was the frequency of adverse events in the two treatment arms.
Secondary outcomes measures were:
• change in clinical nerve function impairment and Clinical Severity Score at days 4, 29, 113 and 337. • time to the next steroid requiring reactional episode or acute nerve function impairment
• the amount of supplementary prednisolone required in addition to the reducing 16 week regimen. A post-hoc physician assessment of neurological outcome was determined in those individuals who had nerve function impairment and had completed the course of treatment.

Results : Forty two individuals were recruited. Twenty-three participants experienced at least one adverse event, twelve (54.5%) in the prednisolone arm and 11 (55%) in the methylprednisolone arm. Seven individuals experienced more than one adverse event. There were no statistically significant differences in the number of individuals experiencing a given adverse event between the two groups of the study. Two individuals (one from each arm of the study) experienced a major adverse event. The risk ratio of having an adverse event (of any type; major or minor) given that the participant received methylprednisolone was 1.0083 (95% CI: 0.5817 to 1.7480; p=0.9764) compared to prednisolone. The physician assessment of neurological outcome demonstrated that 7 (20.6%) individuals who had nerve damage at baseline (of less than six months duration) recovered. Seventeen individuals of 34 (50%) had an improvement in their nerve function. However nine participants (26.5%) had nerve function that was unchanged and one individual's nerve function had deteriorated. The clinical outcome of patients in the two arms of this study was not significantly different in terms of the validated clinical severity scale or a global assessment of neurological examination. The methylprednisolone treated group had significantly less deterioration in

sensory function during the 16 weeks of corticosteroid therapy but this was not maintained to the end of the 48 week follow up period.;

Conclusion : Methylprednisolone appears to no more likely to cause adverse events than prednisolone. The study highlighted that corticosteroid treatment for Type 1 reactions and NFI is sub-optimal even when given in large doses for 16 weeks. ;

Keywords : leprosy reactions corticosteroids;

Category of Presentation: Free Paper

Topic : In leprosy Type 1 reactions changes in severity score are significantly different in treated patients who recover or improve compared to those who do not.

**Authors : SL Walker, RA Hawksworth, S Dhakal, K Mahat, PG Nicholls, DN Lockwood;**

Introduction : Leprosy Type 1 reactions are a significant cause of nerve function impairment in people affected by leprosy. A severity scale for leprosy Type 1 reactions was developed and validated. It can discriminate between mild, moderate and severe disease. It also has good inter-observer reliability.;

Objectives : We wished to assess the ability of a validated severity scale for leprosy Type 1 reactions to differentiate the level of improvement following corticosteroid treatment of Nepali patients with Type 1 reactions.

Methodology : A randomized double blind controlled trial comparing intravenous high dose methylprednisolone and oral prednisolone with prednisolone alone was used. Patients were assessed over 48 weeks on 16 occasions. The clinical severity scale was applied at each assessment and a severity score calculated.
A post-hoc physician assessment of neurological outcome was determined in those individuals who had nerve function impairment and had completed the course of treatment.

Results : Forty two individuals were recruited. Individuals were grouped according to their status with respect to the physician assessment of neurological outcome.
Individuals were grouped into two categories improved and recovered or unchanged and worse.
The median change in nerve score between the baseline and the final recorded assessments were significantly different (Mann Whitney p=0.003).

Conclusion : These results lend further weight to the validity of the severity scale for leprosy Type 1 reactions and support its use in future clinical studies in which improvement in clinical status needs to be quantified.

Keywords : leprosy reactions measurement scale;

Category of Presentation: Free Paper