

## ***Mycobacterium leprae* virulence-associated peptides are indicators of exposure to *M. leprae* in Brazil, Ethiopia and Nepal**

**Kidist Bobosha<sup>1,2</sup>, Sheila Tuyet Tang<sup>3</sup>, Jolien J van der Ploeg-van Schip<sup>1</sup>, Yonas Bekele<sup>2</sup>, Marcia VSB Martins<sup>4</sup>, Ole Lund<sup>3</sup>, Kees LMC Franken<sup>1</sup>, Saraswoti Khadge<sup>5</sup>, Maria Araci de Andrade Pontes<sup>6</sup>, Heitor de Sá Gonçalves<sup>6</sup>, Jemal Hussien<sup>2</sup>, Pratibha Thapa<sup>5</sup>, Chhatra B Kunwar<sup>5</sup>, Deanna A Hagg<sup>5</sup>, Abraham Aseffa<sup>2</sup>, Maria Cristina Vidal Pessolani<sup>4</sup>, Geraldo MB Pereira<sup>7</sup>, Tom HM Ottenhoff<sup>1</sup>, Annemieke Geluk<sup>1/+</sup>**

<sup>1</sup>Department of Infectious Diseases, University Medical Center, Leiden, The Netherlands <sup>2</sup>Armauer Hansen Research Institute, Addis Ababa, Ethiopia <sup>3</sup>Center for Biological Sequence Analysis, Technical University Denmark, Lyngby, Denmark

<sup>4</sup>Laboratório de Microbiologia Celular, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, RJ, Brasil

<sup>5</sup>Mycobacterial Research Laboratory, Anandaban Hospital, Kathmandu, Nepal <sup>6</sup>Centro Dermatológico Dona Libânia, Fortaleza, CE, Brasil

<sup>7</sup>Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

*Silent transmission of Mycobacterium leprae, as evidenced by stable leprosy incidence rates in various countries, remains a health challenge despite the implementation of multidrug therapy worldwide. Therefore, the development of tools for the early diagnosis of M. leprae infection should be emphasised in leprosy research. As part of the continuing effort to identify antigens that have diagnostic potential, unique M. leprae peptides derived from predicted virulence-associated proteins (group IV.A) were identified using advanced genome pattern programs and bioinformatics. Based on human leukocyte antigen (HLA)-binding motifs, we selected 21 peptides that were predicted to be promiscuous HLA-class I T-cell epitopes and eight peptides that were predicted to be HLA-class II restricted T-cell epitopes for field-testing in Brazil, Ethiopia and Nepal. High levels of interferon (IFN)- $\gamma$  were induced when peripheral blood mononuclear cells (PBMCs) from tuberculoid/borderline tuberculoid leprosy patients located in Brazil and Ethiopia were stimulated with the ML2055 p35 peptide. PBMCs that were isolated from healthy endemic controls living in areas with high leprosy prevalence ( $EC_{high}$ ) in Ethiopia also responded to the ML2055 p35 peptide. The Brazilian  $EC_{high}$  group recognised the ML1358 p20 and ML1358 p24 peptides. None of the peptides were recognised by PBMCs from healthy controls living in non-endemic region. In Nepal, mixtures of these peptides induced the production of IFN- $\gamma$  by the PBMCs of leprosy patients and  $EC_{high}$ . Therefore, the M. leprae virulence-associated peptides identified in this study may be useful for identifying exposure to M. leprae in population with differing HLA polymorphisms.*

Key words: *M. leprae* - leprosy - biomarkers - bioinformatics - virulence - peptides

Despite the decrease in the prevalence of leprosy since the introduction of multidrug therapy (MDT) over 30 years ago, new cases are still reported annually at a consistent rate in a number of countries. This observation indicates a continued and significant transmission of leprosy at the population level that presents a challenge for disease control efforts (WHO 2011). The incubation period for *Mycobacterium leprae* and the time that elapses before observable symptoms in an infected individual range between four-10 years, with a maximum of 30 years (Britton & Lockwood 2004). It is hypothesised that most patients are subclinically infected for a considerable period of time before the infection becomes apparent. Therefore, subclinical infection may represent a major source of *M. leprae* transmission.

Early detection of leprosy infection and timely treatment with MDT will help reduce transmission and infection and could prevent damage to nerves, disabilities and deformities. However, there are no diagnostic tests that are currently available that can detect asymptomatic *M. leprae* infection. The development of specific immunodiagnostic tools for leprosy infection requires adequate information about the pathogen-associated antigens. The inability to culture *M. leprae* on artificial media (Truman & Krahenbuhl 2001) has greatly hampered leprosy research for many decades and has prevented the development of specific diagnostic tools for leprosy infection. The recent availability of improved bioinformatics tools and the genome sequence of *M. leprae* have resulted in new possibilities for leprosy research. These advances have enabled the prediction of relevant *M. leprae* proteins and potential human leukocyte antigen (HLA) class I and class II epitopes that can activate T-cells (Geluk et al. 2011). The use of unique *M. leprae* antigens that were identified using post-genomic approaches has resulted in the detection of *M. leprae* protein or peptide-specific T-cell responses. These responses may help identify *M. leprae*-exposed or infected individuals (Geluk et al. 2005, 2009, Spencer et al. 2005, Araoz et al. 2006, Bobosha et al. 2011, 2012).

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+ Corresponding author: a.geluk@lumc.nl

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Antigenic proteins typically contain multiple peptide epitopes. Therefore, antigenic proteins may be preferred diagnostic tools for use in populations with diverse genetic backgrounds. However, compared with the use of antigenic proteins as diagnostic tools, peptides have reduced or absent T-cell cross-reactivity (Geluk et al. 2008, 2009). Therefore, analysing unique *M. leprae* peptides or pools of peptides in different regions that are endemic for leprosy infection could be useful for identifying peptides that have diagnostic potential. The immunogenic and diagnostic significance of the *Mycobacterium tuberculosis* (*Mtb*) proteins ESAT-6 and CFP-10 and peptides derived from the RD-1 region (involved in enhancing virulence) (Arend et al. 2000) have led us to search for similar unique *M. leprae* virulence-associated peptides. In the current study, the complete proteome of *M. leprae* was dissected *in silico* into 20-mer amino acid peptides. Next, unique *M. leprae* peptides derived from group IV.A (virulence) ([sanger.ac.uk/Projects/M\\_leprae/MI\\_gene\\_list\\_hierarchical.shtml](http://sanger.ac.uk/Projects/M_leprae/MI_gene_list_hierarchical.shtml)) that were predicted to bind promiscuously to HLA class I or class II alleles were selected and synthesised. *In vitro* analysis of these synthetic peptides was performed using peripheral blood mononuclear cells (PBMCs) or whole blood isolated from lepromatous leprosy/borderline tuberculoid (TT/BT) and borderline lepromatous/lepromatous leprosy (BL/LL) patients and healthy endemic controls (ECs) who were located in Brazil, Ethiopia and Nepal in areas with high ( $EC_{high}$ ) or low ( $EC_{low}$ ) leprosy prevalence.

**SUBJECTS, MATERIALS AND METHODS**

**Peptide identification strategy** - The peptide identification procedure is depicted in Fig. 1. All of the 20-mer peptides in the *M. leprae* genome (Cole et al. 2001) were identified. A length of 20 amino acids was chosen because this size allows for both HLA class I and class II T-cell epitopes. The 20-mer peptides that had eight or more amino acid overlap in a continuous stretch with sequences from other mycobacteria, compared with complete or nearly complete genomes sequences available in the GenBank sequence database ([ncbi.nlm.nih.gov/GenBank](http://ncbi.nlm.nih.gov/GenBank)), were excluded from this study. The selection procedure is described in more detail below.

**Bacterial genome sequences** - All of the genome sequences that were used in this study were obtained from GenBank. The following six complete mycobacterial genomes were used: *M. leprae* (GenBank Protein accession NC\_002677), *Mtb* strains H37Rv (AL123456 and NC\_000962) and CDC1551 (NC\_002755), *Mycobacterium bovis* (NC\_002945) and *Mycobacterium avium paratuberculosis* (NC\_002944). Other nearly complete mycobacterial genome sequences (unpublished at the time of peptide selection), such as those from *M. avium*, *Mycobacterium smegmatis*, *Mtb* strain 210 ([tigr.org](http://tigr.org)), *Mycobacterium marinum*, *Mycobacterium microti* ([sanger.org](http://sanger.org)), *Mycobacterium paratuberculosis* and *Mycobacterium ulcerans* ([pasteur.fr](http://pasteur.fr)), were also included, using Basic Local Alignment Search Tool (BLAST) to allow a broader understanding of the conservation of the 20-mer sequences across different mycobacterial species.

**Bioinformatics tools** - The complete *M. leprae* genome was divided into 20-mer peptide sequences with an overlap of 19 amino acids using the Genome Patterns program (*M. leprae* list). To check the homology of the *M. leprae* sequences with other, closely related mycobacterial genomes, the Genome Patterns program was also used to generate a list of 20-mer peptide sequences with an overlap of 19 amino acids for the *Mtb* strains H37Rv and CDC1551, *M. bovis* and *M. paratuberculosis* (the *MTB* list). The *M. leprae* list was compared with the *MTB* list and all of the 20-mer peptide sequences that had a continuous stretch of eight or more identical amino acids between the lists were excluded from further analysis. This filtering step resulted in 141,300 20-mer peptide sequences that are unique to the *M. leprae* genome. Any of the 20-mer peptide sequences that were encoded by the 1,116 *M. leprae* pseudogenes were then excluded. This process reduced the *M. leprae* list to 138,938 20-mer peptide sequences derived from 1,546 different *M. leprae* candidate proteins. To reduce the number of candidate peptides for BLAST, we selected peptides that were derived from genes in the functional classification group IV.A (virulence) (included the following 13 genes: ML0360, ML0361, ML0362, ML0885, ML1214, ML1358, ML1811, ML1812, ML2055, ML2208, ML2466, ML2589, ML2711) ([sanger.ac.uk/Projects/M\\_leprae/MI\\_gene\\_list\\_hierarchical.shtml](http://sanger.ac.uk/Projects/M_leprae/MI_gene_list_hierarchical.shtml), currently designated as “genes involved in virulence, detoxification and adaptation” or “genes involved in cell wall and cell processes” on [mycobrowser.epfl.ch/leprosy.html](http://mycobrowser.epfl.ch/leprosy.html)). This step resulted in 886 candidate 20-mer peptide sequences. Next, we used a Perl script entitled “genediff.pl” that compared the genome

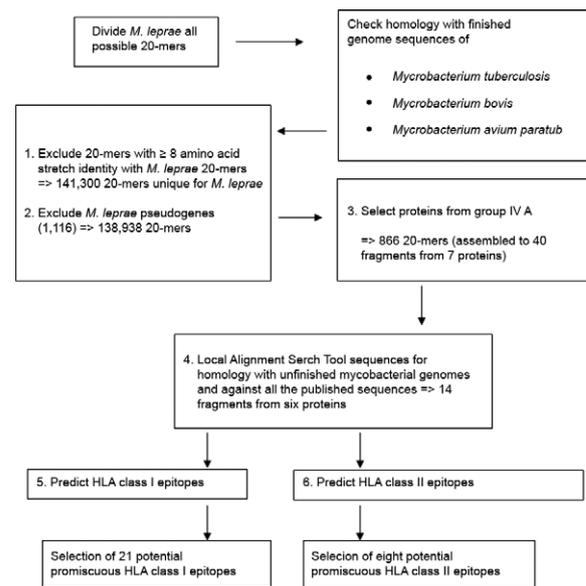


Fig. 1: *Mycobacterium leprae* peptide selection procedure. Identification and selection of predicted *M. leprae* promiscuous human leukocyte antigen (HLA) class I epitopes (n = 21) and predicted *M. leprae* promiscuous HLA class II epitopes (n = 8) derived from *M. leprae* proteins from functional classification group IV.A (virulence) ([sanger.ac.uk/Projects/M\\_leprae/MI\\_gene\\_list\\_hierarchical.shtml](http://sanger.ac.uk/Projects/M_leprae/MI_gene_list_hierarchical.shtml)).

sequences by BLAST analysis (CBS, script used internally) and excluded proteins that were homologous to the human genome and to three *Mtb* homologs. The *M. leprae*-derived 20-mer peptide sequences that overlapped were then assembled (if the peptides were arranged in sequential order within the genome), resulting in a total of 40 protein fragments. BLAST analysis was then used to exclude protein fragments that were also found in the incomplete genome sequences of *M. smegmatis*, *Mtb* strain 210 and *M. microti* OV254 (sanger.org and tigr.org). The assembled *M. leprae* fragments were then analysed by BLAST analysis against all sequences that were available in GenBank (ncbi.nlm.nih.gov/blast/). All of the sequences that had shared more than eight amino acids with the *M. leprae* peptides were again excluded. Of the 40 fragments, 14 peptide fragments from six proteins were determined to be unique to *M. leprae*.

**Prediction of CD4 and CD8-restricted T-cell epitopes** - NetCTL version 1.2 (cbs.dtu.dk/services/NetCTL) was used to predict 9-mer peptide epitopes from the 14 unique *M. leprae* fragments for 12 HLA supertypes (HLA-A1, A2, A3, A24, A26 and HLA-B7, B8, B27, B39, B44, B58, B62) (Larsen et al. 2005). All of the peptides that had a combined score in excess of 1.25 were selected as potential major histocompatibility complex class I ligands. An adapted version of the TEPITOPE program (Singh & Raghava 2003) was used

to predict CD4-restricted T-cell epitopes from the 14 unique *M. leprae* fragments that were identified from six *M. leprae* proteins. The six protein sequences were submitted to the SubCell 1.0 server (cbs.dtu.dk/services/) to predict the subcellular localisation of these proteins in Gram-negative and Gram-positive bacteria. From the resulting sequences, peptides were selected that contained predicted binding sequences for most of the HLA alleles. For this selection, priority was given to peptides that were predicted to bind promiscuously to multiple HLA alleles (Table I). This process resulted in 21 potential CD8-restricted T-cell epitopes and eight potential CD4-restricted T-cell epitopes (Tables I, II). In the event that a positive CD4 T-cell response is induced after stimulation with the 9-mer peptides (potentially a suboptimal length), only the strongest inducers will be identified using this approach.

**Synthetic peptides** - The virulence-associated *M. leprae*-derived peptides that were identified were purchased from Peptide 2.0 Inc (Chantilly, VA, USA). Homogeneity and purity were confirmed by analytical high-performance liquid chromatography and by mass spectrometry. The purity of all of the peptides was  $\geq 80\%$ . All of the impurities that were identified consisted of shorter derivatives of the peptides that were caused by coupling efficiency that was  $< 100\%$  for each round of peptide synthesis. All of the peptides

TABLE I  
Selected *Mycobacterium leprae* virulence-associated peptides  
[human leukocyte antigen (HLA) class I, n = 21]

	Peptide (9-mer)	Starting- position	ML accession	Accession	HLA	HLA	HLA
p15	RAAVVQAAL	262	ML0885	NP_301670.1_245_270	B7	B8	B58
p16	SMDAAVAAL	193	ML1812	NP_302233.1_181_201	A2	B39	-
p17	GIAGSASY	202	ML2055	NP_302372.1_189_211	A1	B62	-
p18	HRKGLWAIL	10	ML2055	NP_302372.1_1_78	B27	B39	-
p19	QMLEASSSV	210	ML1811	NP_302232.1_209_232	A2	-	-
p20	ALDTFGIPV	73	ML1358	NP_301968.1_64_92	A2	-	-
p21	NGIAGSASY	201	ML2055	NP_302372.1_189_211	A26	-	-
p22	KVTVSSVRK	220	ML1811	NP_302232.1_209_232	A3	-	-
p23	TEAVHSAQL	58	ML0885	NP_301670.1_54_76	B44	-	-
p24	KLMGALDTF	69	ML1358	NP_301968.1_64_92	B58	-	-
p25	VASASAFTM	23	ML2055	NP_302372.1_1_78	B58	-	-
p26	AVVASASAF	21	ML2055	NP_302372.1_1_78	B62	-	-
p27	APLPPSTAT	42	ML2055	NP_302372.1_1_78	B7	-	-
p28	GPVPAVATL	220	ML0885	NP_301670.1_220_250	B7	-	-
p29	IPVAGRCCCL	79	ML1358	NP_301968.1_64_92	B7	-	-
p30	RPRRGSVSR	3	ML1812	NP_302233.1_1_20	B7	-	-
p31	LPSADIVPM	172	ML1358	NP_301968.1_158_181	B7	-	-
p32	SASAFTMPL	25	ML2055	NP_302372.1_1_78	B7	-	-
p33	APIPASVSA	274	ML2055	NP_302372.1_257_287	B7	-	-
p34	RPVPVSTAR	204	ML1214	NP_301879.1_173_212	B7	-	-
p35	IPASVSAPA	276	ML2055	NP_302372.1_257_287	B7	-	-

were divided into aliquots to allow the field testing of identical batches of peptides in Brazil, Ethiopia, Nepal and The Netherlands.

***M. leprae* recombinant proteins** - The *M. leprae* genes that encoded the proteins from which the virulence-associated peptides were derived were polymerase chain reaction-amplified from genomic *M. leprae* DNA. The genes were then cloned using the Gateway technology platform (Invitrogen, Carlsbad, CA, USA) using the pDEST17 expression vector, which contains an N-terminal histidine tag (Invitrogen) (Franken et al. 2000). Sequencing was performed on selected clones to confirm the identity of all of the cloned DNA fragments. The recombinant proteins were overexpressed in BL21 *Escherichia coli* (DE3) and purified to remove endotoxins (Franken et al. 2000). To confirm size and purity, each purified recombinant protein was analysed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining and Western blotting using an anti-His antibody (Invitrogen). Endotoxin levels were below 50 endotoxin units per mg of recombinant protein as determined using a limulus amoebocyte lysate QCL-1000 assay (Lonza Inc, Basel, Switzerland). Recombinant proteins were tested in interferon (IFN)- $\gamma$  release assays to exclude the possibility of non-specific T-cell stimulation and cellular toxicity. For this assay, PBMCs from purified protein derivative (PPD)-negative, healthy Dutch donors were recruited at the Blood Bank in Sanquin, Leiden, The Netherlands. None of the healthy control individuals had any known prior contact with leprosy or tuberculosis (TB) patients.

***M. leprae* whole cell sonicate (WCS)** - Irradiated, armadillo-derived *M. leprae* whole cells were probe-sonicated using a Sanyo sonicator to > 95% breakage. These cells were obtained through the National Institutes of Health/National Institute of Allergy and Infectious Diseases Leprosy Research Support Contract N01 AI-25469 from the Colorado State University (available through the Biodefense and Emerging Infections Research Resources Repository available from beiresources.org/TBVTRM-Research Materials/tabid/1431/Default.aspx).

**Study participants** - Human immunodeficiency virus (HIV)-negative individuals were recruited between August 2008-February 2011 from each region of study. In Brazil, 10 TT/BT leprosy patients, 10 healthy controls (EC<sub>low</sub>) living in an area of Fortaleza (Mereiles) with low leprosy prevalence (< 0.2/10,000) and 10 healthy controls (EC<sub>high</sub>) living in other area of Fortaleza (Bom Jardim) with relatively high leprosy prevalence (> 4/10,000;) were recruited. In Ethiopia, 23 leprosy patients (10 TT/BT and 13 BB/BL), 12 house hold contacts (HHCs) of BL/LL patients and 52 healthy controls were recruited. Of the healthy controls, 25 EC<sub>high</sub> were located in a sub-city of Addis Ababa (Kolfe Keranio) that had a prevalence rate of 1.5 per 10,000 (72 in 465,811) and 27 EC<sub>low</sub> were located in areas that had a prevalence rate of 0.36 per 10,000 (10 in 273,310). The endemicity of leprosy for both of the Ethiopian EC groups was determined by the number of new cases of leprosy and the prevalence of leprosy in nearby health centres. In Nepal, seven TT/BT and five BL/LL patients and 20 EC<sub>high</sub> were enrolled in this study. The national prevalence of leprosy in Nepal was 1.1 per 10,000 in 2008/2009 (Annual Report 2008/2009, Leprosy Control Division, Nepal).

In all of the regions, leprosy was diagnosed based on clinical, bacteriological and histological observations. The leprosy patients were classified as TT/BT or BB/BL/LL by a skin biopsy that was evaluated according to the Ridley and Jopling (1996) classification by qualified microbiologists and pathologists. All of the patients who were recruited had been newly diagnosed, were untreated and did not develop leprosy reactions within three months after MDT initiation. ECs were assessed for the absence of clinical signs and symptoms of TB and leprosy. Staff members working in the leprosy centres or TB clinics were excluded from this study.

**24 h whole blood assays (WBA)** - Within 3 h after collection, venous, heparinised blood (450  $\mu$ L/well) was incubated with 50  $\mu$ L of antigen solution (100  $\mu$ g/mL) in 48-well plates at 37°C under 5% CO<sub>2</sub> and 90% relative humidity. After 24 h incubation, 150  $\mu$ L of supernatant was removed from each well and aliquots were frozen at -20°C until further analysis.

TABLE II  
Selected *Mycobacterium leprae* virulence-associated peptides  
[human leukocyte antigen (HLA) class II, n = 8]

	Peptide (9-mer)	Starting- position	ML accession	Accession	HLA	HLA	HLA
p36	VVRDLRLRA	197	ML1358	NP_301968.1_192_213	1_0301	1_1101	1_1501
p37	WAILAIAVV	15	ML2055	NP_302372.1_1_78	1_0101	1_0801	1_1101
p38	ILAIAVVAS	17	ML2055	NP_302372.1_1_78	1_0301	1_0401	1_1101
p39	VRPVPVSTA	203	ML1214	NP_301879.1_173_212	1_0301	1_1101	-
p40	LRADSVLAV	203	ML1358	NP_301968.1_192_213	1_0301	1_0401	-
p41	LQQVPTLPA	199	ML1214	NP_301879.1_173_212	1_1101	-	-
p42	LAIAVVASA	18	ML2055	NP_302372.1_1_78	1_1101	-	-
p43	ISLATVLSA	158	ML1358	NP_301968.1_158_181	1_1101	-	-

*Lymphocyte stimulation tests (LST)* - PBMCs were isolated by Ficoll density centrifugation of venous, heparinised blood and the PBMCs were plated in triplicate cultures ( $2 \times 10^5$  cells/well) in 200  $\mu$ L/well of serum-free adoptive immunotherapy medium (AIM-V) (Invitrogen, Carlsbad, CA) in 96-well round bottom plates (Costar Corporation, Cambridge, USA). Synthetic peptides, recombinant protein, *M. leprae* WCS or PPD (*Mtb*, Statens Serum Institut, Copenhagen, Denmark) were added at final concentrations of 10  $\mu$ g/mL. A positive control of 1  $\mu$ g/mL phytohaemagglutinin (PHA) (Remel, Oxoid, Haarlem, The Netherlands) was also used. After six days of culture at 37°C under 5% CO<sub>2</sub> and 90% relative humidity, 75  $\mu$ L of supernatant was removed from each well. Triplicate samples were pooled and frozen in aliquots at -20°C until further analysis.

*IFN- $\gamma$  ELISA* - IFN- $\gamma$  concentrations were determined by ELISA (U-CyTech, Utrecht, The Netherlands) (Geluk et al. 2005). The cut-off value to designate a positive response was set prior to experimentation at 100 pg/mL. The sensitivity level of the assay was 40 pg/mL. The values for unstimulated cell cultures were typically < 20 pg/mL. Lyophilised supernatant from PHA-stimulated cultures of PBMCs from an anonymous buffycoat (Sanquin, Leiden, The Netherlands) was provided as a positive control supernatant.

*Multi-cytokine and multi-chemokine assay* - The concentrations of 19 chemokines or cytokines [interleukin (IL)-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IFN- $\gamma$ , induced protein-10 (IP-10) (CXCL10), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (CCL2), monokine induced by IFN- $\gamma$  (CXCL9), macrophage inflammatory protein-1 $\beta$  (CCL4) and tumour necrosis factor] in the supernatants from 24 h WBA were measured using the Bio-Plex suspension array system powered by the Luminex xMap multiplex technology (Bio-Rad Laboratories, Veenendaal, The Netherlands) and analysed using the Bio-Plex Manager™ software 6.0 (Bio-Rad Laboratories, Veenendaal, The Netherlands). After pre-wetting the filter with the assay solution, magnetic beads were washed twice with washing solution using 96-well multiscreen filter plates (Millipore), an Aurum™ vacuum manifold and a vacuum pump (Bio-Rad Laboratories, Veenendaal, The Netherlands). Supernatant samples (50  $\mu$ L) were added to the plates and the plates were incubated for 45 min at room temperature (RT) in the dark on a plate shaker at 300 rpm. After three washes, 12.5  $\mu$ L of the detection antibody cocktail was added to each well and the plates were then incubated at RT in the dark for 30 min on a plate shaker. After three washes, 25  $\mu$ L of streptavidin-PE solution was added per well and the plates were incubated for 10 min. After three washes, 80  $\mu$ L of assay buffer was added to each well and the plates were placed in the Bio-Plex System. A minimum of 50 analyte-specific beads were analysed from each well for fluorescence. A fitting curve was applied to each standard according to the manufacturer's manual. The sample concentrations were interpolated from these standard curves. Concentrations

of analytes that were outside the upper or lower limits of quantification were designated as concentrations equal to the limit of quantification for the cytokine or chemokine.

*Statistical analysis* - Differences in cytokine concentration between the test groups were analysed by a two-tailed Mann-Whitney *U*-test for non-parametric distributions using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego California USA) (graphpad.com). *p*-values were corrected for multiple comparisons. The statistical significance level used was *p* < 0.05.

*Ethics* - This study was performed in accordance with the ethical standards of the Helsinki Declaration of 1975 and as revised in 1983. Ethical approval of the study was obtained through the following national or institutional ethics: the Brazilian National Council of Ethics in Research, the National Health Research Ethical Review Committee of Ethiopia and the Nepal Health Research Council. Informed consent was obtained from all individuals before venipuncture.

## RESULTS

*Post-genomic approach for improved, M. leprae-specific CMI test antigens* - Because CD8-restricted epitopes are shorter than CD4-restricted epitopes, reliable bio-informatics methods to predict CD8-restricted epitopes were developed early (e.g., SYFPEITHI) (SYFPEITHI.de). More recently, methods to predict the longer CD4-restricted T-cell epitopes were designed using improved training sets and algorithms. Notably, the PROPRED program (imtech.res.in) has been demonstrated to accurately predict human T-cell epitopes and many of these epitopes have been confirmed experimentally (Sturniolo et al. 1999, Singh & Raghava 2003). Using genomic sequences that are available and these improved bio-informatics tools, we identified unique *M. leprae* candidate antigens that were screened *in silico* to identify potential T-cell epitopes (Fig. 1, Materials and Methods section). Using this post-genomic approach, unique *M. leprae* epitope sequences derived from *M. leprae* functional group IV.A (virulence) proteins were identified. The synthetic peptides encoded by these unique sequences, which are designated in this study as *M. leprae* virulence-associated peptides (Table I), were used to evaluate cellular responses in leprosy patients and healthy controls from Brazil, Ethiopia and Nepal.

*T-cell recognition of M. leprae peptides by non-ECs (NECs)* - To exclude the possibility of nonspecific induction of T-cell responses by the selected *M. leprae* virulence-associated peptides, the peptides were first tested using a six day LST on PBMCs from Dutch healthy controls (NEC). These controls were also analysed in 24 h WBAs using undiluted venous blood. Most individuals were observed to have high responses to PPD (7/8) and *M. leprae* WCS (5/8). None of the NECs had a detectable IFN- $\gamma$  response against any of the individual peptides or the peptide pools (Fig. 2A). Similarly, no response was observed in the 24 h WBAs (Fig. 2B). These data demonstrate the absence of non-specific T-cell responses to the selected peptides.

**T-cell recognition of *M. leprae* peptides in PBMCs from the Brazilian test groups** - The overall aim of our study was to identify new antigens that can specifically indicate *M. leprae* exposure and/or infection. Therefore, the *M. leprae* virulence-associated peptides (Table I) were first tested in a Brazilian population in a six day LST. For this study, TT/BT, healthy EC<sub>high</sub> who lived in an urban area with relatively high leprosy prevalence ( $p > 4/10,000$ ) and EC<sub>low</sub> who lived in the same city in an area with low leprosy prevalence ( $p < 0.2/10,000$ ) were enrolled. In the Brazilian EC<sub>high</sub> group, 50% and 70% of the individuals specifically recognised ML1358 p20 and ML1358 p24 (Fig. 3A), respectively. None of the individuals from the EC<sub>low</sub> group or the TT/BT group produced IFN- $\gamma$  in response to these two peptides (Fig. 3B). In contrast, stimulation with the ML2055 p35 induced considerable levels of IFN- $\gamma$  ( $> 1000$  pg/mL) in 40% of the TT/BT patients. The ML1214 p41, ML1812 p30, ML2055 p31, ML2055 p37, ML2055 p39 and ML2055 p42 peptides induced IFN- $\gamma$  responses in 30-40% of this group, although the median IFN- $\gamma$  concentrations were lower (200 pg/mL) (Fig. 3C).

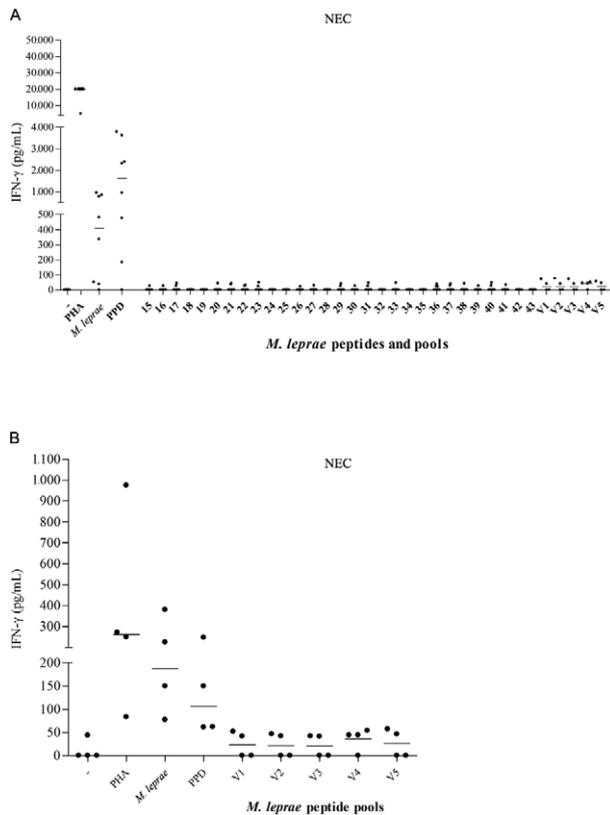


Fig. 2: interferon (IFN)- $\gamma$  responses to *Mycobacterium leprae* peptides in peripheral blood mononuclear cell (PBMC) from non-endemic controls (NEC). IFN- $\gamma$  production (corrected for background values) induced using *M. leprae* virulence-associated peptides or pools thereof (A) in six day PBMC cultures of Dutch healthy controls (n = 8) or as pools in a 24 h whole blood assays (WBA) (B). Pool V1: 15-20; V2: 21-26; V3: 27-32; V4: 33-35, 37-39; V5: 36, 40-43 (numbers indicate peptide sequences depicted in Tables I, II). PHA: phytohaemagglutinin; PPD: purified protein derivative.

**Recognition of *M. leprae* peptides by PBMCs from Ethiopian individuals** - To analyse differences in genetic backgrounds, test groups from different leprosy endemic regions were included in this study. This diversity allowed the analysis of differences in the T-cell response as a result of HLA polymorphisms. Therefore, the *M. leprae* virulence-associated peptides were tested using PBMCs from 12 Ethiopian TT/BT leprosy patients (Fig. 3D) and seven EC (2 EC<sub>high</sub> and 5 EC<sub>low</sub>) (Fig. 3E). The IFN- $\gamma$  concentration in response to the *M. leprae* virulence-associated peptides in both groups was lower compared with the IFN- $\gamma$  concentration in the Brazil cohort. However, the ML2055 p35 peptide induced IFN- $\gamma$  production in 50% of the Ethiopian TT/BT patients (Fig. 3D), which was the case for the Brazilian TT/BT group. In addition, one of the EC<sub>high</sub> individuals responded to eight out of the 28 peptides ( $> 100$  pg/mL) (Fig. 3E). Therefore, the ML2055 p35 peptide is recognised most frequently in the TT/BT patients in Brazil and Ethiopia.

**IFN- $\gamma$  responses in Ethiopian EC<sub>high</sub> and EC<sub>low</sub> in a WBA** - To identify peptides that have the ability to indicate exposure to *M. leprae* using a rapid field-assay, the ML2055 p35, ML2055 p42, ML2055 p37 and ML1358 p24 peptides were selected for comparative analysis in the Ethiopian EC<sub>low</sub> (n = 17) and EC<sub>high</sub> (n = 18) groups using a 24 h WBA. The ML2055 p35 and ML2055 p42 peptides, which were recognised by Brazilian TT/BT patients, also induced significant levels of IFN- $\gamma$  in the Ethiopian EC<sub>high</sub> ( $p = 0.023$  and  $p = 0.020$ , respectively) group compared with the EC<sub>low</sub> group (Fig. 4). The IFN- $\gamma$  levels in response to other *M. leprae* peptides were low and no significant differences were observed between the EC<sub>high</sub> and EC<sub>low</sub> groups.

**IP-10 is a potential biomarker for diagnosis of *M. leprae* exposure** - Although IFN- $\gamma$  is the hallmark effector molecule produced by Th1 cells and is a critical component of the pro-inflammatory immune response, host immunity and immunopathogenicity in response to *M. leprae* infection involve complex interactions between a variety of cells that express different effector and regulatory molecules. Thus, the analysis of multiple biomarkers may be more representative of the immune status of the host and may identify immune patterns that could predispose an individual to *M. leprae* infection. Therefore, aliquots of the 24 h WBA samples from the Ethiopian EC<sub>high</sub> and EC<sub>low</sub> groups were used for the multiplex analysis of 19 additional cytokines and chemokines. The IFN- $\gamma$ -IP-10 (or CXCL10) has been shown to be a useful biomarker for the diagnosis of *Mtb* infection (Singh & Raghava 2003, Ruhwald et al. 2011). Interestingly, the ML2055 p35 protein induced significantly higher levels of IP-10 in the EC<sub>high</sub> group, but not in the EC<sub>low</sub> group ( $p = 0.005$ ) (Fig. 4E). The ML2055 p42 peptide also induced increased levels of IP-10 production in the EC<sub>high</sub> group compared with the EC<sub>low</sub> group, although the difference between these two groups was not significant ( $p = 0.06$ ) (Fig. 4F). None of the other analytes were observed to have significantly different levels between the EC<sub>high</sub>-EC<sub>low</sub> groups (data not shown). Therefore, the elevated IP-10 levels that were induced by the *M. leprae*-

specific antigens in the WBA from EC<sub>high</sub> indicate that this chemokine may have potential for use as a biomarker to differentiate levels of *M. leprae* exposure. Such a marker could be a new diagnostic tool that is similar to what has been reported for TB immunodiagnostic assays (Ruhwald et al. 2007, Aabye et al. 2010).

*IFN-γ production in response to M. leprae recombinant proteins* - The *M. leprae* virulence-associated peptides that induced IFN-γ responses by several individuals were derived from only a few proteins. In particular, p35,

p37 and p42 were derived from ML2055, p20 and p24 were derived from ML1358 and p41 was derived from ML1214. To investigate the immunogenicity of the complete whole antigens, the recombinant proteins ML2055, ML1358 and ML1214 were tested in a 24 h WBA. Ethiopian BB/BL patients, HHCs and ECs (EC<sub>high</sub>: n = 7 and EC<sub>low</sub>: n = 3) (Fig. 5) were tested in this assay. The ML2055 protein induced an IFN-γ response (> 100 pg/mL) in 60% (7 out of 13) of the BB/BL patients and 42% (3 out of 7) of the EC<sub>high</sub> individuals. However, only one

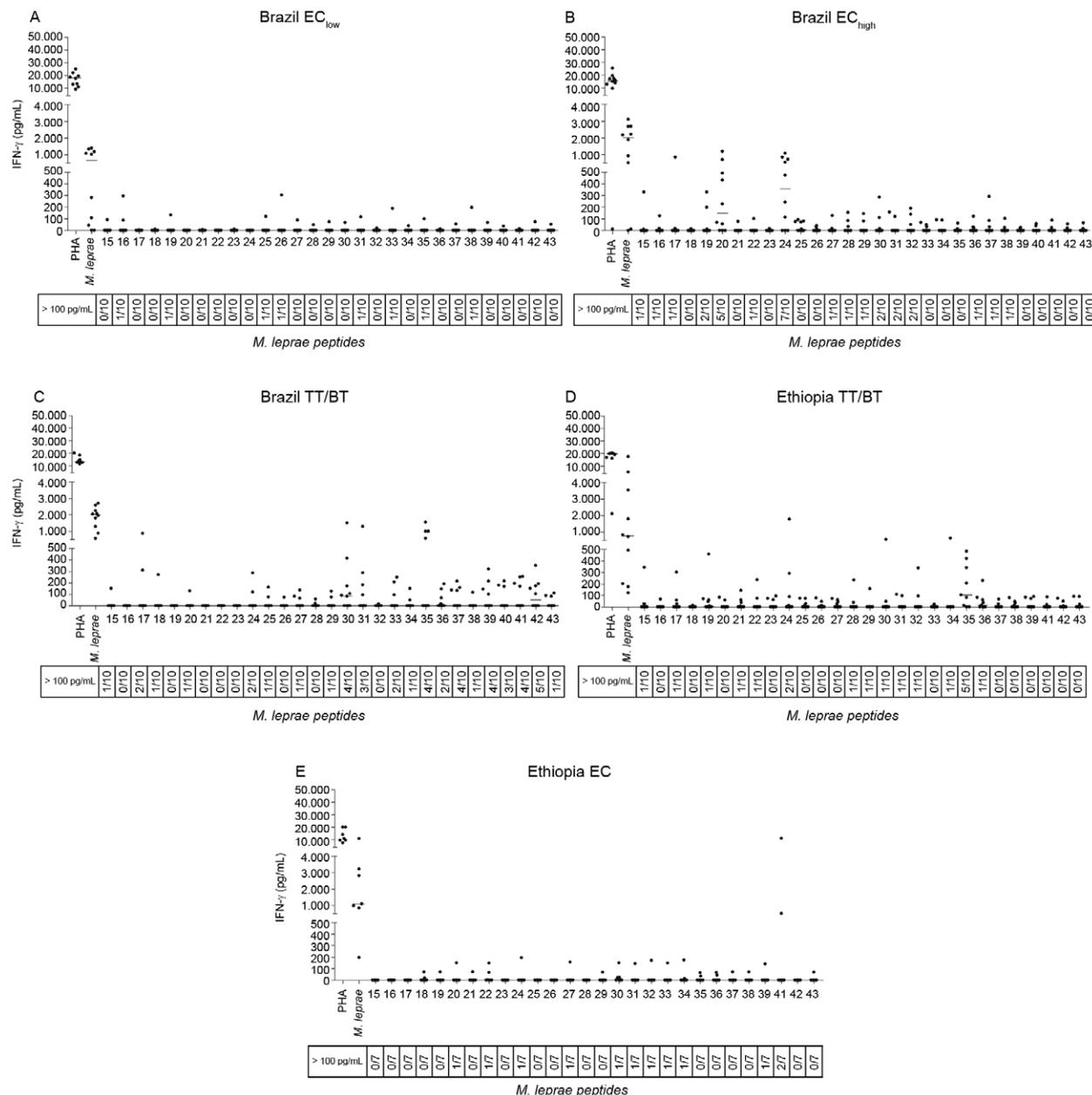


Fig. 3: interferon (IFN)-γ responses to *Mycobacterium leprae* peptides in peripheral blood mononuclear cell (PBMC) from Brazilian and Ethiopian individuals. IFN-γ production (corrected for background values) induced using *M. leprae* virulence-associated peptides in six day PBMC cultures of endemic controls (EC) from areas of Fortaleza, Brazil, with low prevalence (EC<sub>low</sub>) (A) (< 0.2/10,000) (n = 10) and high prevalence (EC<sub>high</sub>) (B) (> 4/10,000) (n = 10) leprosy endemicity, Brazilian lepromatous leprosy/borderline tuberculoid (TT/BT) patients (C) (n = 10), Ethiopian TT/BT patients (D) (n = 10) and Ethiopian EC (E) (n = 7). Median values per test group are indicated by horizontal lines. Background values were < 20 pg/mL.

of the HHCs produced a response against this protein. IFN- $\gamma$  responses against the ML1358 protein were very low in ECs and HHCs, but a significant percentage (60%) of the BB/BL patients responded to this protein. The ML1214 protein induced an IFN- $\gamma$  response in 69% of BB/BL patients, 66% of HHCs and 60% of ECs.

*T-cell responses to pools of M. leprae virulence-associated peptides in Nepal* - Individuals from Nepal were also enrolled in this study to analyse the response by an Asian endemic population. The *M. leprae* virulence-associated peptides were tested in five peptide pools (V1: p15-p20; V2: p21-p26; V3: p27-p32; V4: p33-p35, p37-p39; V5:

p36, p40- p43) using PBMCs from Nepali leprosy patients and ECs. When peptide pools that contained p35, p37 and p42 from ML2055 (V3, V4 and V5) were screened in Nepal, the production of IFN- $\gamma$  was observed from PBMCs of ECs, but there was only a small response by PBMCs from leprosy patients prior to treatment (Fig. 6). Interestingly, after the completion of MDT, three out of the seven BT patients were observed to have increased concentrations of IFN- $\gamma$  production in response to stimulation with the peptide pools (Fig. 6C, F). These findings will need to be confirmed in larger patient cohorts, but these data indicated that the peptides used may have relevance in monitoring therapeutic intervention.

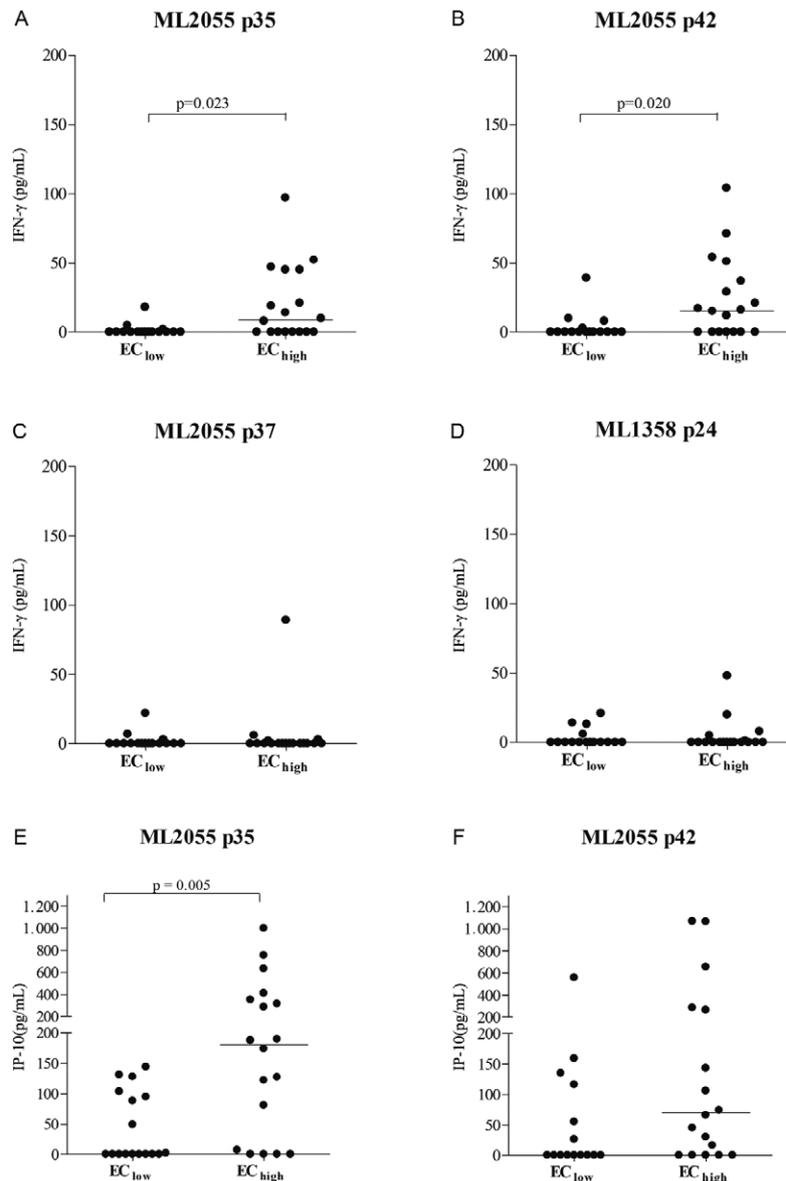


Fig. 4: interferon (IFN)- $\gamma$  and induced protein-10 (IP-10) responses to *Mycobacterium leprae* virulence-associated peptides in 24 h whole blood assays (WBA) in Ethiopian high prevalence (EC<sub>high</sub>) and low prevalence (EC<sub>low</sub>) leprosy IFN- $\gamma$  (A-D) and IP-10 (E, F) production in response to *M. leprae* peptides ML2055 p35 (A, E), ML2055 p42 (B, F), ML2055 p37 (C) and ML1358 p24 (D) in 24 h WBA of EC<sub>low</sub> healthy individuals from areas in Ethiopia with leprosy prevalence of 0.36/10,000 (n = 17) and EC<sub>high</sub> healthy individuals from areas with prevalence of 1.5/10,000 (n = 18). Responses are corrected for background values. Median values per test group are indicated by horizontal lines.

## DISCUSSION

Every year, more than 200,000 people are newly diagnosed with leprosy at health facilities worldwide. The majority of these cases are multibacillary leprosy (MB) patients, which include a considerable proportion of grade 2 cases and paediatric cases (WHO 2011). The lack of tools for the early detection of leprosy and complications in leprosy reactions represent the most important challenges in combating leprosy (Scollard 2005, Scollard et al. 2006).

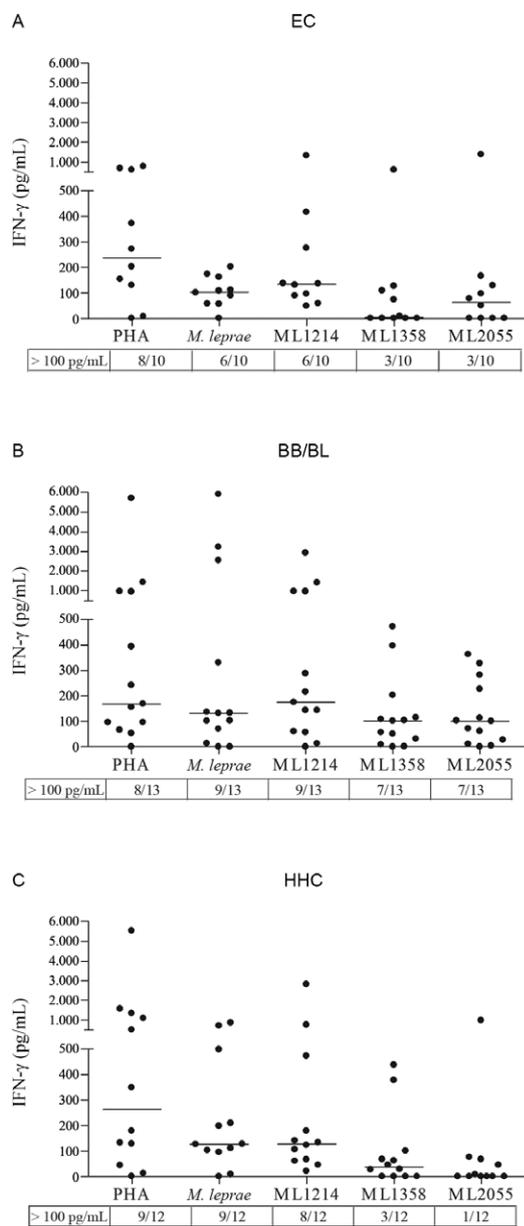


Fig. 5: interferon (IFN)- $\gamma$  responses to newly identified *Mycobacterium leprae* virulence-associated proteins. IFN- $\gamma$  production (corrected for background values) induced using *M. leprae* recombinant proteins ML1214, ML1358 and ML2055 in 24 h whole blood assays from endemic controls (EC) (A) (n = 10), borderline lepromatous/lepomatous leprosy (BL/LL) (B) (n = 13) and house hold contacts (HHCs) (C) (n = 12) in Ethiopia. PHA: phytohaemagglutinin.

The availability of full genome sequences from several organisms and the application of advanced bioinformatics have facilitated the search for unique leprosy antigens (Dockrell et al. 2000, Spencer et al. 2005, Geluk et al. 2008, 2009, 2011, Bobosha et al. 2011). The current study builds upon our previous studies (Geluk et al. 2005, 2009, 2010, Spencer et al. 2005). However, instead of using hypothetical peptides derived from the VI group (*M. leprae* proteins with unknown functions), this study analysed peptides that were derived from virulence-associated *M. leprae* proteins (group IV.A). Twenty-nine *M. leprae* virulence-associated synthetic peptides were selected using a bioinformatics-supported approach to predict unique *M. leprae* sequences. Potential HLA class I and II peptide sequences were derived and subsequently tested in patients from different leprosy endemic regions on three continents to determine the peptides' potential to detect *M. leprae* exposure/infection.

The peptides that induced T-cell reactivity in leprosy patients or healthy individuals living in regions that are hyper endemic for leprosy (EC<sub>high</sub>) were predominantly derived from the following three *M. leprae*-unique proteins: ML2055, ML1358 and ML1214. Consistent with the IFN- $\gamma$  production that was observed in response to the ML2055 peptides, seven out of 13 leprosy patients and three out of seven of the Ethiopian EC<sub>high</sub> group also responded to the ML2055 recombinant protein.

The differences in the *M. leprae* peptide recognition patterns that were observed in this study between leprosy patients and the EC<sub>high</sub> groups compared with the EC<sub>low</sub> groups, implies that the unique peptides have potential use in estimating of the level of *M. leprae* exposure in individuals. This function was also recently described for ML1601-derived peptides (Bobosha et al. 2012). Interestingly, the ML2055 p35 and ML2055 p42 peptides were recognised by both the Brazilian and Ethiopian leprosy patients. Moreover, these two peptides induced significant levels of IFN- $\gamma$  and IP-10 in the Ethiopian EC<sub>high</sub> group, suggesting that the ML2055 p35 and ML2055 p42 peptides can detect *M. leprae* exposure in populations with diverse HLA-alleles. ML2055 has also been reported to induce strong serological responses in lepromatous patients (Sampaio et al. 2011). The low response against ML2055 in the Ethiopian HHC group compared with the EC group could have been a result of an overexposure to mycobacteria. This effect is possible in the HHCs of the MB patients and could have resulted in T cell downregulation, as hypothesised recently (Martins et al. 2012).

We also observed differences in the peptide recognition patterns between the Brazilian EC<sub>high</sub> group (ML1358 p20 and ML1358 p24 peptides) and the Ethiopian EC<sub>high</sub> group (ML2055 p35 and ML2055 p42 peptides). This discrepancy is a reflection of the different HLA polymorphisms in these regions. Both groups of peptides may be useful for indicating *M. leprae* exposure because neither the Brazilian nor the Ethiopian EC<sub>low</sub> groups responded to these peptides. However, a longitudinal analysis of the T-cell responses that were induced by these peptides in a cohort of EC<sub>high</sub> and individuals with household contacts with MB patients at multiple leprosy endemic sites may

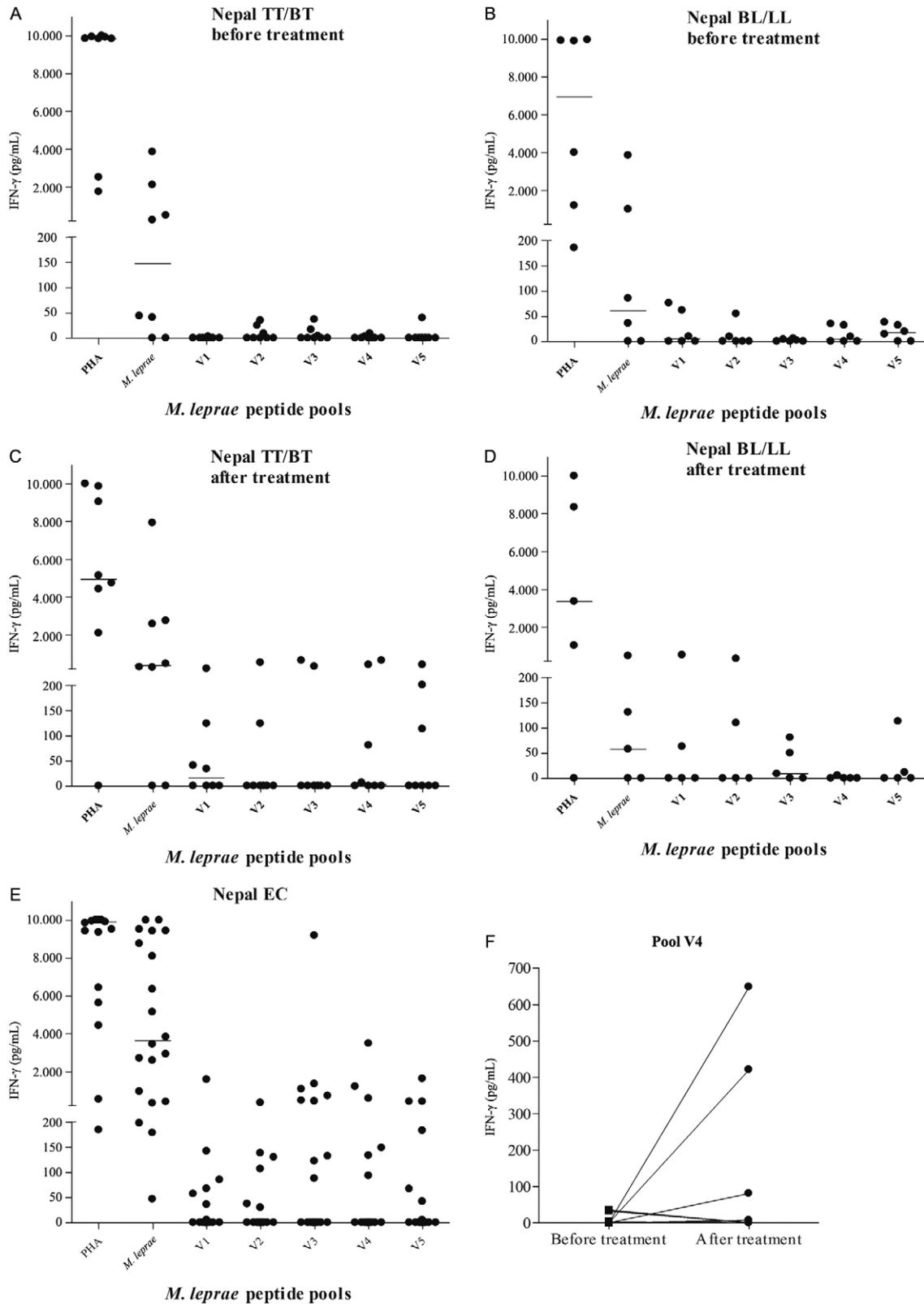


Fig. 6: interferon (IFN)- $\gamma$  responses to *Mycobacterium leprae* virulence-associated peptide pools in peripheral blood mononuclear cell (PBMC) from Nepali individuals. IFN- $\gamma$  production (corrected for background values) induced by pools of *M. leprae* virulence-associated peptides (10  $\mu$ g/mL each) in six days PBMC cultures of borderline tuberculoid (BT) (A) (n = 7) and borderline lepromatous/lepromatous leprosy (BL/LL) (B) (n = 5) patients before and after multidrug therapy (C, D, respectively) and endemic controls (EC) (E) (n = 20). IFN- $\gamma$  responses of all leprosy patients before and after treatment in response to pool V4 (F). Pool V1: p15-p20; V2: p21-p26; V3: p27-p32; V4: p33-p35, p37-p39; V5: p36, p40-p43. PHA: phytohaemagglutinin.

determine whether these peptides can be used to predict disease progression or merely to indicate the level of *M. leprae* exposure.

The benefits of testing peptide pools to detect potential epitopes among several candidate peptides has been reported previously (Geluk et al. 2008). The combination of peptides, as applied in the QuantiFERON®-TB test for TB diagnostics (Chegou et al. 2009), can cover a wider range of HLA alleles compared with using single peptides (Ottenhoff et al. 1991, Geluk et al. 1992, 2005, 2009, Spencer et al. 2005, Geluk & Ottenhoff 2006).

Therefore, the *M. leprae* virulence-associated peptides were additionally tested in four-six peptide pools in Nepali ECs and in patient (TT/BT/BL/LL) groups both before and after treatment. Three peptide pools (V3, V4 and V5) that included immunogenic peptides (ML2055 p35, ML2055 p37 and ML2055 p42) and induced an IFN- $\gamma$  response in the Brazilian and Ethiopian individuals were observed to cause a significant induction of the Th1 response in Nepali ECs (Fig. 6E). In contrast, a small response was detected in leprosy patients prior to MDT (Fig. 6A, B). However, after MDT, certain BT patients displayed a significantly increased IFN- $\gamma$  response in response to the peptide pools and against *M. leprae* (Fig. 6D). This change reflects an improved cellular immune response against *M. leprae* after MDT. The low or absent IFN- $\gamma$  response that was detected for BL/LL patients was consistent with their lepromatous phenotype, which lacked a Th1 immune response.

The immune response against *M. leprae* infection is a collective/synergistic response of various immune cascades that involve both the induction of cytokines and chemokines by innate and adaptive immune cells. IFN- $\gamma$  has been known as an indicator of a Th1 response and the utility of this cytokine will depend on the specificity of the stimulus used in the analysis. In addition to IFN- $\gamma$ , other cytokines and chemokines, such as IP-10, may have potential as markers to distinguish

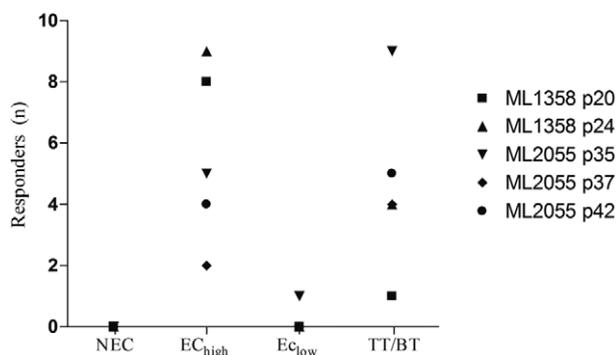


Fig. 7: individuals with interferon (IFN)- $\gamma$  responses to *Mycobacterium leprae* virulence-associated peptides. The total number of individuals that induced IFN- $\gamma$  production in response to five promising *M. leprae* virulence-associated peptides is indicated for each test group: Dutch non-endemic controls (NEC) (n = 8), combined Brazilian and Ethiopian EC<sub>high</sub> (n = 28) living in high leprosy prevalence area, EC<sub>low</sub> (n = 27) living in low leprosy prevalence area, leprosy and lepromatous leprosy/borderline tuberculoid (TT/BT) patients (n = 20).

between different exposure levels and/or leprosy infection (Bobosha et al. 2012) and TB infection (Chegou et al. 2009). In the current study, the induction of IP-10 in Ethiopian EC<sub>high</sub> individuals in response to the ML2055 p35 and p42 peptides confirms the potential use of this chemokine as a biomarker to specifically indicate *M. leprae* exposure. Additionally, IP-10 can be used in HIV infected patients because, in contrast to IFN- $\gamma$ , IP-10 levels were not affected by low CD4 T-cell counts in TB patients with HIV (Aabye et al. 2010). Currently, further studies on the use of IP-10 as a biomarker for the diagnosis of leprosy in HIV<sup>+</sup> individuals are on-going at our Ethiopian test site.

The main advantage arising from the use of synthetic peptides compared with the use of proteins is that peptides less frequently induce T-cell cross reactivity (Spencer et al. 2005). However, because of the HLA-restriction of peptides that are recognised by T-cells, single peptides are not able to cover diverse populations. In this study, we show that the ML2055 p35 and the ML2055 p42 peptides, in addition to the ML1358 p20 and ML1358 p24 peptides, were recognised by patients and EC<sub>high</sub> individuals in both Brazil and Ethiopia. In addition, peptide pools induced an IFN- $\gamma$  response in 30-40% of Nepali ECs. Therefore, similarly to TB diagnostics, analysis of IFN- $\gamma$  and other cytokines, such as IP-10, after stimulation with combinations of *M. leprae* (virulence-associated) peptides will be helpful in developing new tools for the detection of *M. leprae* exposure/infection.

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