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Comparative host transcriptomics as a tool to identify candidate biomarkers for immune reactions in leprosy using meta-analysis

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Abstract

Background: Leprosy is no longer considered an imprecation, as an effective multidrug therapy regimen is available worldwide for its cure. However, its diverse clinical manifestations sometimes involve acute inflammatory reactions. These complications result in irreversible nerve damage, neuritis and anatomical deformities that emerge before, during the treatment or after the completion of treatment. Reversal reaction (Type-I) and erythema nodosum leprosum (Type-II) are the leprosy reactions generally seen in patients with lepromatous and borderline forms of leprosy. At present, there is no accurate diagnostic test available to detect these leprosy reactions.

Objectives: To identify potential biomarkers indicative of Type-I and Type-II leprosy reactions that could help in their early diagnosis.

Methods and Results: Host-transcriptomics investigations have been utilised in this study to decipher a correlation between host-gene expression-based biomarkers and exacerbation of leprosy reactions. We present a comparative analysis of publicly available host transcriptomics datasets (from Gene Expression Omnibus) related to leprosy reactions. Individual datasets were analysed and integration of results was carried out using meta-analysis. Common differentially expressed genes (DEGs) were identified using the frequentist and Bayesian ratio association test methods. We have identified several genes – ADAMTS5, ADAMTS9, IFITM2, IFITM3, KIRREL, ANK3, CD1E, CTSF, DOCK9 and KRT73 to name a few – which can serve as potential biomarkers for Type-II reaction. Similarly, ACP5, APOC1, CCL17, S100B, SLC11A1 among others may likely serve as biomarkers for Type-I reaction.

Limitations: The number of datasets related to leprosy reactions found after the systematic search is less (n = 4) and may limit the accuracy of identified biomarker genes. This could be resolved by including more studies in the data analysis.

Conclusion: We provide a comprehensive list of gene candidates which could be prioritised further in research focusing on immune reactions in leprosy, as they are likely important in understanding its complexities and could be useful in its early diagnosis.

Key words: biomarker, differentially expressed genes, erythema nodosum leprosum, host transcriptomics, reversal reaction

Introduction

Leprosy reactions are acute inflammatory episodes that occur during infection with *Mycobacterium leprae*.[1] These reactions pose a high risk to the infected individuals, resulting in physical deformity followed by nerve damage. Leprosy reactions are classified into two types: Type-I or

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reversal reactions and Type-II or commonly presenting as erythema nodosum leprosum (ENL).[2] Both types of reactions may occur in leprosy patients before and during treatment, but may also occur after the treatment is completed.[3] Type-I reactions occur due to an increase in cell-mediated immunity which results in nerve inflammation at the point of mycobacterial infection. The skin lesions become tender and nerve involvement produces pain, abnormal sensation and functional impairment.[4] Prompt intervention is required to prevent permanent nerve impairment since the expected rate of recovery for nerve function is 60–70%.[5] Type-II reactions occur in multibacillary patients and are immune complex mediated. Patients with Type-II reactions develop tender, erythematous nodules on the face, trunk or legs.[6] The treatment regimen for ENL is quite long and delayed treatment is an important cause of neuropathy and consequent disability.[7] Thus, timely management of leprosy reactions is a big challenge and there is a need to understand the underlying complex immunological processes and gene expression.

Transcriptome profiling can be viewed as a vital approach in disseminating valuable information related to gene expression and associated host response to infection, assessing disease severity and discovering novel gene candidates for diagnosis and prognosis. Due to differences in experimental design and multiple hypotheses in individual studies, these differentially expressed genes (DEGs) should be interpreted accordingly.[8] However, their combined analysis can unravel some common pathological mechanisms. In this study, the meta-analysis of individual datasets paved the way to integrate gene sets from multiple individual studies and work out a common list of DEGs for further biological investigation.[9] In this study, leprosy reaction–specific datasets were analysed and integrated using meta-analysis to identify DEGs that could be considered as candidate biomarkers in leprosy pathogenesis. Further, the list of DEGs was subject to pathway analysis revealing vital processes and their likely association.

Type-I or reversal reaction represents an elevation in cellular immune response which progresses towards the tuberculoid pole of the disease. This type of reaction is common in borderline forms of leprosy and does not occur in the polar forms. The clinical expression of Type-I or reversal reaction includes an increase in the levels of activated lymphocytes which causes the existing skin lesions to become erythematous and oedematous. If it is very severe, then ulceration may occur at times. Peripheral nerves become enlarged and tender due to acute neuritis. It results in rapid loss of sensory and motor functions. The duration of the reversal reaction is often several weeks, but it may extend for many months.

Type-II reaction can be chronic and may occur in half of the patients with lepromatous or borderline lepromatous type of leprosy during the disease. The entire immunology of ENL is not yet completely understood but the condition is aggravated by immune complexes formed with *Mycobacterium leprae* antigens. The clinical expression includes neuritis, iridocyclitis, and painful skin lesions which are erythematous papules and nodules. Chronic ENL is associated with amyloidosis and glomerulonephritis and may cause mortality from renal insufficiency.

Methods

In the present study, we selected four high throughput sequencing and microarray datasets from a whole blood sample and skin biopsy of patients with adverse leprosy reactions from the GEO (Gene Expression Omnibus) database[10] and investigated clinical biomarker candidates for early diagnosis and prognosis of immune reactions of leprosy based on functional and molecular pathway analyses of DEGs. We selected four datasets of whole blood samples from leprosy patients with immune reactions, GSE16844, GSE125943, GSE129033, and GSE74481, using the following keywords: 'reversal reaction', 'leprosy', 'Homo sapiens,' and 'erythema nodosum leprosum'. These datasets were downloaded from the GEO database. The search results were examined and datasets were excluded if they consisted of (i) only the pathogen gene expression, (ii) only a single sample that does not allow comparison, (iii) treatment by any compound, and (iv) studies targeting only non-coding RNAs. Raw data were preferentially downloaded for the selected studies wherever possible; otherwise, the gene expression matrix was used. The datasets were included when (i) they consist of only host gene expression, (ii) they contain samples from either whole blood or skin biopsy and (iii) they target the mRNA. No studies were discarded due to differences in the sequencing platform, publication or experiment date, or unavailability of raw data as it would have further lowered the number of datasets available. A summary of the individual datasets is shown in Table 1. The workflow of the analysis is depicted in Figure 1.

Identification of upregulated/downregulated differentially expressed genes from individual datasets

Upregulated or downregulated DEGs in the selected datasets were identified using the DESeq2 package[11] in RStudio (Version 1.4.1106). Common up- or downregulated DEGs in the datasets were extracted. The cut-off criteria were set as – P-value < 0.05 and standardised $|\log_2 FC| > 1$. Duplicate genes (mapped by Entrezid) were filtered based on the smallest mean expression across all samples in different studies. The resulting P-values were adjusted according to Benjamini and Hochberg's (BH) method.[12] Finally, all the ribosomal protein-coding genes were excluded from the expression matrix of genes.

Common differentially expressed genes from independent studies by ratio association method

To find common DEGs among datasets for related comparisons, a frequentist and Bayesian association ratio analysis was performed. The association of genes with similar comparisons is tested by a ratio measuring the relative increase of genes in common among different studies concerning the number of genes expected by chance, i.e., independence.

Table 1: Description of datasets included in this study.

GEO Accession (Ref)	Organisms	Sample type	Sample group (n)	Platform	Comparison	Design of experiment/Key objective
GSE16844[13]	*Homo sapiens*	Skin biopsy	LL (7), ENL (6)	Affymetrix Human Genome U133 Plus 2.0 Array	LL vs ENL	Mechanism of neutrophil recruitment in skin lesions of leprosy is investigated
GSE125943[14]	Host- *Homo sapiens,* Pathogen- *Mycobacterium leprae*	Skin biopsy	LL (9), TT (6), RR (9)	Illumina HiSeq 2000	LL vs RR	Dual RNA- sequencing was performed on the total RNA from 24 leprosy skin biopsy specimens
GSE129033[15]	*Homo sapiens*	PBMC	ENL (4), RR (5), HC (3)	Illumina HiSeq 2500	HC vs ENL; HC vs RR	RNA sequencing of PBMC enriched for myeloid derived suppressor cells from leprosy patients and HCs
GSE74481[16]	*Homo sapiens*	Skin biopsy	TT (10), BT (10), BB (10), BL (10), LL (4), RR (14), ENL (9), HC (9)	Agilent SurePrint Human GE 8x60K Microarray	HC vs ENL; HC vs RR; LL vs RR; LL vs ENL	Samples from 67 leprosy patients and nine HC individuals were compared for differentially expressed genes

GEO - Gene Expression Omnibus, LL - Lepromatous, ENL - Erythema nodosum leprosum, PBMC - Peripheral blood mononuclear cells, TT - Tuberculoid, BL - Borderline lepromatous, BB - Borderline borderline, BT - Borderline tuberculoid, HC - Healthy control, RR - Reversal reaction.

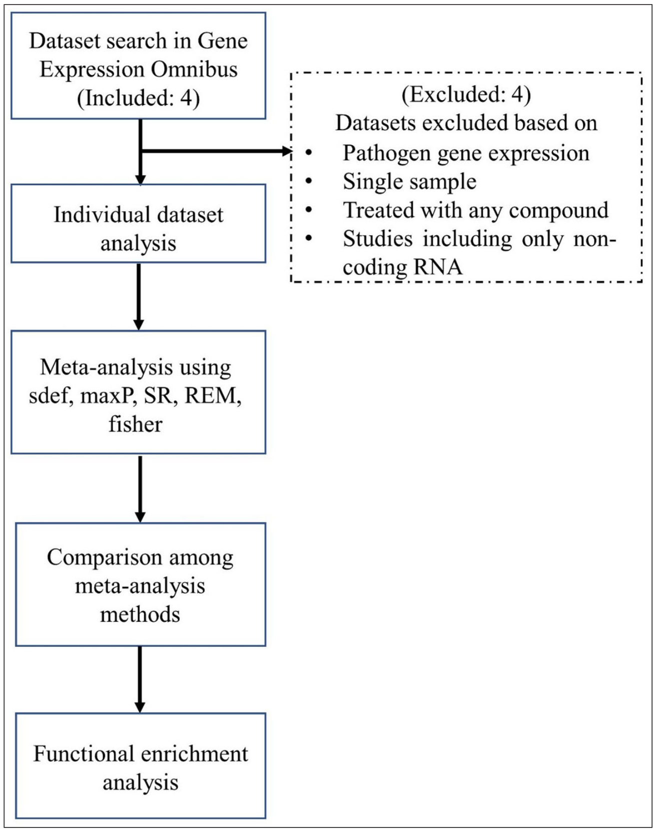
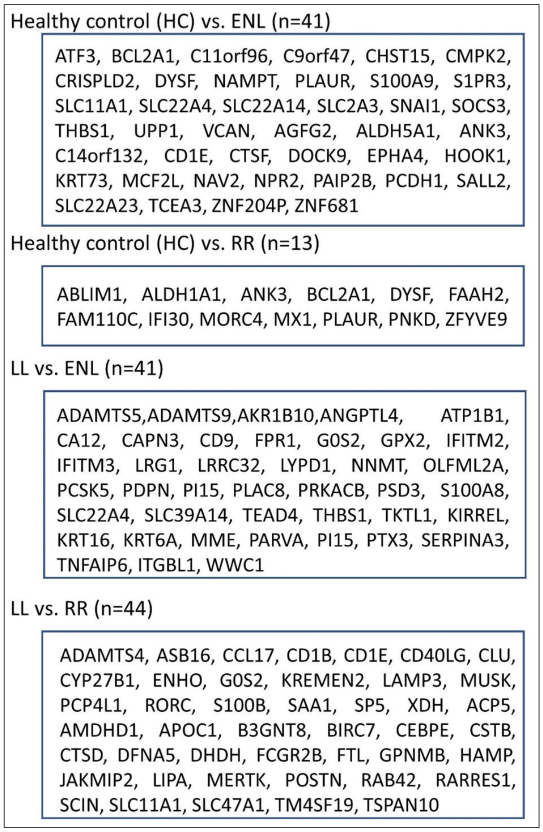


Figure 1a: Flowchart depicting the meta-analysis procedure. (SR - Sum of ranks, REM - Random effects model.)



Healthy control (HC) vs. ENL (n=41)

ATF3, BCL2A1, C11orf96, C9orf47, CHST15, CMPK2, CRISPLD2, DYSF, NAMPT, PLAUR, S100A9, S1PR3, SLC11A1, SLC22A4, SLC22A14, SLC2A3, SNAI1, SOCS3, THBS1, UPP1, VCAN, AGFG2, ALDH5A1, ANK3, C14orf132, CD1E, CTSF, DOCK9, EPHA4, HOOK1, KRT73, MCF2L, NAV2, NPR2, PAIP2B, PCDH1, SALL2, SLC22A23, TCEA3, ZNF204P, ZNF681

Healthy control (HC) vs. RR (n=13)

ABLIM1, ALDH1A1, ANK3, BCL2A1, DYSF, FAAH2, FAM110C, IFI30, MORC4, MX1, PLAUR, PNKD, ZFYVE9

LL vs. ENL (n=41)

ADAMTS5,ADAMTS9,AKR1B10,ANGPTL4, ATP1B1, CA12, CAPN3, CD9, FPR1, G0S2, GPX2, IFITM2, IFITM3, LRG1, LRRC32, LYPD1, NNMT, OLFML2A, PCSK5, PDPN, PI15, PLAC8, PRKACB, PSD3, S100A8, SLC22A4, SLC39A14, TEAD4, THBS1, TKTL1, KIRREL, KRT16, KRT6A, MME, PARVA, PI15, PTX3, SERPINA3, TNFAIP6, ITGBL1, WWC1

LL vs. RR (n=44)

ADAMTS4, ASB16, CCL17, CD1B, CD1E, CD40LG, CLU, CYP27B1, ENHO, G0S2, KREMEN2, LAMP3, MUSK, PCP4L1, RORC, S100B, SAA1, SP5, XDH, ACP5, AMDHD1, APOC1, B3GNT8, BIRC7, CEBPE, CSTB, CTSD, DFNA5, DHDH, FCGR2B, FTL, GPNMB, HAMP, JAKMIP2, LIPA, MERTK, POSTN, RAB42, RARRES1, SCIN, SLC11A1, SLC47A1, TM4SF19, TSPAN10

Figure 1b: Differentially expressed genes selected after the meta-analysis procedure for individual comparisons using multiple methods (sdef, maxP, SR, fisher), LL - Lepromatous leprosy, ENL - Erythema nodosum leprosum, SR - Sum of Ranks, RR - Reversal reaction, HC - Healthy control.

The statistical significance of this ratio is assessed by Monte Carlo permutation which is a model devised in the Bayesian framework. Four categories were created including different studies with similar comparisons based on clinical features to find common DEGs. The following comparisons were created: (i) Healthy control (HC) vs ENL, (ii) HC vs Reversal reaction (RR), (iii) Lepromatous (LL) vs ENL, and (iv) LL vs RR. For each of these comparisons, the ratio of observed versus expected probability of occurrence of genes across different studies was computed based on frequentist and Bayesian methods as proposed in the sdef R package.[17] We have selected HC vs ENL and HC vs RR comparisons to reveal any genes that have been expressed in HCs but may show downregulation in their expression during leprosy progression and further show deviated expression during adverse reactions. Hence, the basal level of expression can be determined.

Meta-analysis

As proposed by the developers of the sdef R package, it aims for fewer false positives when compared to other methods. Thus, a comparative approach was incorporated to compare genes with other standard meta-analysis methods with those obtained from the sdef method. MetaDE R package[18] was used for differential gene expression. MetaDE package incorporates major meta-analysis methods such as Fisher, maximum P-value (maxP), the sum of ranks (SR), rth ordered P-value (roP), and random effects model (REM) which is based on effect sizes and are tested on the datasets used in this study. MetaDE also provides options for gene matching across studies and gene filtering before meta-analysis. In this study, P-value-based methods such as maxP, SR, and roP were used. Combining P-values from different studies has two major advantages: (i) simplicity and extensibility to different outcome variables and (ii) when the outcome variable is not binary then effect size may not be computed; however, P-value association among studies can still be calculated. In REM, the estimated effect size in each study is assumed to come from an underlying true effect size which also includes measurement errors like experimental or sampling error. Further, the studies also contain a random effect that can incorporate unknown cross-study heterogeneities in the model.[19]

Functional enrichment analysis of differentially expressed genes

Functional analysis of DEGs was carried out using the Gene Ontology (GO) database.[20] GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using the Database for Annotation Visualisation and Integrated Discovery (DAVID).[21] The cut-off criteria were set as – adjusted P-value < 0.1 and false discovery rate (FDR) < 0.001. Genes common from each comparison category were tested for enriched function from org.Hs.eg.db v 3.12.0 R package. To further explore the GO of selected genes, R package cluster profiler[22] was used to explore the functions among genes of interest with a threshold value of adjusted P-value < 0.1. GO annotation contains three subcategories – biological process (BP), cellular component (CC), and molecular function (MF) – which identify the biological properties of all gene sets for all organisms.

Assessment of gene lists

The standardised $\log_2$FC, adjusted P-value and False Discovery Rate (FDR) is a vital set of metrics for genes expressed in studies. Thus, the genes found common from similar comparison categories from different studies were tabulated with their median $\log_2$FC (from sdef), adjusted P-value FDR, and tau^2. The DEGs tabulated for HC vs ENL and LL vs ENL are shown in the main text [Tables 2 and 3] while for HC vs RR and LL vs RR are shown in Supplementary materials [Supplementary Table 1 & Supplementary Table 2].

Results

Dataset Search

A systematic search was conducted in GEO using specific keywords to identify leprosy reaction–related datasets. A total of eight datasets were found, of which only four were considered for individual analysis. Datasets with similar comparisons based on clinical features were grouped for analysis.

Identification of common differentially expressed genes using sdef and meta-analysis methods

Individual datasets were processed to identify all the expressed genes. Then, similar comparisons from different datasets were considered to identify DEGs. Four similar comparisons from datasets were considered for ratio association analysis, namely, HC vs ENL, HC vs RR, LL vs ENL, and LL vs RR. Genes common to each study within the specific comparison were used in the ratio association test (sdef). Additional gene expression meta-analysis tools were used as described in the MetaDE R package, such as- rth ordered P-value (roP), sum of ranks (SR), and maximum P-value (maxP), among others. Figure 2 shows the upset plots representing the number of genes selected by each method along with their intersection with a false discovery rate (FDR) < 0.01. maxP and Fisher seems to be a more generous method in the selection of DEGs across comparisons. sdef analysis showed a significant association between the expressed genes in the datasets within each comparison category. Overall, the sdef method seems to be moderate in selection of genes. Other meta-analysis methods are quite conservative as they select fewer genes than the ratio association method. Table 2 represents the list of genes selected with $|\log_2 FC| > 3$ and FDR < 0.001 in the HC vs ENL comparison. A total of 41 genes were selected by all four meta-analyses methods. maxP, fisher, and SR methods selected 29 genes. Top DEGs which are upregulated in HC individuals are ATF3, BCL2A1, C11orf96, C9orf47, CHST15, PLAUR and S100A9 while those more expressed in ENL are – AGFG2, ANK3, CD1E, CTSF, DOCK9, KRT73, NAV2, PCDH1, among others.

Table 3 represents the DEGs selected. A cut-off of $|\log_2 FC| > 2$ and FDR < 0.001 in LL vs ENL is considered. A total of 334 genes were selected by the random effects model (REM) method excluding SR; however, 41 genes were found to be significant by all four methods along with REM. Genes that showed more expression in LL are ATP1B1, CD9, ITGBL1, PRKACB and PSD3 while genes more expressed in ENL are ADAMTS5, ADAMTS9, IFITM2, IFITM3, KIRREL, KRT16, KRT6A, PTX3, SERPINA3, SLC22A4, among others.

DEGs selected by all four methods (Fisher, SR, maxP, and sdef) in HC vs RR and LL vs RR comparison groups are shown in Supplementary Table 1 and Supplementary Table 2, respectively.

Table 2: Genes considered as significant by all meta-analysis methods in HC vs ENL comparison. Cut-off: $|\log_2 FC| > 3$ and $FDR < 0.001$.

Entrez Id	Gene symbol	Log2FC	Adjusted P-value	FDR	Tau^2	Description
3268	AGFG2	-3.03034	2.4E-05	0.0001	0.00	ArfGAP with FG repeats 2
7915	ALDH5A1	-3.97772	4.42E-07	0.0001	0.00	aldehyde dehydrogenase 5 family member A1
288	ANK3	-4.50756	8.6E-05	0.0001	0.00	ankyrin 3
467	ATF3	3.40186	4.83E-05	0.0001	0.00	activating transcription factor 3
597	BCL2A1	3.837651	5.83E-05	0.0001	0.00	BCL2-related protein A1
387763	C11orf96	6.544236	0.001494	0.0001	0.01	chromosome 11 open reading frame 96
256967	C14orf132	-4.95844	0.000537	0.0001	0.01	chromosome 14 open reading frame 132
286223	C9orf47	8.481028	0.000145	0.0001	0.01	chromosome 9 open reading frame 47
913	CD1E	-5.39326	0.000294	0.0001	0.01	CD1e molecule
51363	CHST15	3.29828	9.93E-05	0.0001	0.01	carbohydrate sulphotransferase 15
129607	CMPK2	3.400084	0.000283	0.0001	0.00	cytidine/uridine monophosphate kinase 2
83716	CRISPLD2	3.035294	8.34E-05	0.0006	0.01	cysteine-rich secretory protein LCCL domain containing 2
8722	CTSF	-3.70033	0.000742	0.0001	0.01	cathepsin F
23348	DOCK9	-3.03102	0.001262	0.0001	0.00	dedicator of cytokinesis 9
8291	DYSF	3.740919	0.000209	0.0001	0.00	dysferlin
2043	EPHA4	-3.16614	0.000821	0.0001	0.01	EPH receptor A4
51361	HOOK1	-3.75889	0.000183	0.0001	0.01	hook microtubule tethering protein 1
319101	KRT73	-3.8575	0.001392	0.0001	0.00	keratin 73
23263	MCF2L	-4.78318	0.000257	0.0001	0.01	MCF2 cell line–derived transforming sequence like
10135	NAMPT	3.840097	5.96E-06	0.0001	0.01	nicotinamide phosphoribosyltransferase
89797	NAV2	-3.16788	0.000127	0.0001	0.00	neuron navigator 2
4882	NPR2	-3.46004	0.000107	0.0001	0.01	natriuretic peptide receptor 2
400961	PAIP2B	-3.19456	6.82E-05	0.0001	0.01	poly(A) binding protein interacting protein 2B
5097	PCDH1	-5.97344	7.63E-05	0.0001	0.01	protocadherin 1
5329	PLAUR	4.384652	8.6E-06	0.0001	0.00	plasminogen activator, urokinase receptor
6280	S100A9	3.605774	1.36E-05	0.0001	0.00	S100 calcium-binding protein A9
1903	S1PR3	5.83001	8.21E-05	0.0005	0.00	sphingosine-1-phosphate receptor 3
6297	SALL2	-4.16351	0.000644	0.0001	0.00	Spalt-like transcription factor 2
6556	SLC11A1	4.620372	2.81E-06	0.0001	0.01	solute carrier family 11 member 1
63027	SLC22A23	-3.83493	4.11E-05	0.0006	0.01	solute carrier family 22 member 23
6583	SLC22A4	3.768588	4.36E-06	0.0006	0.01	solute carrier family 22 member 4
144195	SLC2A14	5.311251	0.000118	0.0006	0.01	solute carrier family 2 member 14
6515	SLC2A3	4.481587	6.05E-06	0.0006	0.01	solute carrier family 2 member 3
6615	SNAI1	5.011986	0.001273	0.0001	0.00	snail family transcriptional repressor 1
9021	SOCS3	4.515784	3.13E-05	0.0001	0.00	suppressor of cytokine signalling 3
6920	TCEA3	-3.70372	4.15E-05	0.0001	0.00	transcription elongation factor A3
7057	THBS1	3.819743	5.45E-07	0.0001	0.01	thrombospondin 1
7378	UPP1	3.253603	0.000253	0.0001	0.01	uridine phosphorylase 1
1462	VCAN	3.450314	0.000364	0.0001	0.01	versican
7754	ZNF204P	-4.23346	0.000134	0.0001	0.01	zinc finger protein 204, pseudogene
148213	ZNF681	-3.52729	0.000121	0.0001	0.01	zinc finger protein 681

FDR: False discovery rate; Tau^2: Between-study variance; positive $\log_2$FC shows genes more expressed in HC, Negative $\log_2$FC shows genes more expressed in ENL.
HC - Healthy control, ENL - Erythema nodosum leprosum, FC - Fold change, FDR - False discovery rate, ArfGAP - Adenosine diphosphate ribosylation factor guanosine triphosphatase activating protein 2, EPH - ephrin, MCF2 - Malformations of cortical development, A1, A3, A4, A9 represents protein family member number of this particular protein.

Comparison among groups considered in this study

The number of DEGs from the comparison groups such as HC vs ENL, HC vs RR, LL vs ENL, and LL vs RR were then further compared to reveal genes specific to an individual group such as healthy, LL, ENL, and RR. The DEGs that belong to HC, LL, ENL, and RR were found to be 2536, 2861, 2716 and 2585, respectively. The Venn diagram depicting the genes specific to individual groups and common to different groups is shown in Figure 3.

Gene ontology and functional enrichment analysis

The list of DEGs from different studies across multiple comparisons is used to understand the biological role of these genes. In the HC vs ENL comparison, 41 genes were selected by

Table 3: Genes found to be common between two studies included in the meta-analysis of genes expressed between LL vs ENL comparison. Cut-off: $|\log_2 FC| > 2$ and FDR < 0.001.

Entrez ID	Gene Symbol	Log2FC	Adjusted P-value	FDR	Tau^2	Description
11096	ADAMTS5	–2.49503	0.000030995	0.0001	0.00	ADAM metallopeptidase with thrombospondin type 1 motif 5
56999	ADAMTS9	–3.25833	0.000185	0.0001	0.00	ADAM metallopeptidase with thrombospondin type 1 motif 9
57016	AKR1B10	–4.27945	0.000005835	0.0001	0.00	Aldo-keto reductase family 1-member B10
51129	ANGPTL4	–2.11036	1.9375E-06	0.0001	0.01	Angiopoietin-like 4
481	ATP1B1	2.249928	0.00020495	0.0001	0.01	ATPase Na+/K+ transporting subunit beta 1
771	CA12	–2.25835	0.000131	0.0001	0.00	carbonic anhydrase 12
825	CAPN3	2.138636	0.000489175	0.0001	0.00	calpain 3
928	CD9	2.105496	0.00034002	0.0001	0.00	CD9 molecule
2357	FPR1	–3.68784	0.0009527	0.0001	0.00	formyl peptide receptor 1
50486	G0S2	–2.10941	0.000212861	0.0001	0.19	G0/G1 switch 2
2877	GPX2	–2.65114	0.0010581	0.0001	0.18	glutathione peroxidase 2
10581	IFITM2	–2.35419	0.000326705	0.0006	0.00	interferon–induced transmembrane protein 2
10410	IFITM3	–2.49395	0.00370598	0.0006	0.00	Interferon-induced transmembrane protein 3
9358	ITGBL1	3.842573	0.00110255	0.0001	0.00	integrin subunit beta like 1
55243	KIRREL	–4.18249	0.0000497	0.0001	0.00	kin of IRRE like
3868	KRT16	–3.65565	9.10005E-06	0.0002	0.00	keratin 16
3853	KRT6A	–4.8273	1.59427E-06	0.0003	0.00	keratin 6A
116844	LRG1	–2.48456	0.00018025	0.0001	0.00	Leucine-rich alpha-2-glycoprotein 1
2615	LRRC32	–2.50847	0.0004025	0.0001	0.00	leucine-rich repeat containing 32
116372	LYPD1	–2.25438	0.00031155	0.0001	0.00	LY6/PLAUR domain containing 1
4311	MME	–3.22151	0.000918	0.0001	0.00	membrane metalloendopeptidase
4837	NNMT	–2.81435	0.000094125	0.0001	0.00	nicotinamide N-methyltransferase
169611	OLFML2A	2.29638	0.00023675	0.0001	0.00	Olfactomedin-like 2A
55742	PARVA	–3.0682	0.00020055	0.0001	0.00	parvin alpha
5125	PCSK5	–2.64075	0.00007895	0.0001	0.00	proprotein convertase subtilisin/kexin type 5
10630	PDPN	–2.79629	1.47685E-05	0.0001	0.00	podoplanin
51050	PI15	–3.29239	0.00003225	0.0001	0.00	peptidase inhibitor 15
51316	PLAC8	–2.53294	0.0003115	0.0001	0.00	placenta specific 8
5567	PRKACB	2.030238	0.0025442	0.0001	0.00	protein kinase cAMP–activated catalytic subunit beta
23362	PSD3	2.194707	0.000054475	0.0001	0.00	pleckstrin and Sec7 domain containing protein 3
5806	PTX3	–3.69717	0.000009125	0.0001	0.00	pentraxin 3
7837	PXDN	–2.80582	2.14699E-05	0.0001	0.00	peroxidasin
6279	S100A8	–2.91759	5.90029E-06	0.0001	0.00	S100 calcium-binding protein A8
12	SERPINA3	–3.8388	0.0001885	0.0001	0.00	serpin family A member 3
6583	SLC22A4	–2.0933	0.00009863	0.0008	0.00	solute carrier family 22 member 4
23516	SLC39A14	–2.49318	0.000368546	0.0008	0.00	solute carrier family 39 member 14
7004	TEAD4	–2.86874	0.0002165	0.0001	0.09	TEA domain transcription factor 4
7057	THBS1	–2.07077	0.00327135	0.0001	0.09	thrombospondin 1
8277	TKTL1	–2.14863	0.00009865	0.0001	0.00	transketolase like 1
7130	TNFAIP6	–3.38789	0.000175003	0.0001	0.00	TNF alpha–induced protein 6
23286	WWC1	–4.25724	0.00044245	0.0001	0.00	WW and C2 domain containing protein 1

FDR: False discovery rate; Tau^2: Between-study variance; positive $\log_2$FC shows genes more expressed in LL while negative $\log_2$FC denotes genes more expressed in ENL. LL - Lepromatous leprosy, ENL - Erythema nodosum leprosum, FC - Fold change, FDR - False discovery rate, ADAM - A disintegrin-like and metalloprotease, LY6 - lymphocyte antigen 6, PLAUR - plasminogen activator, Urokinase receptor, AMP - adenosine monophosphate, TEA - Transcriptional enhancer factor, TNF - Tumour necrosis factor, PSD3 - pleckstrin and Sec7 domain containing protein 3, KIRREL - Kirre like nephrin family adhesion molecule, G0/G1 - Gap 0/Gap1.

all four methods with an FDR < 0.01, and standardised $|\log_2 FC| > 3$ was used in the analysis. The top 20 GO-enriched BPs are shown in Figure 4 [Supplementary Table 3]. The expressed genes correspond to processes like 'response to stress', 'negative regulation of apoptotic process', 'cytokine production', 'defense response,' and 'myeloid leukocyte activation'. However, analysis of genes more expressed in HC than ENL showed GO enriched terms such as – 'cellular metabolic process', 'ion binding', 'membrane-bound organelle, and 'intracellular'. Similarly, the ontologies enriched for genes more expressed in ENL than HC are the'cellular macromolecule metabolic process', 'protein metabolic process,' and 'heterocyclic compound binding'. The other enriched terms for genes in HC vs ENL comparison are shown in Supplementary Table 3.

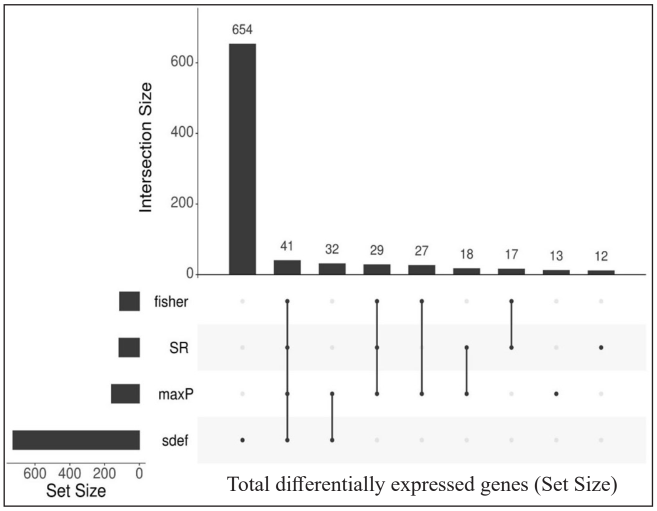


Figure 2a: Upset plots depicting the number of differentially expressed genes (DEGs) from each meta-analysis method and their intersection. The vertical bar represents the total number of expressed genes from different methods (intersection size) and the horizontal bar depicts the total gene size (set size) expressed by the meta-analysis method. DEGs from HC vs ENL comparison from two independent datasets (GSE129033 and GSE74481), 41 genes were identified by all the methods.

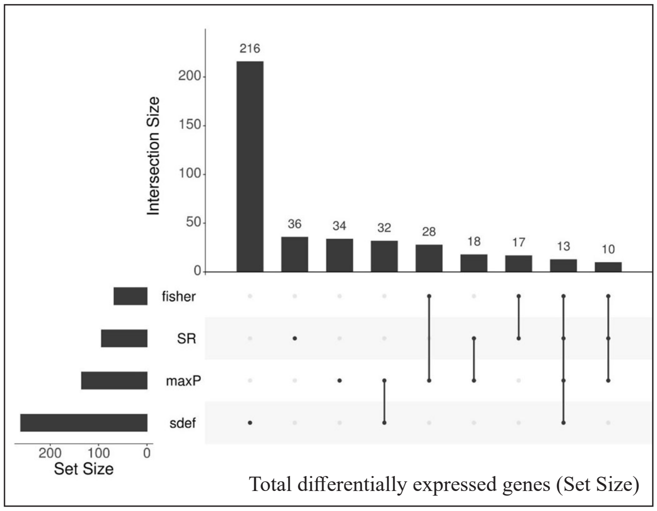


Figure 2b: Upset plots depicting the number of differentially expressed genes from each meta-analysis method and their intersection. Th vertical bar represents the total number of expressed genes from different methods (intersection size) and the horizontal bar depicts the total gene size (set size) expressed by the meta-analysis method. Genes from HC vs RR from two datasets (GSE129033 and GSE74481), 13 genes were found common by meta-analysis methods.

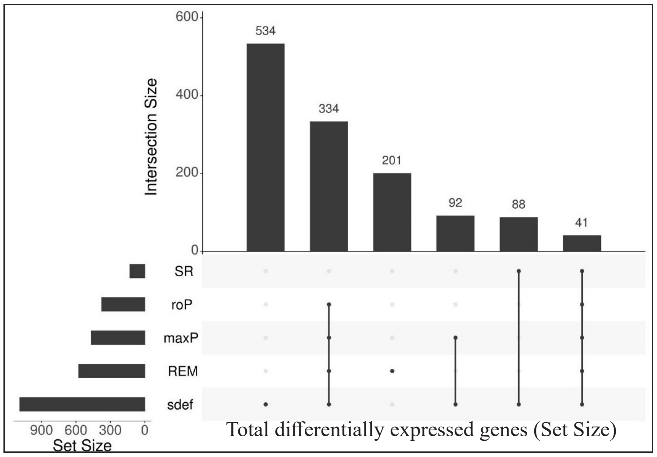


Figure 2c: Upset plots depicting the number of differentially expressed genes from each meta-analysis method and their intersection. The vertical bar represents the total number of expressed genes from different methods (intersection size) and the horizontal bar depicts the total gene size (set size) expressed by the meta-analysis method. Differentially expressed genes resulted from LL vs ENL comparison from two datasets (GSE16844 and GSE74481), 41 DEGs were found.

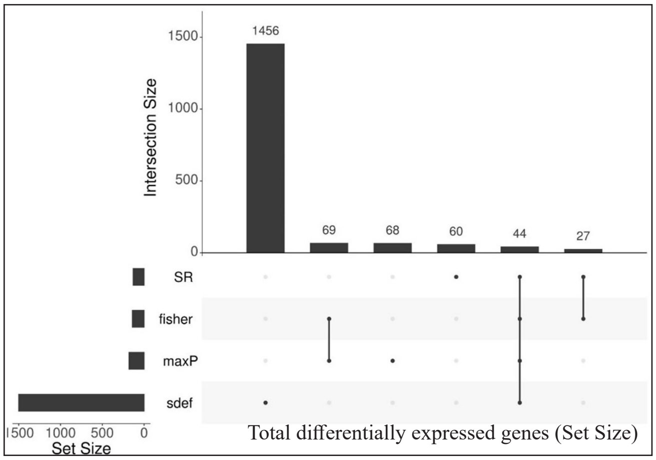


Figure 2d: Upset plots depicting the number of differentially expressed genes (DEGs) from each meta-analysis method and their intersection. The vertical bar represents the total number of expressed genes from different methods (intersection size) and the horizontal bar depicts the total gene size (set size) expressed by the meta-analysis method. DEGs from LL vs RR comparison (GSE125943 and GSE74481), a total of 44 DEGs were selected by all meta-analysis methods.

For LL vs ENL comparison, overall 334 genes were expressed at a cut-off of standardised $|\log_2 FC| > 2$ and FDR < 0.001. However, only 41 genes were found to be significant by all meta-analysis methods. Therefore, these 41 genes were used to understand their BPs. Functional enrichment analysis reveals the GO enriched BPs such as 'cell cycle', 'regulation of ion transport', 'inflammatory response,' and 'response to endogenous stimulus' which are indicative of host response against a pathogen. The top 20 enriched ontologies for genes differentially expressed in LL vs ENL are shown in Figure 4 [Supplementary Table 4]. Genes specific to a particular GO enriched term for HC vs ENL and LL vs ENL comparison sets are shown in Figure 4. Heat plots showing the enriched terms for identified genes based on standardised $\log_2 FC$ for comparison HC vs ENL and LL vs ENL are shown in Figure 5. Figure 6 represents an overview of the meta-analysis of the datasets representing the two types of leprosy reactions: Type-I (Reversal Reactions) and Type-II (Erythema Nodosum Leprosum).

We have identified several DEGs more expressed in RR than in HC such as ABLIM1, ANK3, FAM110C, and

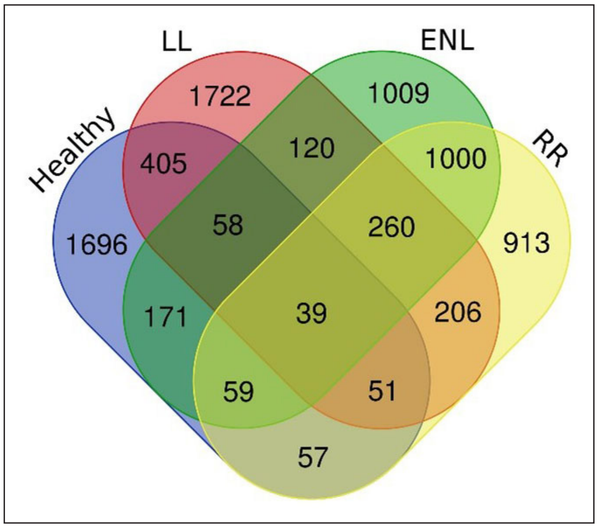


Figure 3: Venn diagram depicting the number of differentially expressed genes specific to each group and common to these groups (healthy control, LL - Lepromatous, ENL - Erythema nodosum leprosum, RR - Reversal reaction).

MORC4 while those less expressed in RR as compared to HC like BCL2A1, IFI30, and PLAUR and they correspond to GO enriched terms like cellular aldehyde metabolic process and cell surface receptor signalling pathway [Supplementary Table 5]. The genes that resulted from LL vs RR, namely, APOC1, CSTB, FTL, SLC11A1, and TSPAN10 are more expressed in RR than LL and not described in the literature earlier. ADAMTS4, CCL17, CD1B, CD1E, and G0S2 among others are more expressed in LL than RR [Supplementary Table 6]. A major class of genes expressed in LL belongs to chemokines and plays a significant role in recruiting neutrophils and lymphocytes to the mycobacterium-infected site.[23] The GO terms for LL vs RR comparison are immune system process cell communication, and signal transduction which are representative of the need for the cell to generate an immune response and would be essential to understand the immunopathogenesis of the disease. Gene ontology- enriched terms for DEGs in LL vs RR and HC vs RR are shown in Supplementary Table 5 and Supplementary Table 6, respectively. CCL17 and CYP27B1 seem to be more expressed in LL patients and are also reported to have no significant correlation with Type-I reaction [24] while MERTK showed higher expression in RR patients [Supplementary Figure 1].

Discussion

Leprosy reactions play a major role in morbidities associated with the disease. These immune reactions lead to nerve damage, and weakness and pose a great risk of injury and deformity.[2] These immune-mediated complications occur in more than 50% of leprosy patients.[6] The pathological mechanisms of these immune reactions are complex and have not yet been completely understood. Comparative host transcriptomics has immense utility in elucidating screening profiles and is capable of efficiently narrowing down the

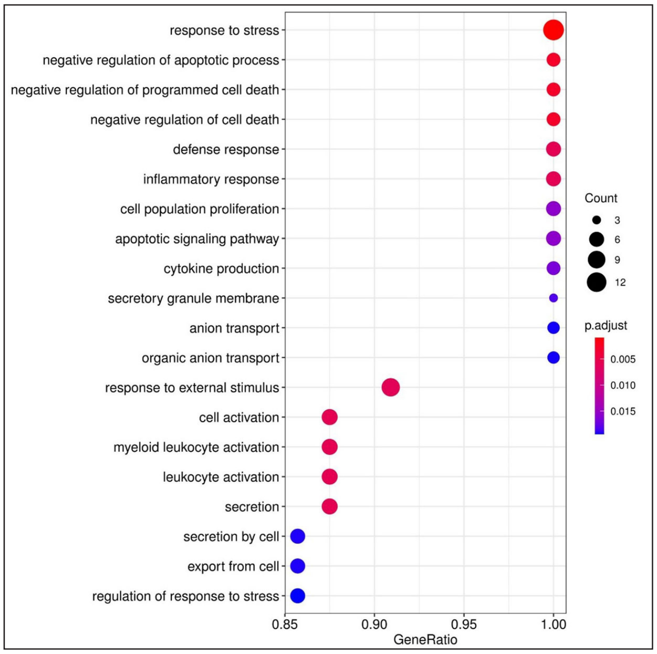


Figure 4a: Dot plots depicting the enriched Gene Ontology (GO) terms. GO terms denote the biological process, cellular component, and molecular function of the expressed genes. The X-axis shows the gene ratio, Y-axis denotes the enriched term. Adjusted P-value < 0.1 is set as a cut-off. The colour scale represents adjusted P-values and the size of the dot represents the gene number. Enriched terms for genes expressed in HC vs ENL comparison.

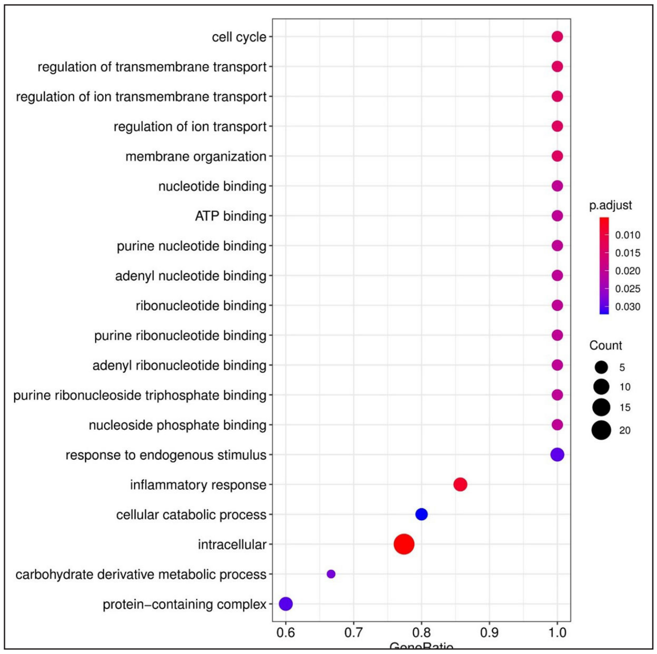


Figure 4b: Dot plots depicting the enriched Gene Ontology (GO) terms. GO terms denote the biological process, cellular component, and molecular function of the expressed genes. The X-axis shows the gene ratio, Y-axis denotes the enriched term. Adjusted P-value < 0.1 is set as a cut-off. The colour scale represents adjusted P-values and the size of the dot represents the gene number. Enriched terms from LL vs ENL comparison, ATP - Adenosine triphosphate.

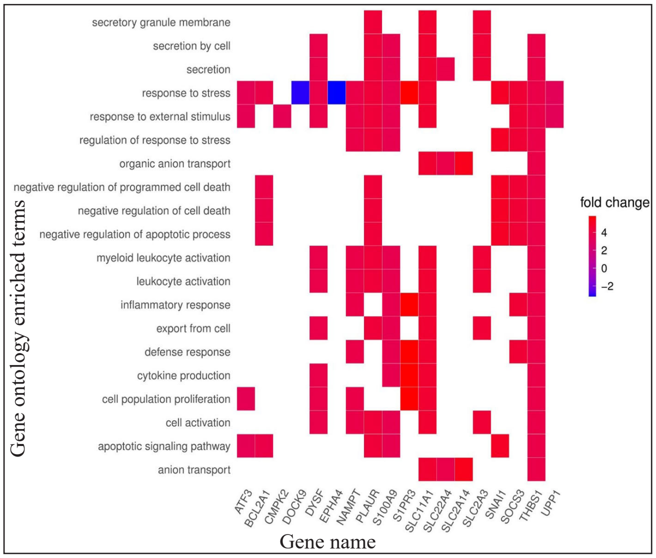


Figure 5a: Heat plots showing enriched genes with specific GO terms according to the standardised $\log_2$FC for HC vs ENL comparison; positive $\log_2$FC denotes genes more expressed in HC individuals while negative $\log_2$FC denotes genes more expressed in ENL patients.

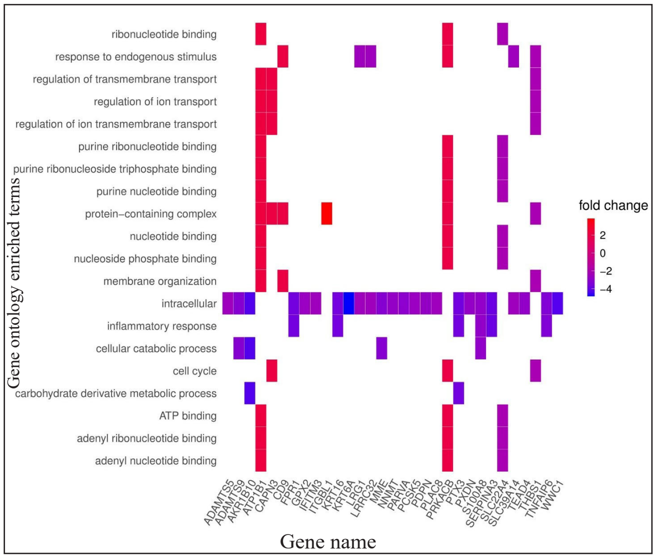


Figure 5b: Heat plots showing enriched genes with specific GO terms according to the standardised $\log_2$FC for LL vs ENL comparison; positive $\log_2$FC denotes genes more expressed in LL while negative $\log_2$FC denotes genes more expressed in ENL patients.

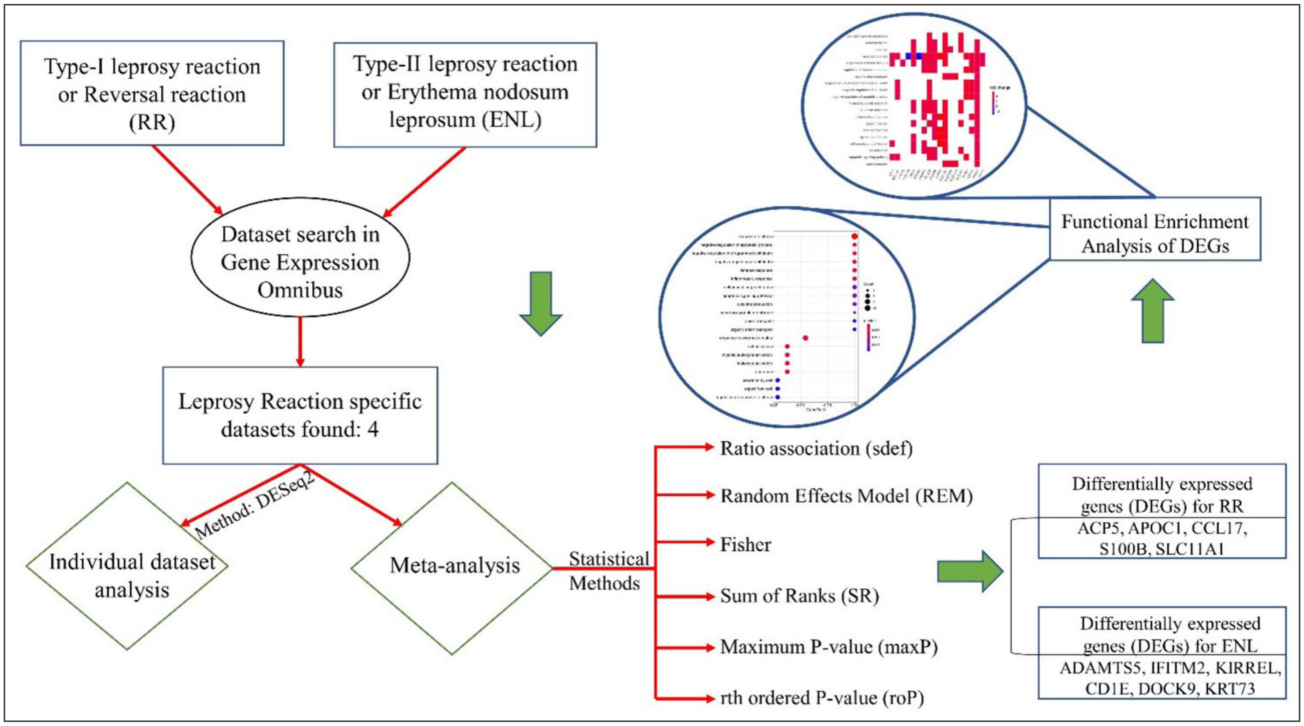


Figure 6: The overview of the meta-analysis of the datasets representing Type-I and Type-II leprosy reactions. RR- Reversal reaction, DEGs - Differentially expressed genes, ENL - Erythema nodosum leprosum.

candidate genes associated with multifactorial diseases like leprosy and investigates the underlying mechanisms of immune reactions for their diagnosis and prognosis. In this study, publicly available datasets from GEO were considered for analysis. The datasets specific to leprosy Type-I and Type-II reactions were identified based on search terms. Individual analysis of these datasets revealed statistically significant DEGs. Integration of these individual studies based on a common comparison pattern was carried out using meta-analysis statistical methods to find common expression patterns across studies. Datasets that resulted from both the microarray and RNA sequencing platforms were included to ensure that no specific dataset is left out merely because of variation in the experimental platform being used. At last, we identified a list of DEGs in each comparison set across different studies which are indicative of their possible role in progression and immune exacerbations in leprosy. Therefore, such common genes can be prioritised as their functional validation would be useful because they are consistently identified across multiple studies.

The criteria used for identifying the differential expression are based on adjusted P-value and standardised fold-change or effect size.[25] However, the hypothesis testing depends on the sample size, and studies with less sample size do not or had fewer differentially expressed genes, and thus chances of getting false-positives and false-negatives are expected. When a gene is identified as differentially expressed in one study, it might not necessarily be statistically significant in other unless that gene is biologically replicated. If a gene is selected by the major meta-analysis methods, such as maxP, SR, Fisher, and sdef, then the chances of it being a false-positive are less likely. Also, if a gene is statistically significant in one study, it might not be necessarily picked up by all methods in a meta-analysis by virtue of differences in P-values or effect size as it is not consistent across studies. This corresponds to the fact that only a small proportion of the genes were selected by these methods over those that only differentially expressed in one or few studies.

Only studying the final set of DEGs from individual studies might limit the discoverability of other potential genes as certain factors contribute to making it biased. For example, the statistical framework used in an individual study might differ; all genes from one study might not be expressed in another on account of different platforms in use, etc. Thus, analysis of individual studies removes these biases and helps to incorporate a common statistical method for them to reach a balanced comparable conclusion.

The meta-analysis methods used in this study are based on the P-value or effect size of expressed genes and thus help to reveal DEGs not identified earlier.[26] The potential role of these genes can be identified using functional analysis or previously defined pathways relevant to the host response to leprosy. Some of the identified genes have been previously reported in studies relevant to leprosy like PTX3 which showed a higher expression in ENL,[27] S100A9 which is more expressed in HC than in the ENL cases and generates an ineffective response to leprosy.[28] GO enrichment analysis of lists of DEGs from HC vs ENL reveals the BPs involved in the inflammatory response, cell population proliferation, and cytokine production. Moreover, a similar profile is seen in GO-enriched BPs from LL vs ENL comparison. It represents terms like inflammatory response, membrane organisation, and regulation of transmembrane transport which connotes genes such as SLC11A1 and showed higher expression in ENL patients.[29]

This study unleashes several potential biomarker candidate genes that may serve the purpose of early detection of reactions in leprosy patients. Over the years, several genes were associated with ENL, for example, Pentraxin-3 (PTX3) which is a protein present in secondary neutrophilic granules and is a potential biomarker of ENL. According to this study, it shows a downregulation in expression from its normal levels. PTX3 can be used to differentiate between episodes of ENL from a reversal reaction. Another gene SLC11A1, a member of the solute carrier family, encodes a protein called natural resistance–associated macrophage protein 1(NRAMP1 or SNP 274C/T) which is associated with leprosy reaction. The presence of the ‘C’ allele becomes a risk factor for Type-I reaction; however, it becomes protective for Type-II reaction. A class of interferons was also identified as a part of candidate biomarkers which constitute a range of cytokines performing multifaceted roles. In this study, IFITM2 and IFITM3 are shown to be upregulated in ENL as also confirmed by other studies.

The gene expression can be validated by techniques such as the qPCR (quantitative polymerase chain reaction). It is quite interesting to note that the analysis of several microarray and RNA sequencing datasets by different people generating analogous results is indicative of a true differential expression. However, proper replication of these findings would be required to rule out any bias.[30]

Conclusion

This study analyses immune reactions in leprosy-specific datasets publicly available in GEO using a standardised approach, thereby revealing the DEGs common among different studies. Integration of DEGs from individual studies using meta-analysis reveals several genes that are vital in understanding the mechanisms of immune reactions and their possible correlation with the host response. We have identified several genes ADAMTS5, ADAMTS9, IFITM2, IFITM3, KIRREL, ANK3, CD1E, CTSF, DOCK9, and KRT73 to name a few which can serve as potential biomarkers for Type-II reaction. For Type-I reaction, DEGs are identified as ACP5, APOC1, CCL17, S100B, and SLC11A1 among others, and may likely serve as biomarkers. Further, the analysed studies imply some important BPs which help in getting insights into the pathogenesis of reactions in leprosy. A combination of biomarkers would be necessary for its early diagnosis or prognosis to be used for clinical applications.

Ethical approval: The Institutional Review Board approval is not required as this article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of patient consent: Patient’s consent not required as there are no patients in this study.

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Use of artificial intelligence (AI)-assisted technology for manuscript preparation: The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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