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Identification of perturbed pathways rendering susceptibility to tuberculosis in type 2 diabetes mellitus patients using BioNSi simulation of integrated networks of implicated human genes

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In type 2 diabetes mellitus (T2DM) patients, chronic hyperglycemia and inflammation underlie susceptibility to tuberculosis (TB) and result in poor TB control. Here, an integrative pathway-based approach is used to investigate perturbed pathways in T2DM patients that render susceptibility to TB. We obtained 36 genes implicated in type 2 diabetes-associated tuberculosis (T2DMTB) from the literature. Gene expression analysis on T2DM patient data (GSE26168) showed that *DEFA1* is differentially expressed at P_{adj}<0.05. The human host TB susceptibility genes *TNFRSF10A*, *MSRA*, *GPR148*, *SLC37A3*, *PXK*, *PROK2*, *REV3L*, *PGM1*, *HIST3H2A*, *PLAC4*, *LETM2*, and *EMP2* and hsa-miR-146a microRNA were also differentially expressed at P_{adj}<0.05. We included all these genes and added the remaining 28 genes from the T2DMTB set and the remaining differentially expressed genes at P_{adj}<0.05 in STRING and obtained a well-connected network with high confidence score (≥0.7). Further, we extracted the KEGG pathways at FDR<0.05 and retained only the diabetes and TB pathways. The network was simulated with BioNSi using gene expression data. It is evident from BioNSi analysis that the NF-kappa B and Toll-like receptor pathways are commonly perturbed with high ranking in multiple gene expression datasets of type 2 diabetes versus healthy controls. The other pathways, necroptosis pathway and FoxO signalling pathway, appear perturbed with high ranking in different gene expression datasets. These pathways likely underlie susceptibility to TB in T2DM patients.

Keywords. BioNSi; differential expression; simulation; text mining; tuberculosis; type 2 diabetes

1. Introduction

Patients with type 2 diabetes mellitus (T2DM) are reportedly at risk of acquiring tuberculosis (TB) (Chaudhry *et al.* 2012; Niazi and Kalra 2012; Kapur and Harries 2013). T2DM develops due to insulin resistance. It is on the rise, affecting an increasing number of people every year worldwide (Faurholt-Jepsen *et al.* 2011). The majority of diabetic patients are of T2DM type (Agarwal *et al.* 2016). Also, 10% of deaths due to TB cases are linked to diabetes in low- and middle-income countries (Faurholt-Jepsen *et al.* 2011). Drugs used to treat TB are reported to cause diabetes-associated conditions. Rifampicin reportedly

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causes hyperglycemic conditions, and isoniazid (INH) is prone to cause peripheral neuropathy, which may worsen diabetic neuropathy (Niazi and Kalra 2012). Harries *et al.* (2016) suggested a collaborative care approach for DM and TB patients to tackle this comorbidity. Tuberculosis comorbidity is associated with poor glycemic control in diabetic patients (Mustafa 2005). A mice model study points to the impact of chronic hyperglycemia in impairment of initiation of adaptive immunity, thereby resulting in a higher load of *Mycobacterium tuberculosis* in the lung (Martens *et al.* 2007).

Diabetic patients are 3.1 times more susceptible to TB infection compared with non-diabetic individuals (Jeon and Murray 2008) and show lower cellular immunity (Karachunskiĭ *et al.* 1997) relative to non-diabetic controls. Because cellular immunity and macrophages offer defence against TB infection, reduced cellular immunity and impairment of Th1 immune response could underlie susceptibility to TB (Tsukaguchi *et al.* 1992; Martens *et al.* 2007). Enhancement of susceptibility to tuberculosis in diabetic patients is related to hyperglycemia and insulin resistance as well as their effects on the functions of macrophages and lymphocytes (Kumar *et al.* 2013; Lopez-Lopez *et al.* 2018). Kumar *et al.* (2013) observed that heightened pro-inflammatory cytokines (IL1B, IL6, and IL18) in diabetic patients result in poor control of tuberculosis infection (Dinarello 2000). Later, they reported significantly reduced expression of cytotoxic markers perforin, granzyme B, and CD107a in $CD8^+$ T cells, whereas in NK cells, expression of CD107a was decreased in T2DMTB patients (Kumar *et al.* 2015a, b). Therefore, an altered repertoire of $CD8^+$ T and NK cells expressing cytotoxic molecules evidently underlies T2DMTB comorbidity (Kumar *et al.* 2015a, b). Syal *et al.* (2015) observed that Vitamin A receptor (RXR) expression is negatively regulated in T2DM patients and tryptophan-aspartate-containing coat protein (TACO) gene expression is positively regulated, which could predispose T2DM patients to TB. Qu *et al.* (2012) identified *HK2* and *CD28* as potential genes for association of TB in diabetic patients.

Gene expression regulation is also achieved post transcriptionally through microRNAs (miRNAs). Latorre *et al.* (2015) selected 4 miRNAs, namely, hsa-miR-150, hsa-miR-21, hsa-miR-29c, and hsa-miR-194, dysregulated in TB versus latent TB infection or TB versus healthy controls, and described a whole-blood-derived miRNA signature enabling diagnosis of TB with over 90% sensitivity and over 87% specificity. These investigations have offered valuable data. The molecular dynamics underlying both T2DM and TB are complex. The studies of both diseases present challenges and a key question arises regarding the susceptibility of the human host towards developing comorbidities. In the context of computational disease modeling, it was realized some years ago that studying groups of patients with common patterns in disease is more tractable than deciphering individual dynamics (Tegnér *et al.* 2009). It was also realized that computational modeling can benefit from machine learning, agent-based modeling, network modeling, and stochastic simulations. Over the years interest has been gaining in computational modeling due to its data integrative power. Computational models offer integrated description of diseases, thereby offering a platform for learning and generating hypotheses for therapeutics (Parton *et al.* 2016; Sancristóbal *et al.* 2018).

In the present work we have used a computational approach to address T2DMTB comorbidity by using an integrative method through the collection of genes implicated in T2DMTB comorbidity, and miRNAs implicated in T2DM and in TB from the published literature. This list was pooled with the differentially expressed host genes susceptible to TB, differentially expressed miRNA, and the remaining host differentially expressed genes. These data were used to identify the network at high confidence score and the pathways involved. Subsequently, we investigated the probable cause of susceptibility to TB in patients with T2DM using pathway simulations and available gene expression data from T2DM patients.

2. Methods

All text analytics were carried out in R 3.4.3 with the CRAN package pubmed.mineR version 1.0.10 (Rani *et al.* 2015).

2.1 *Data collection*

All type 2 diabetes abstracts were downloaded from PubMed and then subjected to R package pubmed.mineR (Rani *et al.* 2015) to extract the gene–disease associations. This set of abstracts, called primary corpus for data mining, was imported in R using the readabs function of pubmed.mineR. Further, secondary corpus with abstracts containing 'TB AND T2DM' was created using the searchabsL function with

specified query terms including 'tuberculosis', 'TB' and 'mtb'. This searchabsL function retrieves abstracts meeting the criteria of containing the given query terms from a large set of abstracts. The secondary corpus created was then used for identification of genes implicated in T2DMTB comorbidity.

2.2 *Entity recognition and their relationships*

2.2.1 *Gene extraction from abstracts:* We used the sentence tokenization function of pubmed.mineR to extract sentences with co-occurrence of two entities: 'genes' and 'disease' terms. The genes list comprised official (Povey *et al.* 2001), and other symbols (aliases, previous symbols), alternative names from UniProt, and gene names from HGNC. For this purpose, the functions used to achieve these retrievals include official_fn, altnamesfun, prevsymbol_fn, alias_fn, and names function of pubmed.mineR. All of these functions output sentences containing gene symbols or names from the abstracts with their corresponding PMID.

2.2.2 *Inclusion and exclusion criteria:* For data mining of genes, we have defined inclusion and exclusion criteria. All the papers considered were those that reported direct measurements from patients or *ex vivo* studies from patient samples or *in vivo* mice studies. No *in vitro* studies were included in this work. Review articles, non-human studies except mice, and non-English articles were excluded.

2.2.3 *Manual curation of associated genes:* False matches arise due to use of identical acronyms by authors signifying other meanings. These were manually identified and discarded. The extracted sentences were examined for 'proof of association' or 'of evidence' in terms of relationships between the entities. In cases where the relationship was not clear, the entire abstract was examined along with full text wherever available from PubMed Central. We analyzed the trends of resulting genes to mark their significance and their annotation with Gene Ontologies (GOs) by using DAVID (Database for Annotation, Visualization and Integrated Discovery) (Dennis *et al.* 2003). The schematic representation of the methodology is given in figure 1. These genes are termed T2DMTB genes.

2.3 *Web server development*

The fully annotated data of T2DMTB genes was plugged into T2DiACoD (Rani *et al.* 2017).

2.4 *Gene enrichment analysis*

To infer potential biological significance of the reported genes, we used DAVID (Dennis *et al.* 2003). The output table was obtained with the following information: Annotation Cluster, Enrichment Score, Category, Term, Count, % (involved genes/total genes), Genes, List Total, Pop Hits, Pop Total, Fold Enrichment, Bonferroni, Benjamini, P-value, and FDR. The enriched annotation categories of genes were considered on the basis of P-value (calculated by Fisher's exact test) as well as FDR $\leq$0.05. Gene ontology enrichment was prepared using EnrichmentMap (Isserlin *et al.* 2014), a cytoscape plugin.

2.5 *Differential expression*

The microarray gene expression (GSE26168) data for blood samples from T2DM patients were collected from NCBI GEO (Barrett *et al.* 2005). The GSE26168 is a super-series and composed of two subseries, GSE21321 and GSE26167. These data contain mRNA and miRNA profiles of T2DM patients without TB. Both mRNA and miRNA samples were studied from GSE21321. Among the mRNA samples, there were 8 controls, 7 impaired fasting glucose, and 9 diabetics. Among the miRNA samples, there were 10 controls, 7 impaired fasting glucose and 9 were diabetics. For miRNAs, the Illumina platform, and for miRNAs, the miRCURY LNA microRNA array platform, were used. This Illumina dataset was processed using the lumi package (Du *et al.* 2008). Normalization was performed using a modified Z-score method based on median absolute deviation (MAD) (Mandić-Rajčević and Colosio 2019). For differential expression, the limma (Ritchie *et al.* 2015) package was used. The differentially expressed mRNAs and miRNAs were selected on the basis of *P*-values <0.05 and P_{adj} <0.05.

2.6 *Integrative analysis*

2.6.1 *Collection of deregulated miRNAs in blood from T2DM and TB patients:* We sought to identify the miRNAs targeting T2DMTB genes to understand their role in the pathogenicity of the comorbidity. We used NetworkAnalyst (Xia *et al.* 2015) to obtain gene–miRNA interactions. This uses TarBase and mirTarBase databases for information retrieval. We extracted common miRNAs deregulated in T2DM as well as in TB in blood. Further literature mining was performed

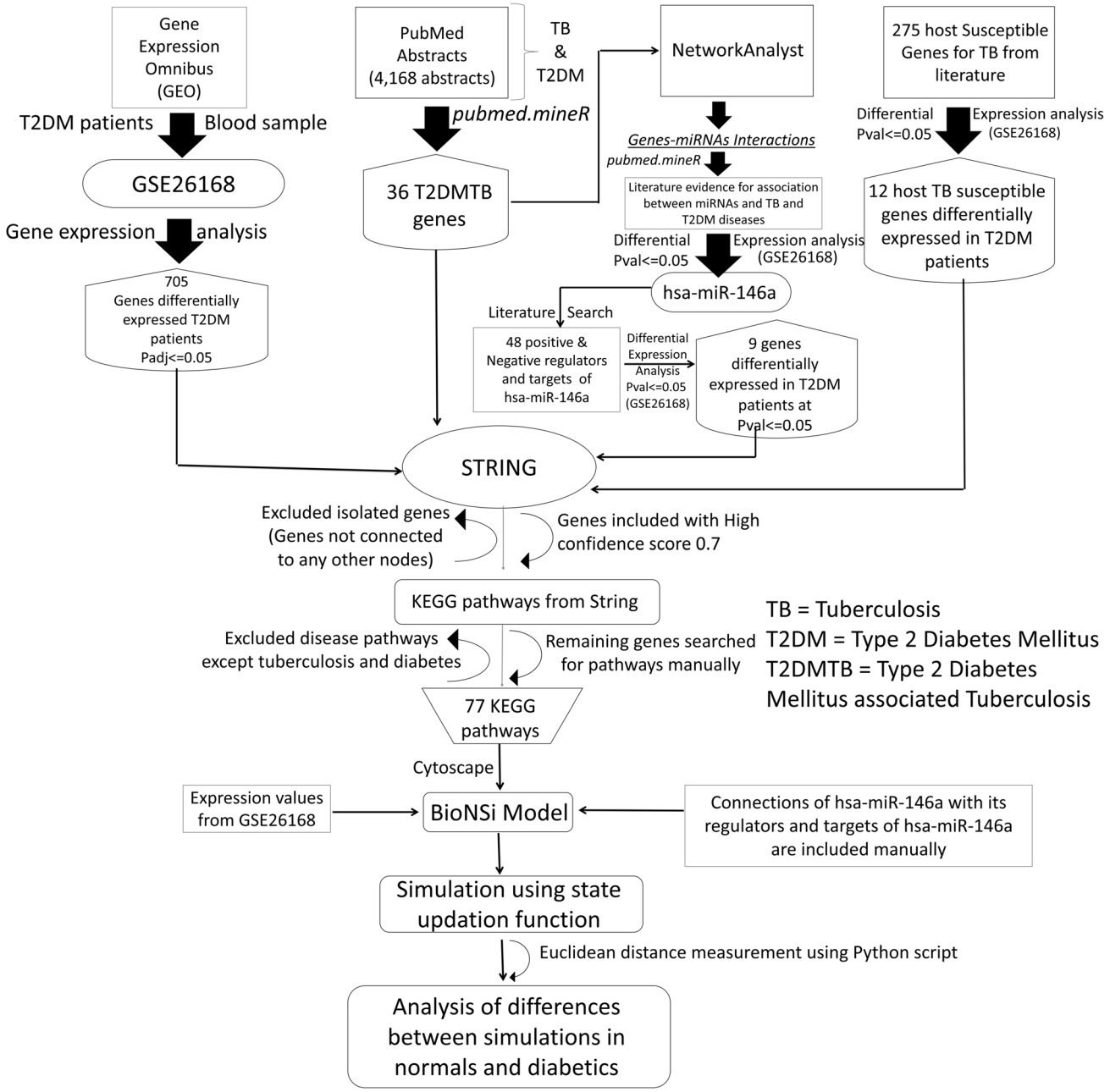

Figure 1. Schematic representation of the methodology. The genes for the final network model are assembled from the multiple sources according to the work flow including text mining and differential expression analysis.

for the evidence of deregulated miRNAs in both conditions.

2.6.2 *Differential expression:* The deregulated miRNAs from the literature (supplementary table 1) were further examined for differential expression in microarray expression of miRNAs from blood samples of T2DM patients (GSE26168).

2.6.3 *Identification of regulators of miRNA-146a and gene targets of miRNA-146a:* To investigate the role of the differentially expressed hsa-miRNA-146a in type 2 diabetic patients, the regulators of miRNA-146a and the gene targets regulated by hsa-miRNA-146a were extracted using text mining from PubMed. Literature mining using pubmed.mineR was performed to extract the human host regulators and targets of miRNA-146a.

2.6.4 *STRING analysis:* All 36 T2DMTB genes were included. Among the genes identified from other approaches, only the genes differentially expressed

were included among the following datasets: between diabetic patients and normal samples (GSE26168), host genes susceptible to TB (12 genes from *in vitro* macrophage studies), regulators of miR-146a (3 genes), and gene targets of miR-146a (8 genes). The invariant genes in human genomes (Pathak *et al.* 2019) were also included and the entire gene set was interrogated using STRING (Szklarczyk *et al.* 2017). Only connections between genes with score ≥0.7 were considered.

2.6.5 *Pathways enrichment:* The STRING output provided a list of 120 significant pathways (FDR<0.05). Our goal was to identify the pathways with potential to render susceptibility to tuberculosis in diabetic patients. We therefore excluded 54 disease pathways from the list. Pathway information for genes that did not give an output by STRING was manually searched for and then included in Biological Network Simulation (BioNSi).

2.6.6 *Biological network simulation:* BioNSi (Yeheskel *et al.* 2017), a biological network simulator, was used to simulate the pathways in normal and T2DM patients. The BioNSi is a discrete biological network simulator tool. Pathways were imported and merged into a large network for simulation. The average MAD Z-score of normalized expression values for normal as well as T2DM patients were provided as input to BioNSi. Based on the expression values of the genes, a state value in the range 0–9 was assigned, where 0 is the minimum and 9 is the maximum state based on the expression values. The simulation was performed with expression data from normal and from diabetic patients separately. Subsequently, the connections between the regulators of and targets of hsa-miR-146a were added manually (supplementary table 2). A total of 29 nodes (genes) in the merged network of pathways were connected to miR-146a. These connections included the 3 differentially expressed regulators and 8 targets of miR-146a. The initial state of miR-146a in diabetics was set to '6', while in normal patients it was set to '9' using the scaling criteria of gene expression values of BioNSi.

The initial states assigned based on gene expression values were updated during the course of simulation using a state update function. We used the default setup in the BioNSi tool for this purpose. The authors of BioNSi have also provided a Python script to calculate the differences between the simulation contours for a given gene and to rank them in terms of the differences in the state values during the course of simulation. This information was used to trace back to the pathways, and we selected the highly perturbed pathways between the disease state and the normal state as the one in which multiple genes in the same pathway showed differences in the states. Following that, the model was validated using expression values from GSE69528 and GSE15932. The GSE69528 contains gene expression values from sepsis patients, namely, 29 samples for *B. pseudomallei* infection, 54 samples for infection of other pathogens, and uninfected healthy and type 2 diabetes patients as controls. We have selectively taken 28 healthy controls (GSM1702888, GSM1702896, GSM1702897, GSM1702898, GSM1702899, GSM1702900, GSM1702901, GSM1702902, GSM1702903, GSM1702904, GSM1702906, GSM1702908, GSM1702909, GSM1702910, GSM1702911, GSM1702912, GSM1702913, GSM1702914, GSM1702920, GSM1702921, GSM1702922, GSM1702923, GSM1702929, GSM1702930, GSM1702931, GSM1702932, GSM1702933, GSM1702934) and 27 subjects with type 2 diabetes (GSM1702889, GSM1702890, GSM1702891, GSM1702892, GSM1702893, GSM1702894, GSM1702895, GSM1702905, GSM1702907, GSM1702915, GSM1702916, GSM1702917, GSM1702918, GSM1702919, GSM1702924, GSM1702925, GSM1702926, GSM1702927, GSM1702928, GSM1702935, GSM1702936, GSM1702937, GSM1702938, GSM1702939, GSM1702940, GSM1702941, GSM1702942) for our analysis. The GSE15932 contained blood samples to find differentially expressed genes specific for pancreatic cancer associated diabetes, which included 8 patients diagnosed as having pancreatic cancer with diabetes, 8 patients of pancreatic cancer without diabetes, 8 patients with diabetes mellitus for >5 years, and 8 healthy controls. The comparison was done using gene expression values from 8 T2DM (GSM399694, GSM399697, GSM399699, GSM399704, GSM399705, GSM399706, GSM399707, GSM399708) vs healthy control groups (GSM399717, GSM399718, GSM399719, GSM399761, GSM399762, GSM399763, GSM399765, GSM399766). We input these gene expression data in the BioNSi model in order to examine the perturbation of genes and pathways using identical parameters.

3. Results

3.1 *Gene retrieval from the literature*

Through literature mining, 36 genes associated with T2DMTB comorbidity were obtained. These associated

Table 1. Gene expression fold change and direction (positively regulated and negatively regulated) of the T2DMTB genes available in the literature and computed using microarray analysis of GSE26168

	CAMP	*CCL2*	*CCL5*	*CD14*	*CD28*	*CORO1A (TACO)*	*DEFA1*	*DEFB4A*	*FPR2*	*GZMB*	*HK2*	*HMOX1*	*IFNG*
Literature based Evidence (Diabetes)	↓ +TB	↓ +TB	↓ +TB	↓ +TB	↓ +TB	↑ +TB	↑ +TB	↓ +TB	↑ +TB	↓ +TB	↓ +TB	↑ +TB	↑ +TB
GSE26168 (Diabetic) (FC)	↑ (1.80)	NC	NC	↑ (1.33)	NC	↑ (1.56)	↑ (2.68)*	NC	NC	NC	NC	NC	NC
	IL1B	*IL10*	*IL12*	*IL17A*	*IL17F*	*IL18*	*IL19*	*IL2*	*IL20*	*IL22*	*IL24*	*IL5*	*IL6*
Literature based Evidence (Diabetes)	↑ +TB	↑ +TB	↓ +TB	↑ +TB	↓ +TB	↑ +TB	↓ +TB	↑ +TB	↓ +TB	↓ +TB	↓ +TB	↑ +TB	↑ +TB
GSE26168 (Diabetic) (FC)	*NC*	NC	NC	NC	↑ (1.22)	NC	NC	NC	NC	NC	NC	NC	NC
	PRF1	*RXRA*	*TLR4*	*TNF*	*VDR*	*LAMP1*	*TIMP4*	*MARCO*	*SOCS1*	*SOCS3*			
Literature based Evidence (Diabetes)	↓ +TB	↓ +TB	↓ +TB	↓ +TB	↓ +TB	↓ +TB	↑ +TB	↓ +TB	↑ +TB	↑ +TB			
GSE26168 (Diabetic) (FC)	NC	NC	↑ (1.20)	NC	NC	↑ (1.55)	NC	NC	NC	↑ (1.11)			

The up-arrow represents positive regulation and down-arrow represents negative regulation in diabetics versus normal. '+TB', data from a study of T2DMTB patients; FC, fold change of T2DM or T2DMTB versus normal; NA, not available; NC, no change in the given expression; *, differentially expressed at P_{adj} <0.05.

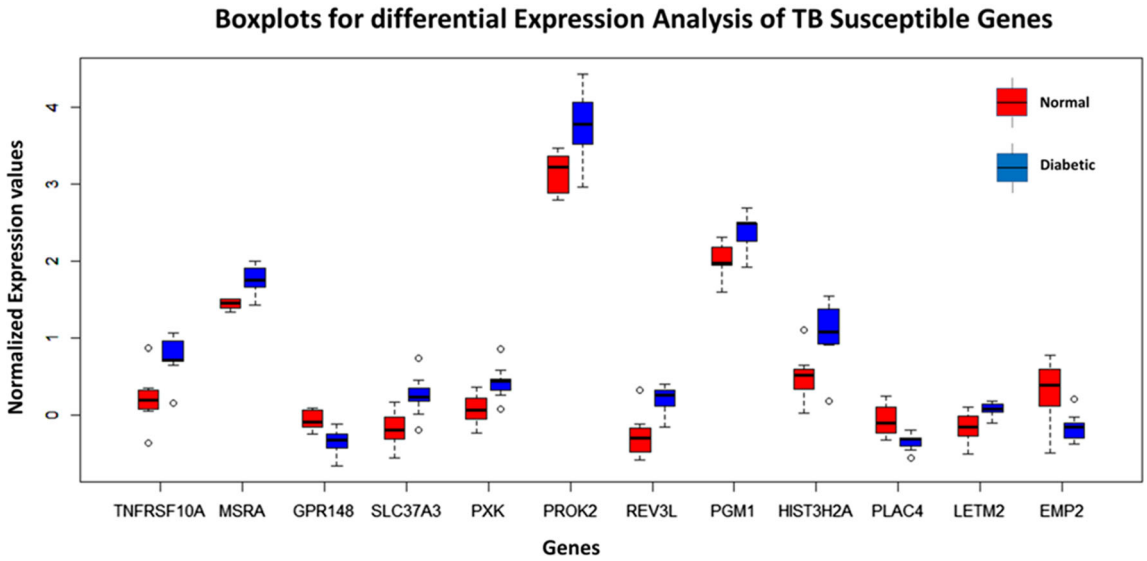

Figure 2. Gene expression of TB-susceptible genes.

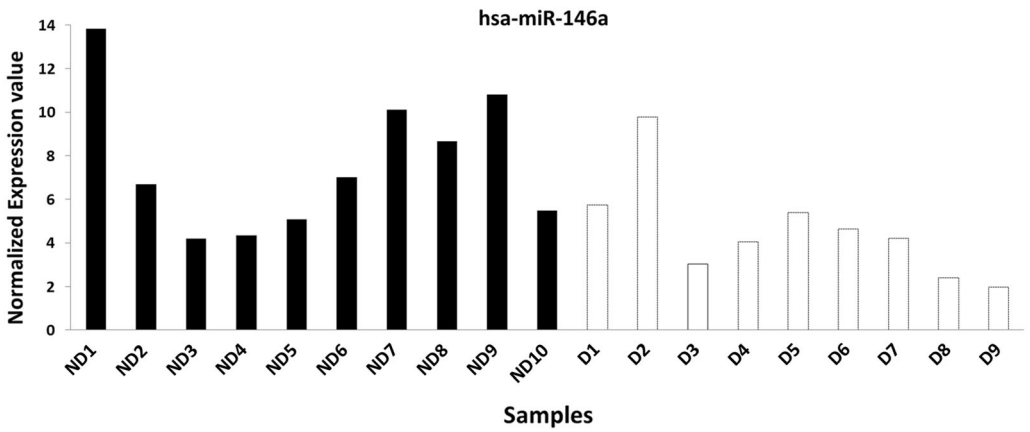

Figure 3. Differential expression of hsa-miR-146a in T2D blood samples from GSE26168. The filled bars represent non-diabetics, and the empty bars represent T2DM patients.

genes along with the evidence are listed in supplementary table 3. Heme oxygenase 1 (*HMOX1*), for example, a mediator of antioxidants and expressed in lung tissue, can distinguish latent from active TB (Andrade *et al.* 2014), and it is reportedly highly expressed in patients with TB and T2DM. Tumor necrosis factor alpha (*TNFα*) offers protection from mycobacterial infection (Pavan Kumar *et al.* 2013), and it is reported to have low expression in patients with T2DM. Interleukin 22 (*IL22*) is reported to protect against the development of metabolic disorder and it is decreased in diabetics with TB (Kumar *et al.* 2015a, b). The increased plasma level of *HMOX1* and decreased plasma levels of *TNF* and of *IL22* result in increased susceptibility of T2DMTB comorbidity (Pavan Kumar *et al.* 2013; Andrade *et al.* 2014; Kumar *et al.* 2015a, b).

3.2 *Gene enrichment analysis*

The GO enrichment data include 26 unique GOs (supplementary table 4). The host immune response to Mtb infection consists of inflammation, cytokine activity, and cellular response. The inflammatory cytokines (IL6, IFNG, TNFα, IL1B) were observed to be positively regulated in mouse models of T2DM with Mtb infection. The cytokines IFNγ, TNFα and IL17A, considered to offer protection against tuberculosis, are all positively regulated in diabetic individuals (Kumar *et al.* 2013; Trentini *et al.* 2016). However, it appears that chronic inflammation underlying T2DM results in poor control of tuberculosis infection. High glucose concentrations positively regulate TLR2 and TLR4 and activate NF-kappa B to induce pro-inflammatory cytokines. Apparently, the production of nitric oxide

Table 2. Genes from non-diabetics with a minimum Euclidean distance of 2 that reached a steady state either prior to or coincident with diabetics and mapped to KEGG pathways

GSE	Pathway	Genes (Euclidean distance)	Score*
GSE26168	Necroptosis	*GLUD1* (4), *TRPM7* (4), *DNM1L* (4), *RIPK3* (4), *GLUL* (4), *TICAM1* (4), *SMPD1* (4), *PGAM5* (4), *MLKL* (4), *TICAM2* (3), *TLR4* (2)	11
GSE26168	NOD-like receptor signaling pathway	*ATG12* (5), *TRPM7* (4), *DNM1L* (4), *RIPK3* (4), *TICAM1* (4), *NLRP1* (2), *TLR4* (2), *ATG16L1* (2)	9
GSE26168	Toll-Like receptor signaling pathway	*TICAM1* (4), *IRAK1-TRAF6* (4), *RIPK1-TRAF6* (4), *TICAM2* (3), *TIRAP-MyD88* (3), *TLR4* (2)	6
GSE26168	NF-kappa B signaling pathway	*TICAM1* (4), *IRAK1-TRAF6* (4), *RIPK1-TRAF6* (4), *TICAM2* (3), *TICAM1-TICAM2* (3), *TLR4* (2)	6
GSE69528	NF-kappa B signaling pathway	*BCL2* (4), *IRAK1-TRAF6* (3), *DDX58* (2), *TLR4* (2), *TICAM2-TICAM1* (2), *TIRAP-MYD88* (2), *LAT* (2)	7

*Score is the number of genes in a given pathway with minimum Euclidean distance of 2.

exhibiting antibacterial activity is also affected in T2DM patients (Herrera *et al.* 2017). Overall, it is apparent that even though T2DM patients can mount an immune response against Mtb infection, the infection is not controlled compared with healthy individuals.

3.3 *Differential expression*

Our goal was to identify whether the T2DMTB genes were differentially expressed between the T2DM patients without reported tuberculosis and normal controls (GSE26168). A total of 705 genes were differentially expressed in the blood sample of diabetic patients at P_{adj}<0.05 (supplementary table 5). Among 8 genes associated with T2DMTB, only the *DEFA1* gene was differentially expressed at P_{adj}<0.05. The genes *CAMP*, *CD14*, *CORO1A*, *LAMP1*, *TLR4*, *IL17F*, and *SOCS3* were differentially expressed at $P \leq 0.05$ (supplementary table 6). The expression changes of T2DMTB genes in diabetic patients (literature data) and in T2DM patients (GSE26168) are shown in table 1. It is evident that most T2DMTB genes were not differentially expressed in T2DM patients without tuberculosis. A few genes, *DEFA1*, *SOCS3,* and *CORO1A*, had altered expression in the same direction in T2DM patients as in T2DMTB patients. Genes with altered expressions between the two disease patients in opposite directions are *CAMP*, *IL17F*, *LAMP1*, *TLR4,* and *CD14*. The remaining genes were not differentially expressed in T2DM patients without tuberculosis. *CORO1A* codes for the protein Coronin 1A, which is retained by mycobacterial phagosomes, thereby preventing fusion with lysosomes (Liu *et al.* 2017). This mechanism prevents intracellular killing of Mtb (Jayachandran *et al.* 2007). The expression of this gene is positively regulated in T2DM patients without tuberculosis in parallel with literature reports. *DEFA1* coding for Defensin Alpha 1 is important for host defence against Mtb (Gonzalez-Curiel *et al.* 2011). Its expression is positively regulated in T2DM patients and parallels the evidence in the literature. On the other hand, *CAMP* is also positively regulated in T2DM patients, contrary to literature reports on T2DMTB patients. Similar is the case with *LAMP1* and *IL17F*. *LAMP1* coding for Lysosomal Associated Membrane Protein 1 is selectively excluded in the process of inhibiting fusion of Mtb phagosomes with lysosomes (Via *et al.* 1997). *IL17F* coding for Interleukin 17F is part of the Th17 immune response. It is reported that Th17 recall responses can be targeted to improve vaccine design against TB (Monin *et al.* 2015). In the case of *SOCS3*, it appears necessary to ensure a balanced inflammatory immune response during experimental TB (Schmok *et al.* 2017), thereby attributing a positive role in protection against Mtb. Taken together, even though the gene expression environment in T2DM patients is favorable for colonization of Mtb, host immune response molecules like defensin and CAMP are positively regulated in T2DM patients without tuberculosis.

3.4 *Human host TB-susceptible genes*

Next, we examined whether in the T2DM patients, the host genes rendering susceptibility to TB (Kumar *et al.* 2010) were differentially expressed. Out of 275 genes, 12 (*TNFRSF10A*, *MSRA*, *GPR148*, *SLC37A3*, *PXK*, *PROK2*, *REV3L*, *PGM1*, *HIST3H2A*, *PLAC4*, *LETM2*, and *EMP2*) were differentially expressed at $P_{adj} \leq 0.05$

Table 3. Pathway distribution of genes from diabetics that exhibited differences and reached steady state before or together with non-diabetics

GSE	Pathway	Genes (Distance)	Score*
GSE26168	Toll-like receptor signaling pathway	*IRAK1* (5), *TRAF6* (4), *IRF5* (3), *IRAK4* (3), *MyD88* (3), *IRAK1-IRAK4* (3)	7
GSE26168	MAPK signaling pathway	*IRAK1* (5), *TRAF6* (4), *IRAK4* (3), *MyD88* (3), *BAK1* (2)	5
GSE26168	NF-Kappa B signaling pathway	*IRAK1* (5), *TRAF6* (4), *IRAK4* (3), *MyD88* (3), *IRAK1-IRAK4* (3)	5
GSE15932	FoxO signaling pathway	*ATM* (3), *BNIP3* (3), *CDKN2D* (3), *CCNG2* (2), *CDKN2B* (2), *IKBKB* (2), *IL7R* (2), *NLK* (2), *SOD2* (2), *TNFSF10* (2)	10
GSE15932	JAK-STAT signaling pathway	*CSF3R* (3), *AOX1* (2), *CISH* (2), *GFAP* (2), *IL7R* (2), *SOCS4* (2), *STAT1-STAT1* (2), *CSF3R-JAK1* (2)	8
GSE15932	NF-kappa B signaling pathway	*TICAM1* (8), *TICAM2* (6), *TLR4* (5), *ATM* (3), *IKBKB* (2), *IRAK1-TRAF6* (8), *TICAM2-TICAM1* (5), *TIRAP-MYD88* (5)	8
GSE15932	Toll-like receptor signaling pathway	*TICAM1* (8), *TICAM2* (6), *TLR4* (5), *IKBKB* (2), *TIRAP-MYD88* (5), *RIPK1-TRAF6* (7)	6
GSE15932	PI3K-Akt signaling pathway	*TLR4* (5), *CSF3R* (3), *IKBKB* (2), *IL7R* (2), *CSF3R-JAK1* (2)	5

*Score is the number of genes in a given pathway with minimum Euclidean distance of 2.

(supplementary table 7). Although the magnitude of differential expression is low (FC in the range 0.72–1.49), the changes are highly statistically significant. A total of 9 genes were positively regulated and 3 genes, *GPR148*, *PLAC4*, and *EMP2*, were negatively regulated in T2DM patients compared with normal controls (figure 2). The data suggest that a small minority (4.36%) of host TB-susceptible genes were differentially expressed in T2DM patients, of which the majority (75%) are positively regulated. It is apparent that these alterations in host TB-susceptible genes expression could create a favorable environment for colonization of Mtb.

3.5 *Network analysis*

3.5.1 *Collection of dysregulated miRNAs in T2DM and TB patients:* Our premise is that dysregulated miRNAs could affect the regulation of expression of target genes, which may lead to T2DMTB comorbidity. We obtained a total of 7 miRNAs (hsa-miR-146a-5p, hsa-miR-155-5p, hsa-miR-125b-5p, hsa-miR-21-5p, hsa-miR-27a-3p, hsa-miR-29b-3p, and hsa-miR-223-3p) from the T2DMTB genes–miRNAs network using NetworkAnalyst (Xia *et al.* 2015) (supplementary figure 1). These miRNAs were further examined for evidence of dysregulation in T2DM and in TB conditions in the literature. The results are shown in supplementary table 1. It is evident that these miRNAs have their role in the regulation of the immune system.

3.5.2 *Differential expression:* Microarray expression analysis revealed the differential expression of above-mentioned miRNAs in T2DM patients. With the criteria of selection based on $P_{adj} \leq 0.05$, only hsa-miR-146a was varied significantly (figure 3). This microarray analysis corroborated a previous study of downregulation of miR-146a in diabetic condition and in children with TB.

3.5.3 *Gene regulators of and genes regulated by miR-146a:* With the aim to elucidate the mechanism, we collected the gene regulators of and genes regulated by hsa-miR-146a using text mining with the help of pubmed.mineR. The data are provided in supplementary table 2. Among the negative regulators of hsa-miR-146a, *SOD2* was differentially expressed at $P_{adj} < 0.05$. Among the positive regulators, *STAT3 a*nd *TGFB1* were differentially expressed in T2DM patients at $P < 0.05$. Among the genes negatively regulated by hsa-miR-146, *MMP9* and *SOD2* were differentially expressed at $P_{adj} < 0.05$, whereas *TLR4*, *CD86*, and *FBXL10* were significantly varied at $P < 0.05$. Among the genes positively regulated by hsa-miR-146a, *PAK1* and *STAT1* were differentially expressed at $P < 0.05$ in T2DM patients. These observations point to the role of hsa-miR-146a in T2DM patients.

3.5.4 *Biological network simulation:* We attempted to elucidate the root pathway for susceptibility of tuberculosis in diabetic patients through BioNSi pathway simulations (supplementary table 8). From the

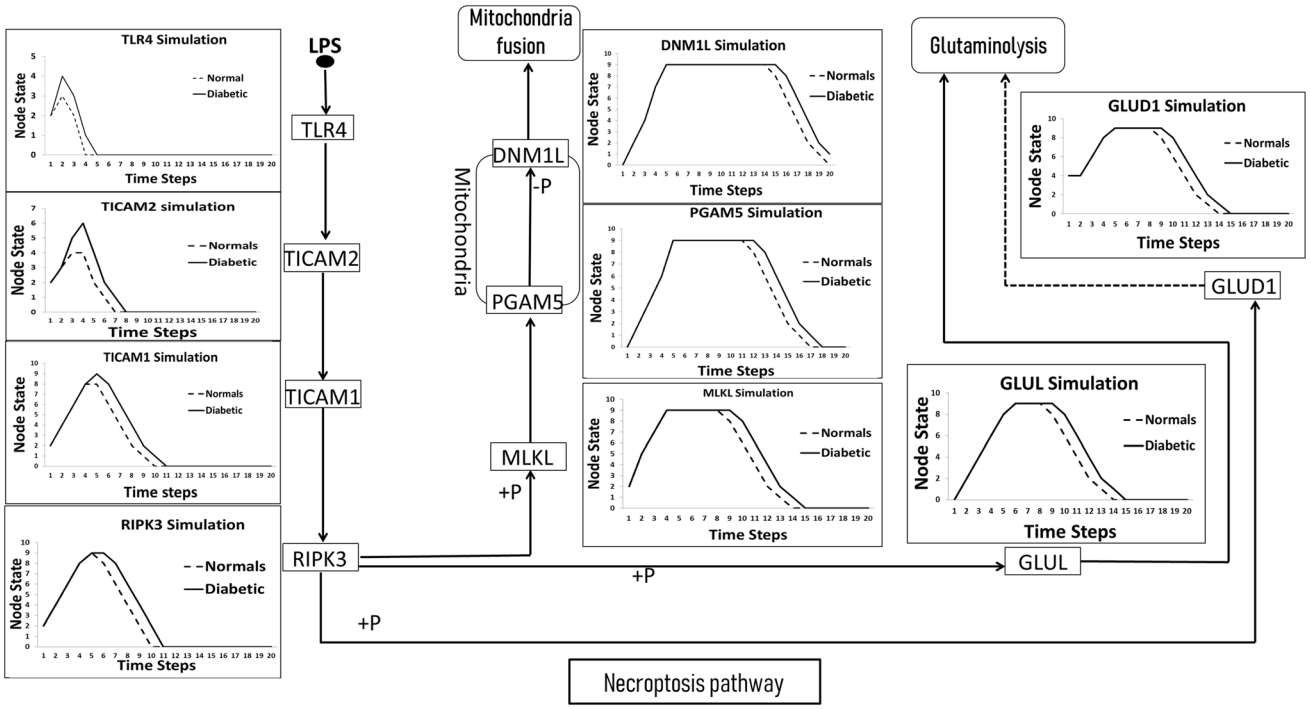

Figure 4. Schematic representation of the most perturbed pathway, the necroptosis pathway (has04217), in simulations using BioNSi. The simulation graphs are shown in square boxes besides each protein in the pathway. +P and −P signify phosphorylation and de-phosphorylation respectively. The arrows represent the regular route of metabolic flow, and the broken arrows represent the alternate route.

integrated analysis, we noted that the downregulation of miRNA-146a in T2DM patients could underlie the mechanism conferring TB susceptibility in T2DM patients. As genes and their regulators are networked in cellular systems, we carried out two simulations, one each with the corresponding expression values from healthy controls and from T2DM patients. Typically, simulations reach a steady state depending on the network connections and the expression values of genes in the network. The exhibited differences in the pattern of the simulation contours could identify the pathways most disturbed due to the changes in the expression values of the genes in the disease conditions. The extent of the disturbance can be quantified through Euclidean distance, and we considered a minimum Euclidean distance of 2.

We obtained 26 genes from healthy controls with a minimum Euclidean distance of 2 in GSE26168. They also reached a steady state either prior to or coincident with T2DM patients (table 2). Under the same criteria, in T2DM patients, 14 genes had a minimum Euclidean distance of 2 and reached steady state either prior to or coincident with healthy controls (table 3). Genes with other than this cut-off distance are given in supplementary table 9a and b. It is evident that the most altered pathway is the necroptosis pathway (figure 4) followed by the NOD-like receptor signaling pathway, Toll-like receptor signaling pathway, NF-kappa B signaling pathway, and MAPK signaling pathway (supplementary figure 2). Quantitatively 11, 9, 6, and 6 genes from the necroptosis pathway, NOD-like receptor signaling pathway, Toll-like receptor signaling pathway, and NF-kappa B signaling pathway, respectively, had a minimum Euclidean distance of 2. Further, during validation of the network model using expression values from gene expression datasets of type 2 diabetes versus healthy controls, we obtained 10 and 33 genes with a minimum Euclidean distance of 2 from GSE69528 and GSE15932 that reached a steady state either prior to or coincident with T2DM patients (table 2; supplementary table 9a). Under the same criteria, 9 (GSE69528) and 12 (GSE15932) genes had a minimum Euclidean distance of 2 and reached steady state either prior to or coincident with healthy controls (table 3; supplementary table 9b). Pathway mapping revealed that the NF-kappa B signaling pathway (7 perturbed genes) is the most perturbed in GSE69528. Likewise, in GSE15932, the most perturbed pathways include the FoxO signaling pathway (10 perturbed genes) followed by the NF-kappa B signaling pathway

(8 perturbed genes) and JAK-STAT signaling pathway (8 perturbed genes), Toll-like receptor signaling pathway (6 perturbed genes), and PI3K-Akt signaling pathway (5 perturbed genes). It was interesting to observe the perturbation of the *TLR4* gene in each simulation. The role of this gene in T2DMTB comorbidity was studied by Li *et al.* (2016). This gene is involved in many of the pathways that were perturbed during BioNSi simulation, namely, necroptosis, NF-kappa B signaling, Toll-like receptor signaling, NOD-like receptor signaling, and PI3K-Akt signaling pathways. As several of these pathways are associated with inflammation, these results uphold the current understanding that chronic inflammation in T2DM patients likely underlies susceptibility to TB.

4. Discussion

Investigations of T2DMTB patients have revealed the role of chronic inflammation in poor TB control (Restrepo 2016). In this work we sought to investigate the perturbed pathways in T2DM patients with potential to render them susceptible to TB. To this end, we carried out integrative analysis with multiple gene sets including 36 T2DMTB genes, 12 differentially expressed host genes in T2DM patients susceptible to TB, 705 differentially expressed genes in T2DM patients, and 1 differentially expressed microRNA associated with T2DM and with TB.

Compared with the gene expression changes in T2DMTB patients, only 8 out of 36 genes showed altered gene expression in T2DM patients. One of these genes, *DEFA1* expression were significantly altered whereas the remaining 7 gene expression alterations were statistically significant only at $P<0.05$. These data clearly show that the expression changes of many more genes were observed when T2DM patients have TB. We closely examined them for identifiable patterns informative of warning signals for *M. tuberculosis* infection in T2DM patients. Genes with the same direction of expression changes compared with T2DM patients were *DEFA1*, *SOCS3*, and *CORO1A*. The remaining genes, *CAMP*, *IL17F*, *LAMP1*, *TLR4*, and *CD14*, exhibited change in expression in opposite directions. It is noteworthy that the defensin gene *DEFA1* and the cathelicidin antimicrobial peptide gene *CAMP* are positively regulated in T2DM patients, signifying that the defence arm is active and therefore could offer protection against microbial infections. These genes are reported as vital in defence against tuberculosis (Via *et al.* 1997; Jayachandran *et al.* 2007; Gonzalez-Curiel *et al.* 2011; Monin *et al.* 2015). Along the same lines, the positive regulation of *SOCS3* is apparently helpful in modulating the inflammation towards fighting TB (Schmok *et al.* 2017). On the other hand, the upregulation of *CORO1A* gene expression in T2DM patients is indicative of setting a favorable environment for colonization of *M. tuberculosis*. Given this mixed signature of changes in gene expression, it is hard to predict susceptibility to TB with high statistical confidence. We can only educate T2DM patients about their potential susceptibility to *M. tuberculosis* infection. We, therefore, attempted to use the pathways approach in order to derive potential signals of susceptibility to *M. tuberculosis* infection. To this end, we used the BioNSi simulation software with gene expression data input from T2DM patients and normal controls in order to shortlist the most perturbed pathways.

The BioNSi software uses a state updating function with starting values scaled in the 0–9 range from the gene expression data. Simulations were carried out for T2DM patients and normal samples separately. The end points corresponded to reaching the steady state. The differences between the gene expression states of T2DM patients and of normal controls were quantified by Euclidean distance. We considered a minimum Euclidean distance of 2 for selecting the perturbed genes.

The network model of pathways for the gene implicated in T2DM-associated comorbidity tuberculosis was initially created using expression values from GSE26168 and later validated using gene expression values from GSE69528 and GSE15932. Further, we have separately extracted perturbed genes from each simulation. After mapping these genes to pathways and ranking these pathways in descending order of scores, we have observed that NF-kappa B and Toll-like receptor are commonly perturbed with high ranking in multiple gene expression datasets of type 2 diabetes versus healthy controls. The other pathways, necroptosis and FoxO signalling pathways, appear perturbed with high ranking in different gene expression datasets. Recently, Pajuelo *et al.* (2020) reported that macrophage necroptosis is exploited by *M. tuberculosis*, which includes NAD^+ depletion, depolarization of mitochondria and impaired ATP synthesis. Therefore, the positive regulation of the necroptosis pathway offers a favorable environment for susceptibility to TB. The results obtained here suggest that the necroptosis pathway is already active in T2DM patients, and therefore, once they are infected, the active necroptosis pathway could enable the dissemination of *M.*

tuberculosis. The NOD-like receptors and Toll-like receptors are pattern recognition receptors that recognize pathogens and serve in innate immunity (Killick *et al.* 2013). The interferon regulatory factor-5 (IRF) transcription factor plays an important role in innate immune responses via the TLR4-MyD88 pathway (Ban *et al.* 2018). The expression of IRF5 is constitutive in macrophages, B cells, and dendritic cells. The alterations observed in the IRF5 pathway through TLR4 signaling suggest its contribution in the pathogenesis of TB in the diabetic condition.

Our analysis is informative regarding monitoring the perturbation of necroptosis pathways in gene expression data from blood of T2DM patients. Necrosome-dependent necrotic cell death is defined as necroptosis and also reported as pathogenic for pulmonary diseases (Mizumura *et al.* 2016). The necrosome consists of receptor interacting protein kinase 1 (RIPK1), receptor interacting protein kinase 2 (RIPK2), receptor interacting protein kinase 3 (RIPK3), and mixed lineage kinase domain-like (MLKL) proteins. It is reported that alterations in the necroptosis pathway may lead to variation in host immune responses against tuberculosis and disease severity (Stutz *et al.* 2018). Further, the alterations in mitochondrial permeability likely lead to primary necroptosis (Dubey 2016). The necroptosis of macrophages allows bacterial pathogens to evade the immune response (Robinson *et al.* 2012). Therefore, taken together, the activation of the necroptosis pathway in T2DM patients could lead to easy survival of the bacteria, thereby rendering them more prone to active tuberculosis. Recent research suggests that altering Treg cells in tuberculosis patients can result in the development of active tuberculosis by downregulating the PI3K-Akt/mTOR pathway (Sharma *et al.* 2009; Zhang *et al.* 2017; Keikha *et al.* 2018). Bouzeyen *et al.* (2019) had reported that FOXO3, which is a master regulator in the FoxO signaling pathway, regulates repression of IL-10 cytokine in Mtb-infected macrophages. Further, T2DMTB patients are observed to have increased levels of IL10 (Kumar *et al.* 2013). Different cytokines of JAK-STAT pathways directly control whether macrophages support or destroy mycobacteria (Harris *et al.* 2007; Cooper *et al.* 2011; O'Garra *et al.* 2013). We were able to appreciate the perturbation of various genes of this pathway through simulation. hsa-miR-146a is downregulated in T2DM patients, thereby linking its role in chronic inflammation (Balasubramanyam *et al.* 2011) through TLR receptor signaling and the NF-kappa B signaling pathways. We noted that none of the 12 host genes susceptible to TB significantly overexpressed in T2DM patients appeared in the list of high-ranking perturbed genes. However, we cannot not rule out as yet undiscovered pathways or interactions of the human host susceptible to TB genes in predisposing T2DM patients to TB.

Although we obtained useful insights through this integrative approach and simulations, our inferences are associated with caveats. First, we used the gene expression dataset from the blood. Most of the measurements pointing to the involvement of genes in the comorbid condition were carried out using human blood samples. The premise here is that blood signatures are a reflection of infection foci. However, whether the signature obtained in this work would match the gene expression patterns in the lungs of T2DM patients remains to be investigated. Nonetheless, we envision that the results reported in this manuscript could guide further investigation in order to enable development of gene expression-based diagnostic signatures and future therapeutics for preventing or controlling TB infection in T2DM patients.

5. Conclusion

In this work we developed a protocol for identifying the top perturbed pathways in T2DM patients with potential for susceptibility to TB. The method followed mining of the literature for extracting associated genes and miRNAs, followed by differential expression analysis, extraction of pathways, integration of miRNA regulators and targets, and simulation using BioNSi from gene expression data. A total of 36 genes were associated with T2DMTB comorbidity. Seven miRNAs interacting with these genes were implicated in T2DM and in TB. *DEFA1* among T2DMTB genes, 12 human host TB susceptibility genes, *TNFRSF10A*, *MSRA*, *GPR148*, *SLC37A3*, *PXK*, *PROK2*, *REV3L*, *PGM1*, *HIST3H2A*, *PLAC4*, *LETM2*, and *EMP*2, and hsa-miR-146a, were observed as significantly differentially expressed in T2DM patients at P_{adj}<0.05. Other genes, such as *CAMP*, *CD14*, *CORO1A*, *LAMP1*, *TLR4*, *IL17F*, and *SOCS3*, were differentially expressed in T2DM patients only at *P*-value <0.05. It is evident from BioNSi analysis that the NF-kappa B and Toll-like receptor pathways are commonly perturbed with high ranking in multiple gene expression datasets of type 2 diabetes versus healthy controls. The other pathways, the necroptosis and FoxO signalling pathways, appear perturbed with high ranking in different gene expression datasets (Rani *et al.* 2022). These

pathways likely underlie susceptibility to tuberculosis in T2DM patients.

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Declarations

Conflict of interest All authors declare no conflicts of interests.

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