Title

Latent Tuberculosis infection in high TB disease burden countries dysregulates cellular and immunological profiles which is further enhanced with uncontrolled hyperglycemia

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Abstract

Background

Tuberculosis (TB) and diabetes mellitus (DM) are both highly prevalent in Pakistan. Latent *Mycobacterium tuberculosis* (Mtb) infection is common however the effect of DM and latent TB infection (LTBI) is less understood. We used RNA arrays to study host transcriptional responses to investigate this.

Methods

Participants were controls (EC) and with DM, sub-classified to LTBI and DM-LTBI. Host blood transcriptomes were studied using microarrays followed by GO, WwikiPathway and rReactome pathway analseis.

Results

Gene expression compared with EC revealed 187 differentially expressed genes (DEGs) associated with LTBI; 182 DEGs with DM and 13 DEGs with DM-LTBI. In LTBI and DM, downregulation of antigen presentation and upregulation of inflammatory genes was evident whilst in DM, mostly immune related genes were downregulated.

Comparison between LTBI-DM and LTBI revealed 321 up- and 12 downregulated DEGs, with upregulated immune response and inflammatory genes whilst a downregulation of genes associated with insulin metabolism and oxidative stress were observed.

The impact of uncontrolled hyperglycemia was seen as downregulation in protein synthesis and oxidative phosphorylation in the host. This effect was further enhanced in those with hyperglycemia within the LTBI-DM group where a shif. Importantly, our observations of dysregulated pathways observed in diabetic individuals were as found in published datasets.

Conclusions

We show here that LTBI and DM synergistically increase host inflammatory and metabolic processes whilst reducing innate immunity. This dysregulation by uncontrolled hyperglyemia highlights increased risk of progression of Mtb infection in this cohort and emphasizes the need for diabetes control in a TB endemic population.

Background

In 2024 there were 10·5 million new TB cases and 1·5 million deaths globally. WHO defines latent TB infection (LTBI) as a state of persistent immune response to stimulation by

Mycobacterium tuberculosis (Mtb) antigens without evidence of clinically manifested active TB, which can be detected by the presence of Mtb specific T cells secreting IFN-■ in peripheral circulation (1). Comorbidities and conditions associated with risk of LTBI reactivation are categorized as high, moderate or low risk. At moderate risk are patients with diabetes mellitus type 2 (DM). In 2014, the global burden of LTBI was 23% (95% uncertainty interval: 20.4%-26.4%). Estimates based on these date estimate TB incidences of 16.5 per 100,000/year in 2035 (2). Pakistan is highly endemic for tuberculosis (TB) with an estimate of 51,000 new cases emerging each year, contributing 61% of the TB cases in Eastern Mediterranean region (1). There is an increasing frequency of diabetes (31% with 34.5 million in 2024) in the adult population according to the International Diabetes Federation (3). DM increases the risk of activation of TB three-fold. Population based surveys show that prevalence of diabetes is also associated with LTBI. A study by Barron et al showed that in the US population, prevalence of LTBI with DM was 2x greater than those who were without DM (4).

Protection against TB is mainly shown to be CD4± T cell driven (5). CThe cytokines including IL-12, IFN-γ, and TNF- ✔ are crucial for induction and maintenance of protective immune responses against Mtb. Cytokines and gene transcriptional analysis reflect the activation, regulation and modulation of innate and adaptive arms of the immune system. Individuals with DM have dysregulated T-cell-activation and signaling as compared with healthy controls (6). Further, genes enriched in immune response processes such as granulocyte and T-lymphocyte activation and those involved in antigen processing are downregulated in individuals with DM, whilst upregulated genes were associated with fatty acid and carbon metabolism (7). LTBI and DM diminished frequency of Th1, Th2 and Th17 has been observed in LTBI-DM subjects (8). Currently, the effect of LTBI and mechanisms associated with progression of Mtb infection due to diabetes are unclear (9).

Here we measured whole blood cytokines stimulated by Mtb-antigens and studied blood transcriptional profiles in individuals with and without a diagnosis of DM to investigate the effect of LTBI. Our study of host blood transcriptomes in the study groups revealed enhanced dysregulation due to the concomitant effect of LTBI and DM with uncontrolled hyperglycemia.

Methods

This is an observational study approved by the Ethical Review Committee of the Aga Khan University (AKU). All research was performed in accordance with relevant guidelines/regulations in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants and/or their legal guardians. Participants were recruited from the AKU Hospital and Jinnah Postgraduate Medical Center, Karachi. Study

groups comprised: individuals aged > 18 years, those from the community, patients attending diabetes clinics and attendants accompanying patients to TB clinics. Exclusion criteria were those with known immunocompromised conditions including HIV, cancer, transplant recipients or with a history of immunosuppressive drugs. We recruited participants into four study groups~~:~~; healthy endemic controls (EC), LTBI, DM and DM with LTBI (DM-LTBI). Complete blood counts (CBC), hemoglobin (Hb), as well as HbA1c levels were tested in all study participants. Patient history regarding any treatments taken for diabetes, hypertension or additional comorbids were documented. The number of individuals taking metformin, sitagliptin, glimepiride, insulin and statins was documented. The DM group was identified at HbA1c $\geq$ 6.5 %. Individuals with HbA1c levels 6.5-8% were classified as controlled (C-DM), whilst those with HbA1c > 8% were classified as having uncontrolled hyperglycemia (H-DM).

IGRA testing

Diagnosis of LTBI was conducted by testing whole blood cells using the QuantiFERON-TB Gold (QFT~~-GIT~~) assay as per manufacturer's instructions (Qiagen, Cellestis, USA). Plasma supernatants from 'nil', 'TB1', 'TB2' and 'mitogen' tubes were tested for IFN-γ using the QFT~~uantiFERON~~ ELISA assay. The TB1 tube contains antigens that stimulate CD4+ T-cells, while TB2 contains additional peptides that stimulate both CD4+ and CD8+ T-cells. LTBI diagnosis was made using QFT software. The cut-off for positivity was $\geq$ 0.35 IU/ml of either TB1 or TB2 values (after subtraction of nil values).

Host blood RNA transcriptome analyses

RNA was extracted from whole blood. 100 ng RNA was used in the Clariom S Array Type gene expression assay as described previously (10). We included assessment of RNA QC and include a sample QC report (S Table 3). In each case ~~it was checked that~~ RNA quality was checked including that concentration, purity and integrity was within the defined acceptable limits. Samples selected for microarray processing were all with 260/280 ratios above 1.5. CEL files were analysed using the TAC Transcriptome Analysis Software Suite (TACS version 2) using the Summarization Method: Gene Level - SST-RMA Pos vs Neg AUC Threshold: 0.7 against Genome Version: hg38 (Homo sapiens). Normalization is performed by the Robust Multi-array Average (RMA) method which runs background correction followed by quantile normalization and summarization (median-polish) to generate a final output of one log2-normalized expression value per probeset per sample. We filtered the data to select significant differentially expressed genes (DEGs) up- or down-regulated (log2 FC (fold change) < -2 or > 2; P value <0.05). Further, hierarchical clustering and volcano plots were made using TACS as well as R.

For accession number generation, array output raw files (CEL files) and processed files (CHP) were submitted to Gene Expression Omnibus (GEO) NCBI and are available as GSE177477 and GSE297003 (**S Table 1**). TACS generated list of DEGs used in the study for the gene enrichment analysis are shown in S Table 2. Specifically, as an example of data QC we have included details for the comparison of LTBI and Control (S Table 3).

Functional gene enrichment analysis

DEGs were analysed using 'R' to perform Gene Ontology (GO) analysis (11) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (12, 13). We used two types of functions from clusterProfiler i.e, enricher function (enrichGO, enrichKEGG) for hypergeometric test and GSEA (gseGO, gseKEGG) function for gene set enrichment analysis on user defined data (14, 15). GO enrichment analysis was carried out employing enrichGO function which requires a gene list as input vector. The results are annotated along three ontologies: Molecular Functions, Biological Processes and Cellular Components with the following parameters: pvalueCutoff = 0.05, pAdjustMethod = "BH" (Benjamani and Hochberg) and qvalueCutoff = 0.05. The enrichKEGG requires a gene-list as input, parameter of pvalueCutoff = 0.05 and organism of interest (homo sapiens "hsa"). Gene set enrichment analysis was performed on GO terms using gseGO which requires gene-list in the form of input vector, organism of interest (database: org.Hs.eg.db), pvalueCutoff = 0.05, minGSSize (minimal size of genes annotated by Ontology term for testing) = 10 and maxGSSize (maximum number of genes annotated for testing) = 800. gseKEGG function is similar with respect to input parameters (genelist, organism = hsa, minGSSize, maxGSSize and pvalueCutoff), applied on KEGG database (with permission). Additional analysis was performed on wiki-pathways, using enrichWP (organism = "Homo sapiens") and gseWP (organism = "Homo sapiens"). Reactome pathway analysis was also performed as it can analyse multiple datasets simultaneously for comparative pathway analysis. The function used was gsePathway (geneList, pvalueCutoff = 0.2, pAdjustMethod = "BH"). For visualization of results related R packages such as GOplot, enrichplot, DOSE and pathview were used to generate pathway maps, dotplots, heatmaps and barplot (15-17).

To further validate the results of enrichment and functional analysis, a cell deconvolution analysis was performed on the DM-LTBi cohort (n=10), by classifying them in HDM=4 and CDM=6. The normalized data of the cohort was obtained from TACS using probe normalization method (RMA). The sample signal table was generated and used as input in the gedit(Gene Expression Deconvolution Interactive Tool) (18). The reference matrix used were LM22 (19) and ImmunoState (20). The heatmap of cell types was computed on the basis of minimum entropy.

Results

Description of study groups

We studied gender and age matched participants (51 with normoglycemia and 49 with hyperglycemia) subclassified as endemic controls (EC, n=22), LTBI (n=29), DM (n=30) and DM-LTBI (n=19), **S Fig 1**. Study subjects were aged 41.27 ± 13.09 years (**Table 1**). Whilst gender distribution was comparable between all groups, those with diabetes were significantly older ($p<0.0001$). Hemoglobin levels, whole blood cell counts (WBC) and neutrophil/lymphocyte ratio (NLR) were comparable between the groups studied. HbA1c levels

were comparable between DM-LTBI and DM (p=0.078). Most hyperglycemic participants (93.3% DM, 89.5% DM-LTBI) were on treatment however, 60% of DM and 42.1% of DM-LTBI study subjects had H-DM. An equivalent number of individuals were taking anti-hypertensive medications and statins in DM and DM-LTB groups.

Details of the anti-diabetic treatments taken are given in S Table 3. All 49 patients with diabetes were on treatment but data was not available for six individuals. They were taking different medications either separately or in combination; 38 were on metformin, 25 were on sitagliptin, 17 were on glimepiride, and 6 were using insulin. Twelve patients with diabetes were on a single antidiabetic drug and of these four had C-DM and 8 had H-DM. Thirty-one patients took combination treatments with two or three drugs. Fourteen patients used sitagliptin with metformin; 3 had C-DM, and 11 had H-DM. Nine patients took a combination of sitagliptin, metformin, and glimepiride, but all had H-DM. Further, six patients used insulin and two had C-DM and four had D-DM. There was no difference in the anti-diabetic drug combinations used by those in the H-DM and C-DM groups. Furthermore, the usage of medications between those in the DM-LTBI and DM groups was not significantly different.

Assessment of IFN-[redacted] in whole blood stimulated with Mtb antigens

Measurement of IFN-[redacted] after stimulation with Mtb specific antigens is indicative of host Mtb infection status (21). IFN-[redacted] levels stimulated by TB1 and TB2 were found to differ, p<0.001 between study groups (**Fig 1**). As expected, TB1- and TB2- induced IFN-[redacted] was greater in LTBI than EC groups (p<0.0001). Also, IFN-[redacted] levels were raised in LTBI (p<0.0001) and DM-LTBI (p<0.0001) as compared with DM groups. IFN-[redacted] levels induced by TB-1 and TB-2 correlate positively in each of the groups: EC (Spearman's rank rho 0.807, p<0.0001), LTBI (SR rho 0.961, p<0.0001), DM (SR rho 0.909, p<0.0001) and DM-LTBI (SR rho 0.994, p<0.0001) groups (data not shown).

Investigating the impact of MTB infection and hyperglycemia on host blood transcriptional profiles

We investigated host blood transcription profiles to understand immunological and cellular profiles in study participants. Between EC, LTBI, DM and DM-LTBI groups, 359 Differentially Expressed Genes (DEGs) were observed. DEGs between EC and other groups are listed in **S Table 2.** Compared to EC, there were 187 DEGs against LTBI, with 4 Up- (2.1%) and 183 down- regulated genes, **Fig 2A**. There were 182 DEGs against DM (25 up- (13.7%) and 127 down- regulated genes) and then 13 DEGs between DM-LTBI and EC groups (6 up- and 7 down-regulated genes), **Fig 2B**.

Analysis of abovementioned DEGs through a Venn diagram (**Fig 2C)** revealed 165 common DEGs between LTBI and EC, 160 DEGs between DM and EC and 12 DEGs between DM-LTBI and EC groups. Twenty-two

down-regulated DEGs were similar to LTBI/ EC, and DM/ EC analyses, **Fig 2D**. These included immune response genes (*CHI3L1* which plays a role in Th2 inflammation; *PECAM1*, a cell adhesion and signaling molecule; *ADGRE2*, involved in mast cell responses; *CHMP2A*, involved in endocytosis and, *DDX56* an Interferon Stimulated Gene, *ISG*). Genes associated with mRNA processing (*SNRPN*), ribonucleases (*RNASET2*), as well as cellular metabolism (*TALDO1*, involved in the pentose phosphate pathway; *SLC2A3*, a *GLUT* membrane transporter) were downregulated.

Downregulation of innate immune response related genes in LTBI

We focused further on the transcriptomic comparison between individuals in the LTBI and EC groups. Specifically, as an example of a dataset comparison, we have included details for sample QC and list of DEGs the comparison of the LTBI and Control groups (Tables S4-5).

In LTBI, we found upregulated *USP17* genes (de-ubiquitinating enzymes involved in apoptosis), *OR51F2* (G protein-coupled receptor signaling) and *YEATS2* (activates TAK1/NF-kappaB pathway), **Fig 3A**. HLA genes (*HLA-DRB5*, *HLADQA1*) were most downregulated. Also downregulated~~lowered~~ were *JCHAIN*, *UBE2W*, *TLR10*, *CHI3L1* and *PI3*. Dotplot analysis of GSE-GO biological processes and molecular functions (**Fig 3B**) and the heatmap of DEGs **(Fig 3C)** further emphasized how affected DEGs were involved in antigen presentation and protein modification.

There were only 13 DEGs between DM-LTBI vs EC, so gene ontology pathway analysis could not be run. However, upregulated genes in DM-LTBI were associated with innate immunity (*CLEC12A*), processing of proteins and lipids (*PAM16*, *MAN2A2*, *PIGC*) oxidative (*CORO7*) and angiogenic (*SVBP*) function. Down-regulated genes (*DPP4*, *FAM102A*, *TTN*, *ERO1A*, *IL6ST*, *LEF1*) were associated with blood sugar control and insulin secretion.

Transcriptional profiles of diabetic individuals display enhanced inflammatory and reduced innate immune responses

DEGs between DM and EC visualized in a volcano plot **(Fig 4A)** show up-regulated genes include *ADAM29* (a disintegrin and metalloprotease domain) and *CHMP7* (involved in endosomal sorting). Downregulated genes include *SORL1* (associated with endolysosomal processing), *GNLY*, *CRLF3* and *CACUL1* (associated with anti-obesity treatment), *ADGRE2* and *CHI3L1*. Dotplot analysis of the GSE Reactome for these data further supported suppression of neutrophil degranulation, innate immune and immune pathways in the DM group (**Fig 4B**). Additionally, the heat map of the GSE-Reactome identifies downregulated genes associated with innate responses (*GNLY*, *PRF1*) and neutrophil function (*KLRC3*), **Fig 4C.**

Impact of diabetes on the transcriptome of those with LTBI

Next, a comparison of DM-LTBI and LTBI revealed 333 DEGs with 321 (96.3%) upregulated genes. *HLA-DRB5* was the most upregulated, followed by *S100A8* (involved in leukocyte recruitment). Other upregulated genes were, *PI3*, *HEMGN* (hematopoietic cell differentiation), *GYPA* (erythrocyte protein) and *BLVRB* (regulator of hematopoiesis and intermediary metabolism) (**Fig5A).** Downregulated genes included *ND6* (role in mitochondrial function), *ABCD2* (involved degradation of fatty acids) and *PSMC3* (an ATPase subunit).

GSEPathway (Reactome) analysis revealed that in those with DM-LTBI there was upregulation of innate (Toll like receptor, neutrophil degranulation) and adaptive immune responses (antigen presentation pathways), inflammatory processes related to interferon signaling and cytokine signaling (**Fig 5B**.) These pathways are further described through the KEGG GSE heatmap showing inflammation and innate host immunity (*IL-1R2*, *TLR1*, *TLR4*, *TLR8, TREM1*, *JAK2* and *MMP9*) and antigen presentation (*HLA-DQA1*, *HLA-DRA*) to be differentially regulated (**Fig 5C).**

We also compared transcriptional profiles ~~conversely,~~ by comparing individuals with DM-LTBI and those with diabetes only. Analysis of host transcripts between DM-LTBI and DM groups revealed 195 DEGs, with 150 upregulated and 45 downregulated genes (**S Table 2**). Upregulated genes in DM-LTBI were mostly related to innate immunity pathways and 38 of them overlapped with those raised in comparison with LTBI. However, the fold change of DEGs between DM-LTBI and DM (using the ~~our~~ cut-off used) was insufficient to give results in GO pathway analyses.

Gender and age-based analysis in those with diabetes

We investigated the effect of gender and age on host transcriptional profiles of study subjects with DM (DM-LTBI and DM). There were 11 males and 11 females, respectively. Analysis revealed 58 DEGs with 20 upregulated~~Up-~~ genes. GSE biological pathway analysis revealed the ~~one~~ suppressed pathway related to macrometabolic processes (**S Fig 3**). Presence of sex-specific signatures were present particularly related to the Y-chromosome identifying DDX3Y (DEAD box protein crucial for RNA metabolism and translation), EIF1A4 (eukaryotic translation initiation factor), RPS4Y1 (encodes ribosomal protein S4 crucial for protein synthesis), USP9Y (involved in protein deubiquitination and ~~is~~ responsible for male fertility), UTY (encodes protein with TPR motifs involved in protein-protein interaction), and PRKY (pseudogene). Genes associated with the contractile ring and intraflagellar transport were observed such as ACTN4, MYH9, MSN (encodes moesin protein critical for immune synapse formation), and IFT52 (cilial biosynthesis and maintenance). Transcription regulators like HIPK1 (phosphorylation of homeodomain transcription factors), MED15 (encodes subunit of a mediating complex that regulates transcriptional signaling), NCOA6 (multifunctional coactivator), RTF1

(encodes a component of PAF1 complex regulating transcription elongation), and ZMAT2 (pre-mRNA splicing). CX3CR1 a sex-specific gene was found to be downregulated. Biological pathway analysis identified athe hemoglobin signal (haptoglobin-hemoglobin complex, hemoglobin alpha binding) and reflects the sex-based differences in erythroid transcript contribution (males and females have different erythroid biology).

Furthermore, as there is a significant difference in age of the DM cohort and(DM, DM-LTBI) cohorts, an analysis was required to check the impact of age on these responses. Wwe compared DEGs comparing those aged below 50 years with those aged 50 years and over (**S Fig 4**). This analysis showed 68 DEGs with 28 Upup-regulated genes. Due to the limited number of differentially expressed genes, enrichment analysis using ClusterProfiler could not be reliably performed. Therefore, we used g:Profiler (22) package in R, which is suitable for smaller gene lists, to conduct functional enrichment analysis. Enrichment pathway analysis performed using KEGG, reactome and WikiPathways revealed the dysregulation of Coronavirus disease, has-mir, axon guidance and cytoplasmic ribosomal protein pathways. The highest gene count corresponded to has-mir-590-3p which is a precursor miRNA (mir-590) and regulates genes associated with synaptic maturation. Followed by nervous system development and axon guidance displayed in the REACTOME category. The COVID-19 KEGG hit likely points towards pathways overlapping with innate immunity or other inflammatory genes.

Impact of hyperglycemia on the host and in those with LTBI

Although most study participants with diabetes were on treatment, only 41% of individuals had controlled blood sugar (C-DM). We first focused on the effect of H-DM comparing profiles with C-DM, irrespective of LTBI status. A PCA analysis showed separation between profiles of H-DM and C-DM groups, **(SFig 5A)** with 13 DEGs, as shown in the unsupervised hierarchical heatmap, **SFig 5B**. The 10 up-regulated genes included those associated with lymphocyte activation (*AKAP7*, *IL6ST*, *BANK1*, *FCER2*) and the three down-regulated genes were markers of innate responses (*HLA-DRB1*, *ST8S1A4*, *VNN2*).

We then investigated uncontrolled hyperglycemia in those with LTBI whereby a comparison of H-DM and C-DM participants revealed 333 DEGs with 321 (96%) Up- and 12 Down-regulated genes. A volcano plot depicts upregulated genes to include ribosomal proteins (*RPL5/7/21/34* and *RPS17*), small nucleolar RNAs (*SNORD21/66*) and those associated with cell growth and signaling (*CASP8AP2*, *ACOT13*, *NAT1*), **Fig 6A**. Gene enrichment and GO biological processes analysis depicts upregulation of pathways related to leucocyte migration, cell motility and differentiation, as well as catabolic processes in this cohort**.** We observed downregulation of ribosome biogenesis, RNA processing, ribonucleoprotein complex biosynthesis and peptide processing, **Fig 6B**.

We used investigated using an cell-deconvolution analysis to identify which cellular types were identified in the sample groups analysed. We also include the sample signal data for the same DM-LTBi-HDM and DM-LTBi-

CDM individuals (Table S6). These data were used via Gedit3 tool to deconvolute as per cell type analysis using different reference databases. We used Immunostates as well as LM22 Immune signature analysis and obtained partners distinct to the comparison of H-DM and C-DM subsets (S Tables 7-8). Immunostates analysis revealed upregulation of a neutrophil signature across all samples, consistent with a DM phenotype. Further, CD8-positive alpha-beta-T cells were present (**Fig 6C)**. LM22 revealed that tissue-wise expression profiles showed dysregulation in monocytes and T cell (CD8 and CD4-naive) fractions. Of note downregulation of M2 macrophages was evident in samples with H-DM (**Fig. 6D**).

Comparison of transcriptional data with published gene sets

To validate our LTBI group and analysis, we checked for RISK genes of active TB in DEGs associated with LTBI, S Table 2. Warasinkse et al. 3- gene, Zak et al. 4–gene, Penn-Nicholson et al. 6- gene and Mulenga et al. 11- gene signatures (**S Table 9**), (23-27), were all absent in these DEGs. We also compared LTBI associated DEGs with those reported in a UK- based study of IGRA+ individuals by Broderick et al. (28). We found four commonly downregulated genes; *FAM174A* (prognostic markers in cancer progression), *KIAA0226L* (involved in endocytic trafficking), *PECAM1* (leucocyte trafficking and inflammation) and *RASSF2* (tumor suppressor gene).

Next, we compared our DM associated DEGs with transcripts identified in diabetic individuals in the multi-country study by Eckold et al. (29), and found 42 DEGs common with our list (**S Table 10A)**. Enriched GO biological pathway analysis of these common DEGs demonstrated their role related to cellular signaling, protein secretion and platelet formation (**S Fig 5A**). Notably, these data (**S Fig 5B**), included the downregulated genes *SORL1*, *CACULI* earlier noted in **Fig 4**. We also compared H-DM DM-LTBI associated DEGs with the Eckold et al. study (29). Here 100 DEGs (12 up- and 88 d-own-regulated) were common between the data sets (**S Table 10B**). Further, GSE GO biological pathway analysis highlights the activation of protein biosynthesis and metabolic processes and gene expression in individuals with H-DM (**S Fig 6A**), with key ribosome associated proteins (RPS and RPL family). WikiPpathway analysis depicts the impact of diabetes on oxidative phosphorylation in the mitochondria (**S Fig 6B**).

Discussion

Control of hyperglycemia remains a challenge in resource limited settings where diagnosis of diabetes and access to treatment remains difficult. In high TB burden settings, there is a compounding effect of diabetes together with need to investigate its impact in the context of latent TB infections. We observed dysregulation of inflammatory markers in those with LTBI and in the presence of diabetes there was alteration of metabolic

pathways leading to disrupted glycemic control with downregulated immune-related genes. Our data highlights the impact of uncontrolled hyperglycemia with dysfunction of cellular pathways such as oxidative phosphorylation in the host with LTBI and DM.

Study participants were ~~age and~~ gender matched however, those with diabetes were comparatively older, consistent with the natural distribution of diabetes which is more prevalent in older individuals (30). Neutrophil counts were significantly higher in those with diabetes (DM and DM-LTBI), corroborating previous reports of increased neutrophil levels in diabetic subjects (29).

In the study cohort, individuals with diabetes were receiving routine medications prescribed by the endocrinologist including insulin or metformin alone, with each other or in combination with other antihyperglycemic agents. These drugs such as metformin might have a modulatory impact on immune and metabolic pathways. The relatively small sample size and variation in the diabetic medications used did not allow for multivariate analysis based on antidiabetic treatment.

Studies of blood transcriptional profiles revealed that in LTBI, there was upregulation of *USP17*, which is associated with inflammation and endolysosomal trafficking (31), and a~~. A~~lso of, *YEATS,* associated with activation of TAK1/NF-kappaB pathway shown to influence cellular processes in TB infections (32). Additionally, inflammatory markers and host antigen processing pathways were downregulated. Notably, increased *CHI3L1* and *PI3* expressions are associated with disease severity in patients with TB. *CHI3L1* recruits immune cells, influence cell apoptosis and proliferation signals, in response to microbial infection (33). Similar to LTBI associated DEGs reported by Broderick et al. (28), we found downregulation of genes associated with inflammatory and cell recruitment processes, which might be necessary to maintain the latency of infection.

In those with diabetes we observed upregulation of inflammatory genes, changes in the metabolic pathways as well as increased host protein synthesis, with downregulation of innate immunity related genes **(Fig 4)**. Decreased NK cell activity is also associated with impaired metabolic pathways and increasing glucose concentrations and greater infection risk (34). Genes found to be commonly downregulated in LTBI/EC and DM/EC group comparisons **(Fig 2)** were related to immune response pathways as well as others related to gene regulation (*SNRPN* and *RNASET2*) and metabolic pathways (*TALDO1*, *SLC2A3*). Increased *ADAM29* is associated with DM-associated pathology. Downregulation of *SORL1* and *ADGRE2*, which affect insulin signaling and adipogenesis (35, 36), and of *CACUL1,* ~~is~~ a cell stress response gene (37), highlights the cellular and metabolic dysfunction in diabetic individuals.

The upregulation of *HLA-DRB5* points to an enhanced immune defect in DM-LTBI given that this marker is associated with progression to TB. Similarly, *S100A8* induces proinflammatory cytokines and chemokines and is increased in active TB (38). Our findings fit with literature showing that individuals with diabetes~~ies~~ with latent TB infection have decreased frequencies of γδ T cells, Type I and Type 17 cytokines, and CD8 T cell

markers (perforin, granzyme B, granulysin) (39). The shift in inflammatory markers is supported by reports that people with LTBI and DM display a shift in frequencies of classical monocytes (M1) to non-classical monocytes (M2) (40). In DM-LTBI, upregulation of *HEMGN* and *GYPA* indicates dysregulation of hematopoietic function in this group, whilst upregulation of *BLVRB* involved with intermediary metabolism and insulin signaling supporting dysregulation of glucogenesis associated with diabetes mellitus. Whilst downregulation of *ND6* and *PSMC3* alterations in mitochondrial function and oxidative metabolism **(Fig 5)**.

BLVRB reduces biliverdin to bilirubin in a NADPH-dependent mechanism in a final step in heme metabolism. In the case of BLVRB deficiency there is a change in cellular pathophysiology with an increase in oxidative stress and a shift in macrophages to the M2 phenotype (41, 42). Hence, the observed increase in BLVRB in DM-LTBI suggests that there may be a shift to an alternate macrophage phenotype in this cohort. The observation of increase in ribosomal protein expression also fits with the hypothesis of increased cellular stress in this group.

It was important to conduct a sex-based analysis to see if there was an effect within the diabetic cohort studied. We found differential regulation of genes associated with male-sex such as those on the Y chromosome and male sexual function. Further, the hemoglobin pathway was found upregulated whichith fits with increased erythroid cells in males. CX3CR1 a fractile chemokine was found to be downregulated in males.

In comparison of older and younger than 50 years age groups, we found cytoplasmic ribosomal pathways associated with our age-related analysis. As protein synthesis is associated with effective cellular responses this fits with the increased in the COVID-19 KEGG which is associated with innate immunity and inflammatory genes. These may suggest increased innate immune activation in the younger age group.

Sex-specific biological traits along with environmental factors also modulate an individual's susceptibility to obesity and type 2 diabetes (43). IHowever, in summary our data showed that gender-based differences exist amongst those with diabetes but these did not overlap with the ind inflammatory signature shift observed in the analysis of DM-LTBI and DM groups.

Chronic hyperglycemia associated with diabetes results in increased inflammation and disease associated pathophysiology (44). The association of lymphocyte activation in H-DM (*AKAP7* and *IL-6*) fits with worse outcomes in diabetes (45, 46). Conversely, downregulation of *FCER2* fits with lowered immunity and failure to kill microbial organisms (47).

In particular, the upregulation of innate immune genes *IL-1R2*, *TLR1*, *TLR4*, *NLRP3* and *MMP9* in H-DM with LTBI denotes affected inflammation and oxidative stress in this group (**Fig 6**). This may further induce the secretion of other cytokines and interfere with beta cell function (48, 49). *MMP9* has also been shown to be associated with dissemination of granulomas (50). We observed upregulation of *MMP9* in DM-LTBI as

compared with DM. Previous studies have shown increased expression of *MMP9* in monocytes/macrophages (51). *MMP-9* degrade type IV collagen, fibronectin and elastin in the lung allowing migration of immune cells at the site of infection. Altered MMP9 in the DM-LTBI group suggests increased inflammation and extracellular matrix remodelling-. Increased expression of TLR-pathway associated genes (TLR1, 4 and 8) were observed in DM-LTBI group. Elevated expression of TLR have been documented in both TB and diabetes showing increased pathogen recognition as well as a marker for chronic inflammation (52). Altered level of TLRs are also shown to be associated with TB resistance and more severe TB outcomes. Higher TLR4 expression in TB with DM have shown increased expression of NFKb resulting in persistent inflammation explaining increased pathology in such cases (53).

Conversely, we found NAT downregulated in the DM-LTBI cohort and this could contribute to worsening outcomes as *NAT1* is known to contribute to insulin sensitivity in diabetics (54, 55). NAT1 is included as a prognostic marker for breast cancer and in patients receiving chemotherapy low NAT1 expression has been associated with a significant decrease in 5 year survival (56). The effect of *NAT1* on drug-metabolism results in its association with oxidative phosphorylation in related genes (e.g. NDUFB6). Therefore, NAT1 levels can also play a role in progression of MTB infections, especially in the presence of diabetes where cellular stress levels are increased.

Importantly, deconvoluted cell-specific analysis using reference datasets revealed that in the Immunostates generated heatmap there was a neutrophil-driven inflammatory signature in individuals of the H-DM subsets. This fits with the impact shown of neutrophils function in type 2 diabetes and increase in RAGE products (57). Similarly, cell specific analysis using the leucocyte matrix 22 immune-cell signature revealed an M2 macrophage response highlighted an immunoregulatory phenotype characterized by levels of IL-10 and TGF-β and reduced expression of IL-12 (58). As an M2 phenotype is less capable of maintaining effective containment of latent MTB, the LM22 deconvolution supports ourare findings that diabetes particularly in the H-DM group triggers a shift towards impaired macrophage function.

Our results describing the effect of diabetes on those with LTBI including downregulation of innate immune and cancer markers, were supported through identification of common gene sets identified in TANDEM study cohorts from South Africa and Romania (29). Additionally, in our H-DM DM-LTBI associated DEGs we found common genes associated with dysregulated protein biosynthetic, metabolic and oxidative phosphorylation pathways.

Our data identify key pathways which may be used as targets for host-directed therapies in the diabetic host with LTBI. Host directed therapies for tuberculosis can add value to anti-tuberculous treatment regimens. Inhibiting *MMP* activity has shown to enhance frontline TB drug delivery and/or retention in the infected tissues through improving blood vessel integrity (59). *TLR* modulators can play a significant role in re-defining

immune responses to control chronic inflammatory responses. Upregulation of vitamin D receptor following ligation of TLRs induces antimicrobial peptides such as cathelicidins and defensins (60). TLR modulators can play a significant role in re-defining immune responses to control chronic inflammatory responses. Hence v~~V~~itamin D can be used as a therapeutic marker which may enhance the immune responses and might be helpful in favourable disease outcome.

It is a limitation that this was a cross-sectional study and we did not follow the individuals to record if any progressed to active TB or, could not measure clinical outcomes. It is also a limitation that we were unable to conduct any protein validation studies such as through Western blots, or use flow cytometric analysis to characterize cell populations. Additional limitations include the absence of full treatment protocols of the individuals in the diabetes group. Previous studies have shown synergistic effects of metformin and rifampicin in reducing intracellular growth of Mtb~~*M. tuberculosis*~~. Retrospective analysis of clinical trial data demonstrated that patients with TB and DM show better clinical outcomes with fewer lung cavities and decreased proportion of individuals progressing to advanced disease post treatment with metformin (61). As a result, metformin is being promoted as a candidate for therapeutic prevention and adjunctive treatment approaches in TB (62). However, as these medications are not standardized across the treatment cohort and due to their heterogenous use we could not determine their specific effects on host transcriptomic changes. In addition, we also did not have chest X-ray information on our study participants, rule out any granulomas within the LTBI cohort. The group sizes were too small to enable the DM group participants to be studied by their diabetes treatment regimen.

In summary, we observed dysregulated immunity in individuals with DM-LTBI which is compounded by uncontrolled hyperglycemia. Such immune modulation is likely to increase susceptibility of the host to TB but would extend to a broad range of pathogens. Of note, individuals with LTBI are not usually given prophylaxis in Pakistan due to limited availability of treatment provided by the National TB Program, Pakistan for high-risk individuals such as, patients living with HIV, household contacts of smear positive TB patients and those initiating biologics treatment using anti-inflammatory drugs.

In conclusion, our data highlights the risks of ineffective diabetes management in a high TB burden setting, where there is a high likelihood of LTBI amongst the population. Our data reinforces the importance of bi-directional screening for LTBI and DM followed by effective diabetes management to reduce risk of progression to active TB.

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Authors contributions

ZH, designed the study; KIM, MY, JA, AA: implementation and execution; MY, JA: data analysis; NR, QM, NA, MI, BJ: recruitment of patients; KIM, ZH: drafted the initial manuscript; KIM, ZH, RH, HMD, MR, JMC: manuscript revision with critical input. All authors reviewed and approved the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding this work.

Data availability statement

All datasets generated and/or analysed during the current study are included in this published article and its supplementary information file.

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Figure Legends

Figure 1. IFN-[redacted] induced by MTB antigen stimulation of whole blood higher in IGRA positive than IGRA negative individuals. Whole blood cells from study subjects in each group endemic controls (EC, n=22), those with latent TB (LTBI, n=29), diabetes (DM) and with diabetics and LTBI (DM-LTBI, n=19) were stimulated using the QuantiFERON assay. IFN-[redacted] levels shown are calculated after values of nil were subtracted from Mtb Ag1 and Ag2 stubes respectively. The Kruskall-Wallis (KW) test was conducted to compare the results across the four groups. The Mann-Whitney U (MWU) test was used to compare responses between two groups. $p \leq 0.05$ were considered significantly different.

Figure 2. Investigating transcriptional differences between healthy individuals, those with diabetes and with LTBI. Whole blood RNA transcripts from study participants in EC (n=128), LTBI (n=16), DM (n=12) and DM-LTBI (n=10) groups were analysed. A, Principal component analysis was used to visualize the clustering of samples in each group seen as EC (blue), LTBI (red), DM (purple) and LTBI-DM (green). B, A barplot depicts the number of differentially expressed genes (DEGs) between groups as Up-regulated (orange), Down-regulated (blue) genes respectively. DEGs were filtered at based on an absolute fold change (FC) of ≥ 2 or ≤ -2 and $p < 0.05$. C, The Venn diagram compared 359 DEGs between comparisons of LTBI (187), DM (182) and DM-LTBI (12) with EC groups. D, Twenty-two common DEGs found between LTBI and EC, as compared with DM and EC groups are depicted.

Figure 3. Reduced innate immunity related genes in LTBI as compared with controls. The figure depicts comparison between transcriptomes of participants of LTBI (n=16) and EC (n=12) groups whereby 187 DEGs, 4 Up and 183 Down-DEGs were observed. A, Volcano plot analysis depicts the DEGs with showing Up-genes are shown in red and Down-genes in green. DEGs identified, based on a FC of ≥ 2 or ≤ -2 and $p < 0.05$. B, Dotplot of GSE-GO biological processes and molecular functions show pathways associated with the DEGs. First two are activated and last two entries in the dotplot are downregulated. C, Heatmap of GSE-GO biological processes and molecular functions. ~~(~~B~~)~~, showing genes involved in the important processes.

Figure 4. Reduced innate immunity related genes in DM as compared with controls. The figure depicts comparison between transcriptomes of participants of DM (n=12) and EC (n=12) groups whereby 25 Up and 157 Down-DEGs were observed. A, Volcano plot analysis depicts the DEGs with showing Up-genes are shown in red and Down-genes in green. DEGs identified, based on a FC of ≥ 2 or ≤ -2 and $p < 0.05$. The grey area of the volcano plot represents genes that were not differentially expressed between groups. The inset zoom shows further details of the DEGs. B, Dotplot of GSE Reactome showing suppression of neutrophil degradation and immune response. C, heat map of GSE-Reactome highlighting genes involved in the pathways.

Figure 5. Dysregulated immune and metabolic pathways in those with diabetes and LTBI. The DEGs were compared between DM-LTBI and LTBI groups. Significant differential expressions are represented by a FC of

≥ 2 or ≤ -2 and p <0.05. A, Shows volcano plot analysis of the DEGs. B, Depicts GSE Reactome. C. DEGS were analysed through the heatmap of GSE Reactome pathways.

Figure 6. Analysis of DEGs in the diabetic cohort based on LTBi infection and also hyperglycemic levels. The analysis shows comparison between individuals who have DM-LTBI in the the uncontrolled (H-DM, n=4) as compared with controlled (C-DM, n=6) groups. A, The DEGs labelled in volcano plot showing significant differential expression using a threshold of an absolute fold change ≥2 and p value < 0.05. B, Dotplot showing list of activated and suppressed biological processes enriched using GSE GO ontology, highlighted on the basis of p-adjusted value. C, Cell-type proportion estimated heatmap generated using the ImmunoStates reference matrix applied on the normalized data of the same cohort, each row corresponds to a specific immune cell, and each column is our study sample. Color intensity and numeric values indicate the estimated relative proportion of each immune cell type in each sample. Hierarchical clustering groups samples and cell types based on similarity in immune composition. D, Heatmap of immune cells generated using Leukocyte Matrix 22 (LM22) database as a reference.