

Distinct blood transcriptomic signature of treatment in latent tuberculosis infected individuals at risk of developing active disease

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ABSTRACT

Although only a small fraction will ever develop the active form of tuberculosis (ATB) disease, chemoprophylaxis treatment in latent TB infected (LTBI) individuals is an effective strategy to control pathogen transmission. Characterizing immune responses in LTBI upon chemoprophylactic treatment is important to facilitate treatment monitoring, and thus improve TB control strategies. Here, we studied changes in the blood transcriptome in a cohort of 42 LTBI and 8 ATB participants who received anti-TB therapy. Based on the expression of previously published gene signatures of progression to ATB, we stratified the LTBI cohort in two groups and examined if individuals deemed to be at elevated risk of developing ATB before treatment (LTBI-Risk) differed from others (LTBI-Other). We found that LTBI-Risk and LTBI-Other groups were associated with two distinct transcriptomic treatment signatures, with the LTBI-Risk signature resembling that of treated ATB patients. Notably, overlapping genes between LTBI-Risk and ATB treatment signatures were associated with risk of progression to ATB and interferon (IFN) signaling, and were selectively downregulated upon treatment in the LTBI-Risk but not the LTBI-Other group. Our results suggest that transcriptomic reprogramming following treatment of LTBI is heterogeneous and can be used to distinguish LTBI-Risk individuals from the LTBI cohort at large.

1. Introduction

Tuberculosis (TB), caused by the pathogen *Mycobacterium tuberculosis* (*Mtb*), is one of the leading cause of death by a single infectious agent, killing approximately 1.4 million individuals each year [1]. The historic binary classification of patients into those with 'latent TB infection' (LTBI) and 'active TB disease' (ATB), the latter associated with clinical symptoms and morbidity, does not capture the biological

heterogeneity and overlap between infection states [2,3]. Indeed, the 'clinical state' of LTBI is heterogeneous, and includes individuals who may have eliminated the pathogen, as well as individuals with incipient or subclinical active disease [4].

A key target of TB control strategies is the prevention of *Mtb* (re-) activation in individuals with LTBI using chemoprophylactic treatment. It is estimated that approximately a quarter of the world's population had LTBI [5], representing a considerable reservoir for active disease

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development and therefore transmission. The estimated lifetime risk of individuals with LTBI progressing to ATB is 12% [6], but varies widely depending on several factors (e.g. age and immunosuppression). Thus, although not all LTBI individuals will benefit from therapy, this approach represents an effective way to eliminate *Mtb* in this population and reduce the possibility of transmission to others. Treatment of LTBI typically consists of either three months of daily rifampicin and isoniazid combination therapy or six months of daily isoniazid monotherapy [7,8]. These treatments can have severe side effects, such as hepatotoxicity which can be fatal. More recently, shorter and lighter regimens such as isoniazid and rifapentine weekly for three months, or daily for one month have proven as efficacious, with higher treatment completion and lower adverse reaction rates, when compared to six or nine months of daily isoniazid [9–12]. There is no “gold standard” test for LTBI which is currently diagnosed by either a tuberculin skin test or by measuring IFN γ release in response to *Mtb*-derived T cell antigens in an Interferon Gamma Release Assay (IGRA). Since these tests only test *Mtb* immune sensitization, rather than ongoing infection, they correlate poorly with progression to active disease [13] and with chemoprophylactic treatment success [14,15]. Monitoring immune responses to TB preventive therapy might therefore be beneficial to patients, not only to potentially shorten therapy (and reduce the risk of drug-related adverse events) but also to confirm treatment success, and more globally to better understand LTBI heterogeneity and identify the individuals who derive the most benefit from treatment. Even if shorter and simpler regimens are safer, it is logistically and economically not possible to treat the entire LTBI population (especially in high endemic areas where the risk of re-infection is high).

Whole blood transcriptomics is increasingly used in the field of TB research to identify both diagnostic and mechanistic immune signatures of infection and disease, highlighting the heterogeneous spectrum of TB [16]. Whole blood gene signatures have recently shown potential to predict ATB progression in LTBI [17–22]. With respect to anti-TB therapy, blood transcriptomic changes upon treatment in ATB patients have also been used to monitor treatment efficacy, and identify the individuals at risk of relapse [21,23–27]. More recently, one study has investigated transcriptomic changes in whole blood in response to treatment among individuals with LTBI following *Mtb*-specific *in vitro* stimulation [28]. Authors identified a dichotomous transcriptomic profile following *in vitro* stimulation across all participants, and hypothesized it might distinguish between LTBI individuals with viable *Mtb* bacilli versus those who have controlled or eliminated the bacteria.

In this study, we monitored direct *ex vivo* blood transcriptomic changes following treatment in a cohort of 42 individuals with LTBI. We hypothesized that individuals with LTBI at risk of developing ATB are immunologically more similar to those with ATB compared to the majority of non-progressing LTBI individuals, and will thus display blood transcriptomic changes following treatment that are similar to those in treated ATB individuals. Establishing such immune heterogeneity in treatment signatures might enable us to better understand the spectrum of LTBI and facilitate chemoprophylactic TB treatment monitoring.

2. Material and methods

2.1. Ethics, consent and permissions

Participants recruited for this study were enrolled under ethical approval granted by the National Research Ethics Service, Heart of England NHS Foundation Trust (2012107RM), Committee South Central - Oxford C (12/SC/0299) and the Ministry of Defence Research Ethics Committee (MoDREC 237/PPE/11), respectively. All samples were obtained for specific use in this study. All clinical investigations were conducted according to the principles of the Declaration of Helsinki, and all participants provided written informed consent prior to participation.

2.2. Study design

This is a longitudinal study of individuals with LTBI or ATB receiving anti-TB treatment, with blood samples collected before and after therapy. Participants were enrolled at one of three clinical sites in Birmingham, Oxford or Catterick, UK. Enrollment of all participant groups was done simultaneously at the three clinical sites. ATB were recruited as symptomatic individuals seeking care at the clinical sites. LTBI were recruited as IGRA positive seeking care at the clinical sites, but were not household contact of ATB participants. For the LTBI group, participants with prior or recent history of ATB were excluded. ATB was defined as either a suspected (based on clinical suspicion and radiological and/or histological evidence) or microbiologically confirmed (by *Mtb* culture) new diagnosis of pulmonary or extrapulmonary TB disease, in the absence of any other significant co-morbidity. LTBI status was confirmed in participants with a positive IGRA (QuantiFERON-TB Gold In-Tube, Cellestis, or T-SPOT.TB, Oxford Immunotec) and the absence of clinical and radiographic signs of ATB or any other significant co-morbidity. Uninfected control participants (TBneg cohort) were recruited as healthy controls at the same three clinical sites in whom ATB and LTBI were excluded and had no past medical history of ATB or evidence of previous exposure to *Mtb*, as confirmed by a negative IGRA test. All participants were HIV-negative adults (≥ 18 years). Anti-TB treatment was based on UK standard regimens of combination drug therapy [29]. For the LTBI cohort, treatment was 3 months of daily rifampicin and isoniazid. For the ATB cohort, treatment consisted of 2 months of daily rifampicin, isoniazid, pyrazinamide, and ethambutol, followed by 4 months of daily rifampicin and isoniazid. All ATB participants successfully completed the 6 months treatment course, based on resolution of clinical symptoms and sputum conversion to negativity (where applicable). There was no occurrence of relapse or recurrence. Because all three clinical sites have a low *Mtb* incidence setting, it was presumed that the risk of re-infection was negligible for both LTBI and ATB participants. For the LTBI cohort, the post-treatment sample was obtained 4–10 months after the pre-treatment sample, corresponding to at least one month and up to 7 months after completion of treatment. For the ATB cohort, the post-treatment sample was obtained 7–13 months after the pre-treatment sample, corresponding to at least one month and up to 7 months after completion of treatment. For all samples, 2.5 mL of blood was collected directly into a PAXgene® tube (BD Biosciences), mixed, and stored at -80°C until processing.

2.3. Full blood count assay

Full blood count (FBC) was performed on whole blood in UKAS-accredited clinical laboratories using standard procedures. Each feature is expressed in its standard reference unit, and values were compared between TB cohorts using the non-parametric Mann-Whitney test.

2.4. Microarray analysis

Total intracellular RNA was extracted from PAXgene® samples using the Blood RNA Kit (Qiagen) according to the manufacturer's instructions. Globin mRNA was depleted from total RNA extractions using a biotin/streptavidin/magnetic bead-based assay, according to the manufacturer's instructions (GLOBINclear Kit, Ambion). The depleted RNA was then amplified and biotin-labeled using the TotalPrep RNA Amplification Kit (Illumina), followed by hybridization onto Illumina HumanHT-12 (v4.0) expression beadchips according to the manufacturer's instructions. Arrays were scanned with an Illumina bead array reader confocal scanner. The idat Illumina HumanHT12-V4 microarray files were converted to raw expression data using the Bioconductor package beadarray v2.38 [30] in R. The raw data was quantile normalized and log₂ transformed using the Bioconductor package lumi v2.40 [31] in R. A comprehensive annotation was performed using

annotations provided by biomaRt v2.40.5, the Gene Expression Omnibus (GEO), and platform specific annotation obtained from Illumina. If there was not a consensus among annotations, biomaRt was given preference given its regular updates, followed by GEO, and lastly the platform specific annotation. For non-specific filtering median, coefficient of variation and inter-quartile range for all probes across all samples were calculated. The probes accounting for the bottom 50% in each measurement were overlapped, and those probes that fell in the bottom 50% of all measurements ($n = 20,147$) were removed from further analysis, leaving a final set of 27,960 probes of the initial 48,107 probes. Microarray data has been deposited to NCBI's Gene Expression Omnibus (GEO) database repository and is available under GEO accession number **GSE152532**.

2.5. Bioinformatic analysis

Batch effect was removed using the Combat function from the Bioconductor package *sva* v3.36 [32] in R (Fig. S1). Differential expression analysis was performed on combat normalized data using the Bioconductor package *limma* v3.44.1 [33] in R and probes as entities, and the Benjamini and Hochberg's method for p-value adjustment. Differentially expressed probes were then converted to corresponding genes for biological pathways enrichment and protein-protein interaction analyses, and for defining signature overlaps between cohorts. When differentially expressed probes mapped to the same gene, it was ensured that directionality was similar. If no known gene was associated to the probe, then the entity was ignored. Gene signature scores were calculated using a standard z-score formula, by summing the normalized expression values for all upregulated genes (or probes if a gene was mapped to several probes) and subtracting the sum of the normalized expression of all downregulated genes (or probes if a gene was mapped to several probes) present in the signature, and correcting for the average and standard deviation of the sums across all samples, as well as the number of samples. Heatmaps and PCA components were extracted from normalized expression values using the bioinformatic software analysis *Qlucore omics explorer*. Expression of gene signatures in immune blood cell types were calculated with the *CellTypeScore* tool in DICE database (<http://dice-database.org/>). The DICE database contains gene expression data generated from 13 immune cell types (and 2 activation conditions) isolated from peripheral blood mononuclear cells of 91 healthy individuals [34]. For a given gene list, the *CellTypeScore* tool sum the normalized expression values of each individual gene constituting the list for each of the cell types/stimulation conditions. Pathway analysis was performed with the online platform *enrichR* [35, 36] and the *BioPlanet* resource database [37]. Functional protein-protein association networks were defined by querying the online STRING database (version 11.0) using a minimum required interaction score of 0.4 (medium confidence). Statistical significance of overlap between treatment signatures was calculated using the hypergeometric distribution test and considering all 27,960 probes that passed the initial filtering as the total number of probes. All R scripts used to analyze the data have been deposited to GitHub and are publicly available (https://github.com/akulsinghania/burel_et_al_LTBI_heterogeneity).

3. Results

We enrolled a total of 69 LTBI and 17 ATB participants into the study. Blood samples were obtained before treatment for all participants (LTBI_{pre} and ATB_{pre} cohorts), and post anti-TB therapy for 42/69 LTBI and 8/17 ATB participants (LTBI_{post} and ATB_{post} cohorts). A blood sample was also obtained from 11 Mtb uninfected individuals (TBneg) that were recruited at the same sites. Participant demographics metadata are available in Table S1 and summarized for each cohort in Table S2. The TBneg cohort was age-matched to the LTBI cohort (mean age 27), whereas the ATB cohort was older (mean age 34) (Table S2). In

terms of sex, the TBneg cohort was about equally split between female and male (55% female), whereas both LTBI and ATB cohorts had a lower percent of female (38% and 18% female, respectively) (Table S2). The TBneg cohort was predominantly composed of individuals of European ethnicity (55%) compared to LTBI (10%) or ATB (12%) (Table S2). For the ATB cohort, about a third (29%) of participants had microbiologically confirmed presence of Mtb (Table S2). For each blood sample, RNA content was assessed for gene expression using microarrays. Full blood counts were also obtained from blood samples collected prior to treatment for 7/11 TBneg, 15/17 ATB and 64/69 LTBI participants.

3.1. Stratification of the LTBI cohort based on previously published gene signatures of risk of progression to ATB

To identify individuals within the LTBI cohort that were potentially at increased risk of progression to active disease, we stratified the cohort based on their transcriptomic status pre-treatment using 'risk of progression to ATB' gene signatures previously identified [17–21]. The seminal study by Zak et al. reported a 16-gene signature (termed Zak16 henceforth) that predicted progression from LTBI to ATB up to one year before reactivation [17]. We assessed the expression of the Zak16 signature pre-treatment in the three cohorts: LTBI_{pre}, ATB_{pre} and TBneg. As expected, the Zak16 signature could discriminate between ATB_{pre} and LTBI_{pre} (AUC of 0.86, $p < 0.0001$), as well as ATB_{pre} and TBneg (AUC of 0.87, $p = 0.001$), but not between LTBI_{pre} and TBneg (AUC of 0.56, $p = 0.51$) (Fig. 1A). For each sample, we calculated a Zak16 gene signature z-score by summing the normalized expression values of all probes mapped to each gene present in the Zak16 signature using a z-score standard formula. The ATB_{pre} cohort had an increased Zak16 z-score compared to LTBI_{pre} or TBneg participants, and the LTBI_{pre} cohort showed the highest inter-individual variability (Fig. 1B). Based on the maximum Zak16 z-score value in the TBneg cohort, the LTBI_{pre} cohort was sub-divided into 2 groups: a LTBI-Risk group ($n = 10$, 14%), comprising LTBI participants with a score expression higher than the maximum value of the TBneg cohort; and a LTBI-Other group ($n = 59$, 86%), encompassing the remaining LTBI participants (Fig. 1B). The level of expression of all genes included in the Zak16 signature was highly similar within a given LTBI individual (Fig. 1C), indicating that all genes contributed to the calculated z-scores. Since Zak16 is not the only published TB progression signature, we also looked for expression of Sweeney3 [18,19], BATF2 [20] and RISK6 [21] signatures to ensure reproducibility. All three signatures included at least one gene overlapping with the Zak16 signature (Fig. S2A), and performed equally well at discriminating ATB_{pre} from LTBI_{pre} (AUC of 0.92, $p < 0.0001$ for Sweeney3; AUC of 0.83, $p < 0.0001$ for BATF2; AUC of 0.80, $p = 0.0002$ for RISK6) or ATB_{pre} from TBneg (AUC of 0.90, $p = 0.0003$ for Sweeney3; AUC of 0.84, $p = 0.003$ for BATF2; AUC of 0.87, $p = 0.001$ for RISK6) (Fig. S2B). Expression of the Zak16 signature was also highly correlated with all three signatures ($p < 0.0001$ and spearman correlation coefficient r of 0.85, 0.91 and 0.68 for Sweeney3, BATF2, and RISK6, respectively) (Fig. S2C). All 10 individuals identified as LTBI-Risk according to the Zak16 signature showed an increased z-score compared to the LTBI-Other group (Fig. S2D) and were also ranked in the top 10 highest z-score for at least two of the three other signatures. Six of the 10 genes making up the three other signatures showed a high level of correlation of expression within each individual (BATF2; DUSP3 and GBP5 in Sweeney3; FCGR1B, GBP2 and SERPING1 in RISK6, Fig. S2E) including all overlapping genes with Zak16 (Fig. S2A). We applied the Zak16 signature classification for subsequent analyses as it is the only signature presenting an overlap with all three others, and thus the most representative of the literature.

Full blood counts from samples collected prior to treatment indicated significant differences between all four cohorts (Fig. 1D). ATB has been previously associated with increased monocyte counts and M/L ratio compared to LTBI [38,39]. We found that monocyte counts and monocyte to lymphocyte ratio (M/L ratio) were increased in ATB compared to

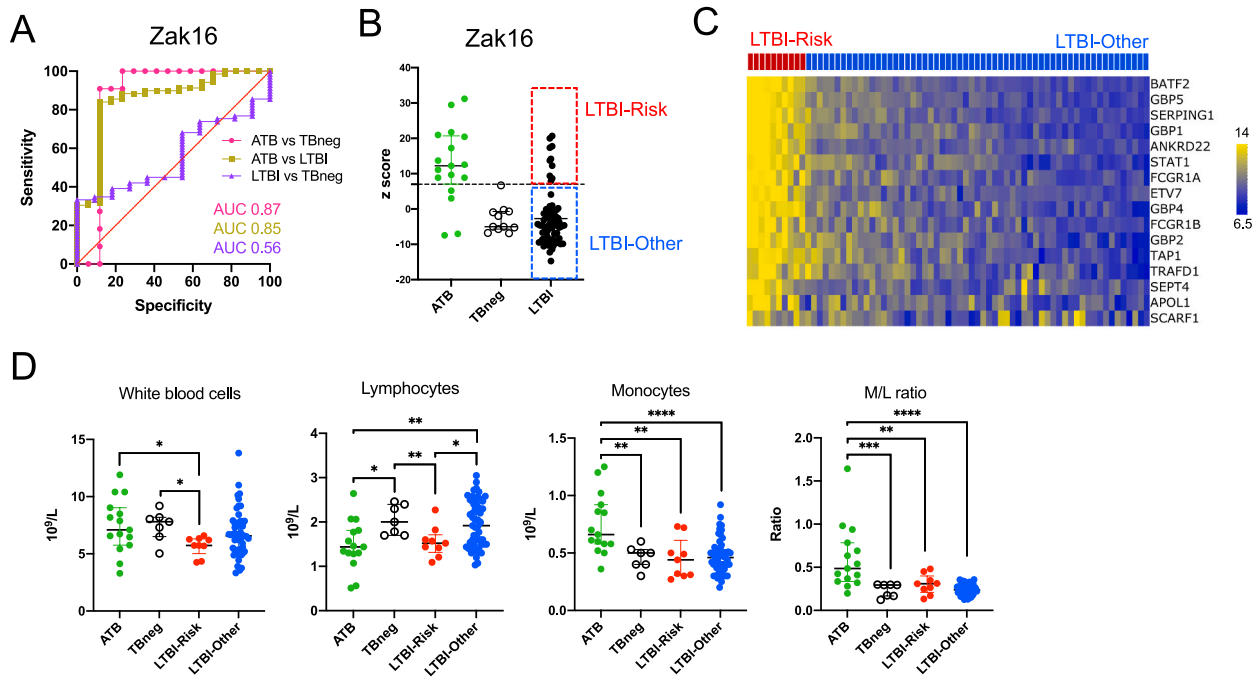


Fig. 1. Identification of LTBI participants at higher risk progression based on the expression of a previously published ‘risk of progression to ATB’ signature prior the start of treatment. A) Receiver operating characteristic (ROC) curves and B) individual z-scores for the Zak16 gene signature [17] in blood samples collected prior to treatment in ATB (n = 17), LTBI (n = 69) and TBneg (n = 11) individuals. The upper value in the TBneg cohort was used to stratify the LTBI cohort into LTBI-Risk (n = 10), and LTBI-Other (n = 59) groups. C) Heatmap representing the normalized expression of individuals genes of the Zak16 gene signature in blood samples collected prior to treatment in LTBI-Risk and LTBI-Other individuals. If a gene was mapped to several probes, average expression of all probes was shown. D) Differences in full blood counts parameters in blood samples collected prior to treatment in ATB (n = 15), TBneg (n = 7), LTBI-Risk (n = 9) and LTBI-Other (n = 55). Statistical differences between cohorts were defined with the non-parametric unpaired Mann-Whitney test (*p < 0.05; **p < 0.01; ***p < 0.001).

TBneg and LTBI cohorts and that lymphocyte counts were reduced in ATB compared to TBneg or LTBI-Other cohorts (Fig. 1D). Interestingly, the LTBI-Risk group also showed a reduction in lymphocyte counts, as well as total white blood cells counts compared to TBneg or LTBI-Other cohorts (Fig. 1D). Thus, in terms of white blood cell composition, it appears that LTBI-Risk participants share some similarities with the ATB cohort. No quantitative differences were observed for polymorphonuclear cells or platelets between any of the cohorts (Fig. S3).

3.2. Identification of an ATB treatment signature

In order to identify transcriptomic changes in the LTBI groups upon treatment and define its overlap with ATB, we first identified the treatment signature in our ATB cohort. Several studies have reported whole blood gene signatures of treatment in ATB [23,24,26]. To ensure that our experimental data and analysis strategy were consistent with previous findings, we set out to identify the treatment signature of the ATB cohort by comparing ATB_{post} and ATB_{pre} samples (ATB_Δ) and define its overlap to previously published signatures. Despite the low number of participants (n = 8, including 3 microbiologically confirmed cases), we found significant changes upon treatment with a total of 262 differentially expressed probes (which mapped to 238 unique genes) (adjusted p < 0.1, Fig. 2A and 2B, Table S3). The expression pattern of the ATB treatment gene signature was similar in ATB cases that were either confirmed (n = 3) or suspected (n = 5) for both upregulated and downregulated genes (Fig. 2A and C). No differences were observed for participants with pulmonary (n = 6) vs extra-pulmonary (n = 2) ATB (data not shown). Genes that were upregulated after treatment in ATB were associated with the highest cumulative expression level in NK cells (Fig. 2D) and were significantly enriched for one pathway (tyrosine kinase activity upon TCR activation, Fig. 2E). In contrast, genes that were downregulated after treatment in ATB were strongly associated with

classical and non-classical monocytes (Fig. 2F) and significantly enriched for several cytokine signaling pathways including IFN γ , IL-5, and IL-12 (Fig. 2G). We compared our ATB_Δ treatment signature (designated Burel238) to previously published host transcriptomic signatures following anti-TB therapy in ATB patients (Bloom267²³, Cliff62²⁴, and Thompson9 [26]) and found a significant overlap between our ATB_Δ treatment signature and these signatures (Fig. 2H, Table S4). This confirmed that our approach for defining an ATB-treatment signature identifies sets of genes that are comparable to those identified in previous studies.

3.3. Identification of LTBI-Risk and LTBI-Other treatment signatures

Next, we investigated the transcriptional changes associated with anti-TB therapy in the LTBI cohort by identifying differentially expressed genes between LTBI_{post} and LTBI_{pre} samples in both LTBI-Risk and LTBI-Other cohorts (LTBI-Risk_Δ and LTBI-Other_Δ treatment signatures) for which a paired blood sample was available (6/10 for LTBI-Risk and 39/59 LTBI-Other participants). There was only one differentially expressed probe when comparing post and pre-treatment samples in the LTBI-Risk group using the same cutoff that was used for the ATB cohort (adjusted p < 0.1, Table S5). The probe was mapped to the gene GBP6, which has been repeatedly associated with whole blood transcriptomic signatures of ATB [23,40,41]. In the LTBI-Other group, the same cutoff identified seven probes (which mapped to seven genes) as differentially expressed, all upregulated (Table S6). These results indicate that anti-TB therapy in LTBI leads to minor changes in the blood transcriptome compared to the ATB cohort.

Since our study aimed to identify global similarities in blood transcriptome changes upon treatment in LTBI and ATB, it sought to identify a higher number of genes that were dysregulated upon treatment in the LTBI groups. We therefore modified the statistical threshold to a non-

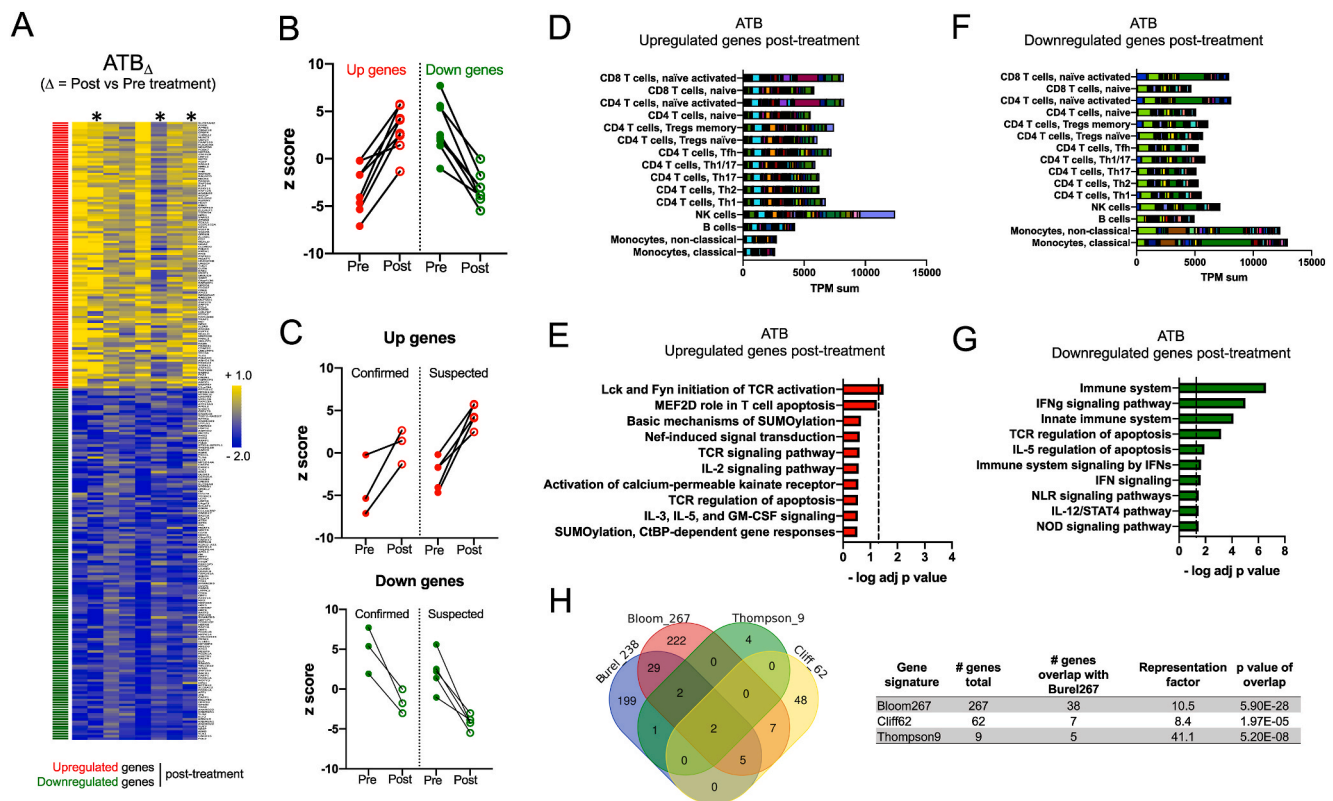


Fig. 2. Blood transcriptomic signature of treatment in the ATB cohort is associated with NK cells, monocytes and IFN signaling. A) Heatmap representing the log fold change expression upon treatment of the upregulated (red) and downregulated (green) gene-mapped probes in the ATB Δ signature derived from differential expression analysis (adjusted p value < 0.1) between post- and pre-treatment paired blood samples in the ATB cohort ($n = 8$) (Table S3). Asterisks show microbiologically confirmed cases. B) Individual z-scores pre and post treatment for the upregulated and downregulated genes in the ATB Δ signature in all ATB participants ($n = 8$) and C) microbiologically confirmed ($n = 3$) vs suspected ($n = 5$) ATB participants. D) Immune cell type-specific expression and E) top-10 biological pathways enriched in the upregulated genes of the ATB Δ signature. F) Immune cell type-specific expression and G) top-10 biological pathways enriched in the downregulated genes of the ATB Δ signature. (D,F) For immune cell type-specific expression, each bar consists of stacked sub-bars showing the TPM normalized expression of every gene in corresponding cell type, extracted from the DICE database [34] (<http://dice-database.org/>). (E,G) Biological pathways were ranked with increasing adjusted p value and dotted line represents significance threshold (adjusted p value < 0.05). H) Overlap between our newly identified ATB Δ treatment signature (Burel238) and previously reported signatures associated with anti-TB therapy [23,24,26]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

adjusted p value lower than 0.01 and an absolute log fold change higher than 0.2. This fold change threshold was selected to compensate for the lowest stringency of the non-adjusted p value in comparison to the adjusted p value, and eliminate half of the differentially expressed genes meeting the new criteria of non-adjusted $p < 0.01$. With this modified threshold, we identified 140 differentially expressed probes (which mapped to 125 genes) following treatment in the LTBI-Risk group (Fig. 3A, Tables S5) and 195 differentially expressed probes (which mapped to 185 genes) after treatment in the LTBI-Other group (Fig. 4A, Table S6).

The LTBI-Risk Δ signature contained mostly genes that were downregulated upon treatment (Fig. 3A). Despite a variation in magnitude, all participants showed a relatively homogeneous expression profile except for one participant who did not show any changes in expression upon treatment for the downregulated genes (Fig. 3B). Upregulated genes were associated with the highest cumulative expression level in classical and non-classical monocytes (Fig. 3C) and no statistically significant enrichment for biological pathways (Fig. 3D). Downregulated genes were associated with the highest cumulative expression level in activated T cells (Fig. 3E) and significant enrichment for IFN signaling pathway (Fig. 3F).

In contrast, the LTBI-Other Δ signature showed much higher heterogeneity across participants and the majority of genes were upregulated upon treatment (Fig. 4A and B). Interestingly, some participants showed an antagonistic pattern with downregulation of the expected

upregulated genes and vice-versa (Fig. 4A and B). Further analysis identified that age, but not sex, ethnicity, or time since completion of treatment was significantly positively correlated with the LTBI-Other Δ signature score (Fig. S4). This observation highlights the significant heterogeneity within the LTBI cohort and suggests the LTBI-Other group could be further divided into several subgroups, in particular age groups. Upregulated genes in the LTBI-Other Δ signature had the highest cumulative expression level in NK cells, followed by non-classical monocytes (Fig. 4C). Biological pathways significantly enriched in the upregulated genes upon treatment included platelet-associated pathways (response to elevated platelet cytosolic calcium; platelet activation, signaling and aggregation; platelet aggregation (plug formation)) and adhesion/migration pathways (focal adhesion; leukocyte trans-endothelial migration; integrin cell surface interactions) (Fig. 4D). Downregulated genes had the highest cumulative expression level in B cells (Fig. 4E) and significant enrichment for ribosomal gene associated pathways (top 8 pathways, Fig. 4F).

3.4. Significant overlap between LTBI-Risk and ATB treatment signatures

To investigate if treatment-induced transcriptional changes in LTBI-Risk or LTBI-Other cohort resembled those seen in ATB, we defined the overlap between the treatment signatures in all three cohorts: ATB Δ (Fig. 2A, Table S3), LTBI-Risk Δ (Fig. 3A, Table S5) and LTBI-Other Δ (Fig. 4A, Table S6). There were four genes shared between LTBI-Risk Δ

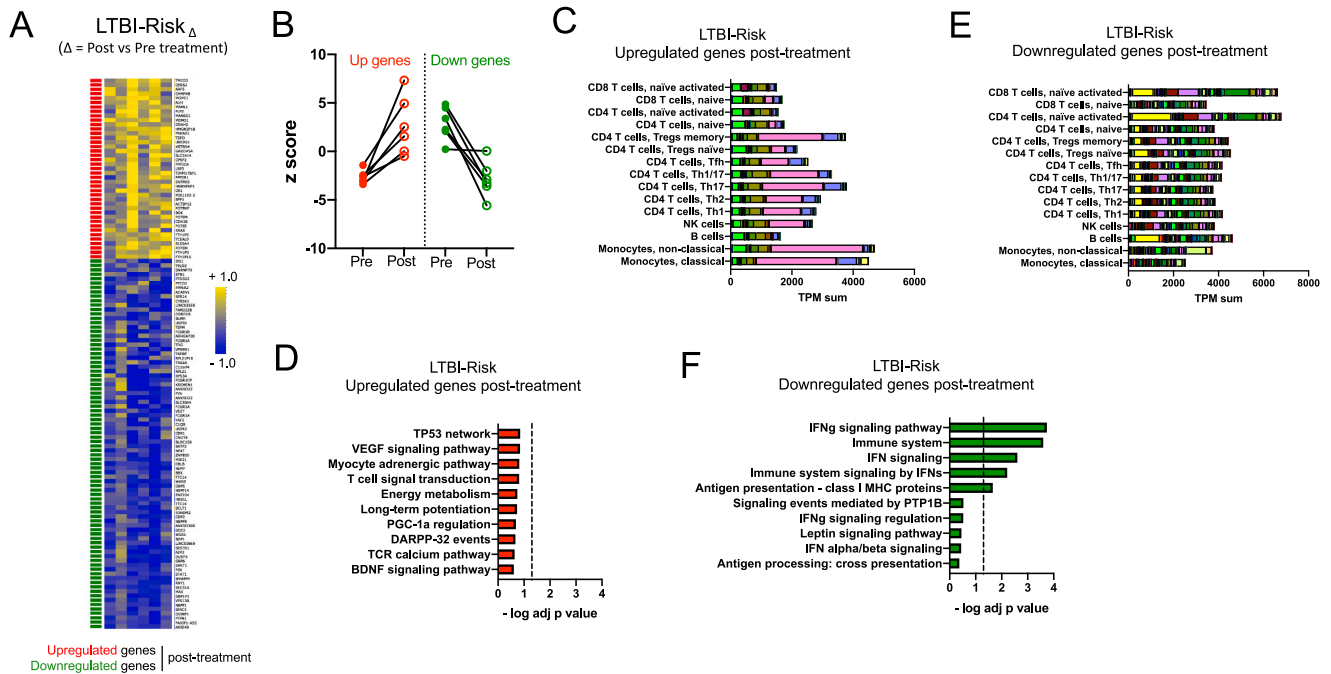


Fig. 3. Blood transcriptomic signature of treatment in the LTBI-Risk cohort is associated with activated T cells and IFN signaling. A) Heatmap representing the log fold change expression upon treatment and B) individual z-scores pre and post treatment for the upregulated (red) and downregulated (green) gene-mapped probes in the LTBI-Risk_Δ signature derived from differential expression analysis (non-adjusted *p* value < 0.01, absolute log fold change > 0.2) between post-and pre-treatment paired blood samples in the LTBI-Risk cohort (n = 6) (Table S5). C) Immune cell type-specific expression and D) top-10 biological pathways enriched in the upregulated genes of the LTBI-Risk_Δ signature. E) Immune cell type-specific expression and F) top-10 biological pathways enriched in the downregulated genes of the LTBI-Risk_Δ signature. (C,E) For immune cell type-specific expression, each bar consists of stacked sub-bars showing the TPM normalized expression of every gene in corresponding cell type, extracted from the DICE database [34] (<http://dice-database.org/>). (D,F) Biological pathways were ranked with increasing adjusted *p* value and dotted line represents significance threshold (adjusted *p* value < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

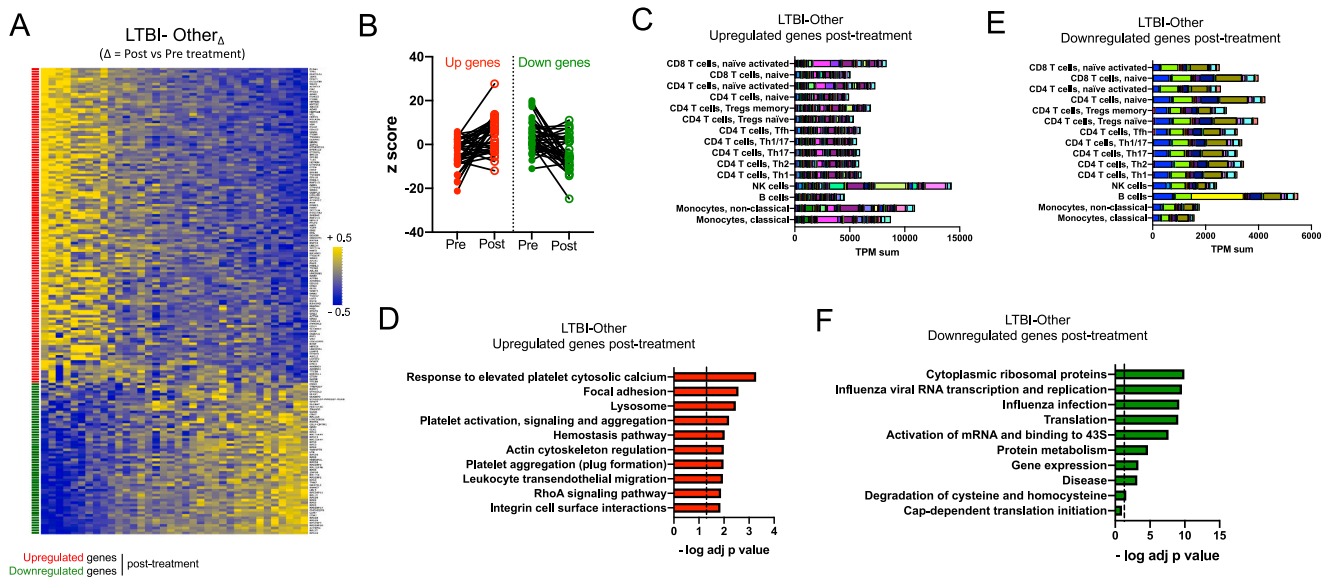


Fig. 4. Blood transcriptomic signature of treatment in the LTBI-Other cohort is associated with NK cells, B cells and platelet-related pathways. A) Heatmap representing the log fold change expression upon treatment and B) individual z-scores pre and post treatment for the upregulated (red) and downregulated (green) gene-mapped probes in the LTBI-Other_Δ signature derived from differential expression analysis (non-adjusted *p* value < 0.01, absolute log fold change > 0.2) between post-and pre-treatment paired blood samples in the LTBI-Other cohort (n = 36) (Table S6). C) Immune cell type-specific expression and D) top-10 biological pathways enriched in the upregulated genes of the LTBI-Other_Δ signature. E) Immune cell type-specific expression and F) top-10 biological pathways enriched in the downregulated genes of the LTBI-Other_Δ signature. (C,E) For immune cell type-specific expression, each bar consists of stacked sub-bars showing the TPM normalized expression of every gene in corresponding cell type, extracted from the DICE database [34] (<http://dice-database.org/>). (D,F) Biological pathways were ranked with increasing adjusted *p* value and dotted line represents significance threshold (adjusted *p* value < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and LTBI-Other_Δ (R3HDM2, SEC31A, SNRNP70, WARS) and five genes shared between LTBI-Other_Δ and ATB_Δ (DISC1, IL2RB, SP100, TTC38, TUT7B) (Fig. 5A). Neither of these two overlaps was significant. In contrast, a significant overlap was found between LTBI-Risk_Δ and ATB_Δ, with 21 genes shared (adjusted $p < 5e-17$, Fig. 5A). All 21 genes were downregulated upon treatment in both LTBI-Risk and ATB cohorts (Fig. 5B and C). Interestingly, the LTBI-Other cohort presented an opposite pattern with prevailing upregulation of all these genes upon treatment (Fig. 5B and C). The 21 genes were associated with the highest cumulative expression level in T cells (Fig. 5D), particularly activated T cells, and were significantly enriched for IFN signaling pathway (Fig. 5E). Thus, transcriptomic changes upon anti-TB therapy in the LTBI-Risk group resemble those occurring in ATB, with shared down-regulated genes associated with IFN signaling.

3.5. Expression changes of ‘risk of progression to active TB’ signatures during treatment in LTBI

We identified that the 21 overlapping genes shared between LTBI-Risk_Δ and ATB_Δ contained several genes from ‘risk of progression to ATB’ signatures (ANKRD22, BATF2, DUSP3, FCGR1A/B and GBP5, Fig. 5B), which we used to stratify the LTBI cohort into LTBI-Risk and LTBI-Other groups prior the start of treatment (Fig. 1A and Fig. S2). This is not unexpected, since the Zak16 signature expression has been shown to be reduced upon treatment in ATB [17]. This prompted us to further analyze expression changes of individual genes from risk progression signatures upon anti-TB therapy in LTBI.

We classified genes present in any of the four progression signatures (Zak16 [17], Sweeney3 [18,19], BATF2 [20] and RISK6 [21]) as either downregulated, upregulated or not significantly changed upon anti-TB therapy in the LTBI-Risk or LTBI-Other cohorts. Out of the total number of genes present in any of the four progression signatures, 8 were downregulated upon treatment in the LTBI-Risk group but not the LTBI-Other group (Fig. 6A, Fig. S5). The remaining genes were not

differentially expressed upon treatment in either the LTBI-Risk or the LTBI-Other group, according to our criteria of significance ($p < 0.01$ and absolute log fold change > 0.2 ; Fig. 6A, Fig. S6). Genes from the progression signatures that were selectively downregulated in the LTBI-Risk group showed significant enrichment for IFN signaling (Fig. 6B) and for functional protein-protein associations (p value of enrichment = $1.1e-16$, Fig. 6C) in particular between IFN signaling associated genes. In contrast, no statistically significant enrichment for biological pathways or functional protein-protein associations was found in the ‘not significant’ genes upon treatment (Fig. 6D and E). Overall, this shows that several genes from previously described progression to ATB signatures, in particular those that are significantly associated with IFN signaling, have a reduced expression upon TB-preventive treatment in LTBI-Risk but not LTBI-Other participants.

4. Discussion

We studied changes in the host blood transcriptome following anti-TB therapy in LTBI to investigate immune heterogeneity in response to treatment in this cohort. We stratified LTBI participants into two groups based on Zak16, the seminal ‘risk of progression to ATB’ gene signature reported in LTBI [17] (and which correlated well with subsequent signatures [18–21]): LTBI-Risk and LTBI-Other, displaying high and low expression of the Zak16 gene signature prior to treatment, respectively. We identified 10 out of 69 LTBI participants (14%) as LTBI-Risk. This prevalence is much higher than the expected rate of progression from LTBI to ATB [22,42], therefore it is unlikely that all of the LTBI-Risk individuals would develop active disease in their lifetime. A distinct classification might have been obtained with another method to calculate the gene signature score, and set the threshold for positivity. We used a standard z-score approach (see methods) without gene pairing to calculate gene signature scores in our study. This differs from the methods that were used for the calculation of Zak16 [17] and RISK6 [21] signature scores, which used customized gene pairing approaches,

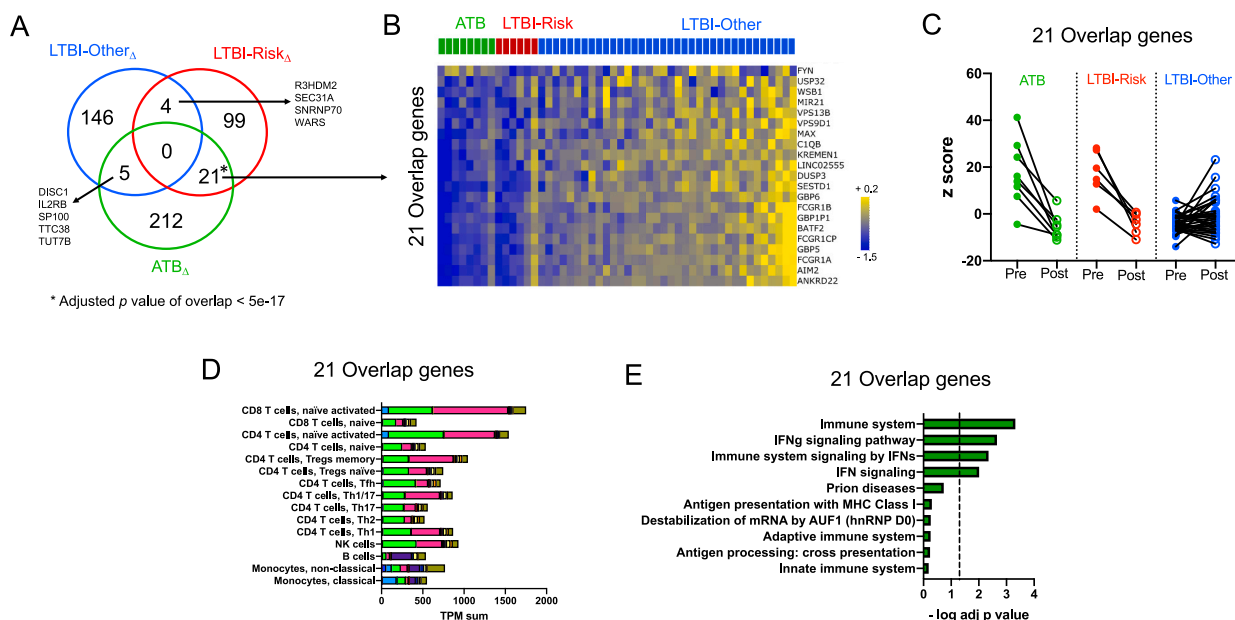


Fig. 5. Blood transcriptomic signatures of treatment in the LTBI-Risk cohort significantly overlaps with the ATB cohort. A) Venn-diagram representing overlaps between individual genes from the ATB_Δ, LTBI-Risk_Δ and LTBI-Other_Δ treatment signatures as defined in Figs. 2–4. B) Heatmap representing the log fold change expression upon treatment and C) individual z-scores pre and post treatment for the 21 overlapping genes between the LTBI-Risk_Δ and ATB_Δ treatment signatures across LTBI-Risk, LTBI-Other and ATB cohorts. For B), if a gene was mapped to several probes, average expression of all probes was shown. D) Immune cell type-specific expression in the 21 overlapping genes between the LTBI-Risk_Δ and ATB_Δ treatment signatures. Each bar consists of stacked sub-bars showing the TPM normalized expression of every gene in corresponding cell type, extracted from the DICE database [34] (<http://dice-database.org/>). E) top-10 biological pathways enriched in the 21 overlapping genes between the LTBI-Risk_Δ and ATB_Δ treatment signatures. Biological pathways were ranked with increasing adjusted p value and dotted line represents significance threshold (adjusted p value < 0.05).

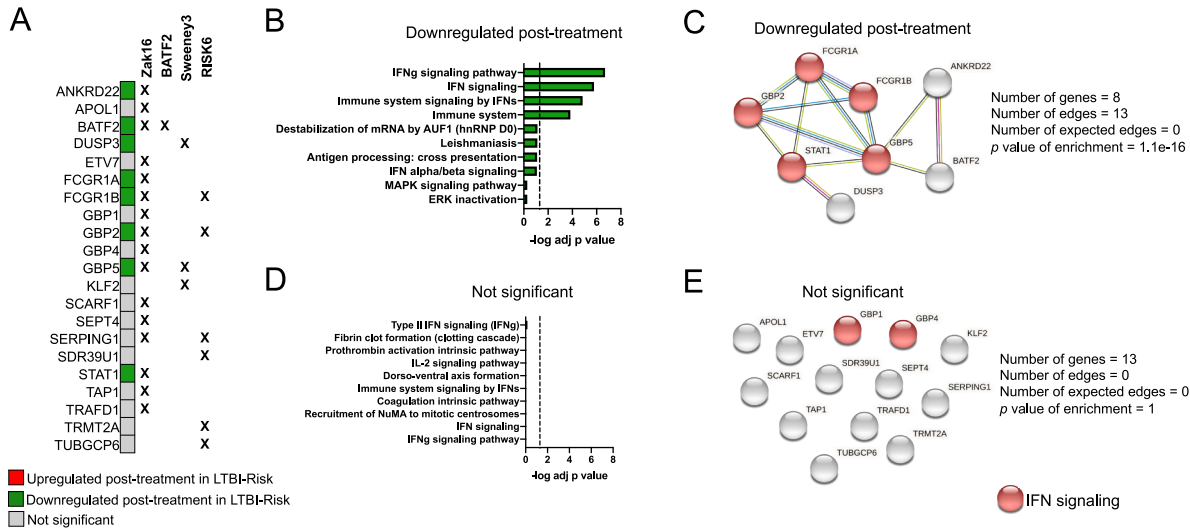


Fig. 6. Genes from ‘risk of progression to ATB’ signatures that are associated with IFN signaling are downregulated upon anti-TB treatment in LTBI-Risk but not LTBI-Other participants. A) Individual classification of genes within the ‘risk of progression to ATB’ signatures (reported in [17,18,20,21]) based on whether they are differentially expressed in the LTBI-Risk or the LTBI-Other group upon treatment (LTBI-Risk Δ and LTBI-Other Δ treatment signatures defined in Figs. 3 and 4). None of the genes were differentially expressed in the LTBI-Other group upon treatment. B) top-10 biological pathways enriched and C) functional protein-protein association network in the downregulated genes upon treatment in the LTBI-Risk group. D) top-10 biological pathways enriched and E) functional protein-protein association network in genes that are not significantly dysregulated upon treatment in either the LTBI-Risk or LTBI-Other groups. (B,D) Biological pathways were ranked with increasing adjusted *p* value and dotted line represents significance threshold (adjusted *p* value < 0.05). (C,E) Functional protein-protein association networks were defined by querying the online STRING database (version 11.0).

but allowed us to use a consistent method across all gene signatures analyzed in this study (i.e. previously defined and newly discovered). As for the threshold, we used the highest value within the TBneg cohort to divide the LTBI cohort into LTBI-Risk and LTBI-Other, which is subjective to our study. This is based on the hypothesis that the TBneg cohort contain exclusively *Mtb* uninfected individuals. Thus, the maximum value of the TBneg cohort is the maximum value that would be expected for individuals with no evidence of current or past *Mtb* infection, and any higher value potentially reflects incipient disease (LTBI-Risk). We elected this approach as the Zak16 signature was originally measured with RNAseq and qRT-PCR, and using a different method (as mentioned above) [17], thus cut-offs used in that study could not be directly translated to our microarray expression data.

Using our classification, we identified that LTBI-Risk individuals displayed a whole blood transcriptomic signature following preventative therapy that overlapped significantly with that observed among the ATB cohort in response to treatment, but differed from the remaining LTBI cohort. We contend that such overlap is due to immunological similarities in response to treatment and corroborates the hypothesis that the LTBI-Risk cohort includes individuals with incipient disease. Our findings strike a chord with those of a very recent study in a small LTBI population which reported stratification of the cohort into two distinct populations based on their whole blood transcriptome upon treatment [28]. The authors suggested that this dichotomy might differentiate between individuals with viable bacilli from immunological sensitization without viable infection [28]. There was no overlap between their and our treatment signatures, likely due to the fact that their gene expression data was generated after *Mtb*-specific *in vitro* stimulation rather than measured directly *ex vivo* in whole blood. In addition, we also found full blood count differences between LTBI-Risk and LTBI-Other cohorts. Similar to ATB, the LTBI-Risk group showed reduced lymphocyte counts compared to TBneg or LTBI-Other groups. Taken together, our findings support our hypothesis that the LTBI-Risk cohort is at higher risk for progression to active disease compared to the LTBI cohort at large, and thus resembles more closely ATB

participants. More importantly, they suggest that monitoring blood transcriptomic changes upon treatment might help to identify which individuals have incipient disease and viable bacilli from those who do not, and thus identify which LTBI individuals might more benefit from chemoprophylaxis treatment.

The LTBI-Risk Δ treatment signature contained a significant overlap with ‘risk of progression to ATB’ signatures. Looking at four predictive gene signatures (Zak16 [17], Sweeney3 [18,19], BATF2 [20] and RISK6 [21]), we have shown that in the subset of LTBI-Risk individuals (who do express high level of these genes prior to treatment), several of the ‘risk of progression to ATB’ genes are downregulated upon anti-TB therapy. These genes were significantly enriched for IFN signaling and functional protein-protein associations, in comparison to those that are not significantly affected by treatment. The IFN signaling pathway has been repeatedly associated with host RNA signatures in TB [23,24,40,43], and in our study, it was also significantly enriched in downregulated genes in both LTBI-Risk Δ and ATB Δ treatment signatures. This likely reflects the higher activated state of both innate and adaptive immune cells present in the blood of clinically infected individuals and incipient cases, which is then switched off upon treatment and highlights the importance of this pathway in LTBI progression to ATB. The presence of an IFN signature in the blood has been repeatedly associated with other respiratory infections including *Influenza* [44], *SARS-CoV-2* [45], but also auto-immune diseases [46]. Thus, an alternative explanation for the elevated expression of the IFN pathway in the blood of LTBI-Risk participants could be the presence of another underlying infection or illness at the time of enrollment.

In addition to IFN associated genes, downregulated genes in the ATB Δ treatment signature showed the highest cumulative expression in monocytes, which is consistent with previous findings that monocyte absolute counts and monocyte to lymphocyte ratio are prospective markers of active TB risk [47,48], and that these parameters are reduced following TB therapy in ATB [39]. Conversely, upregulated genes in the ATB and the LTBI-Other treatment signature showed highest expression in NK cells. NK cells have been previously associated with protective

immune responses to TB [49], and their frequency and cytolytic potential is increased in LTBI individuals compared to ATB or TBneg cohorts [50]. Upon TB treatment, NK cells are increased in frequency [50] and display increased activation and cytokine production [51]. Taken together, these results suggest monocytes and NK cells might play a key role in *Mtb* immune responses. Alternatively, this may reflect changes in frequency of white blood cell populations over time in response to TB treatment, rather than qualitative changes within these subsets.

Regardless of whether dysregulated gene expression profiles in the blood upon treatment have a quantitative or qualitative origin, our study shows its potential for monitoring immune responses associated with chemoprophylactic treatment of TB. There are currently no clinical biomarkers for monitoring the success of anti-TB therapy in LTBI. Previous studies have found no evidence that IGRA tests are a reliable metric for monitoring LTBI treatment [14,15]. A recent study has reported the presence of *Mtb* DNA in CD34⁺ PBMC, which was reduced upon TB chemoprophylaxis therapy in HIV infected individuals [52]. Importantly, their marker correlated poorly with IGRA status, suggesting it might reflect “true” LTBI rather than *Mtb* sensitization. The main limitation of this test is the requirement of a large volume of blood (100 mL) which would be challenging to implement in low resource countries with high *Mtb* prevalence. Nevertheless, it highlights the promise of blood-based biomarkers other than IGRA for monitoring TB therapy in LTBI. One gene expression study has found a handful of genes that are universally downregulated in LTBI upon treatment, but did not consider LTBI as a heterologous population, and limited their analysis to 10 genes [53]. Only one study has previously investigated blood transcriptomic changes upon LTBI treatment on a genome-wide scale in 18 participants, but relied on *Mtb in vitro* stimulation to identify gene signatures [28]. Thus, our genome-wide longitudinal investigation of the *ex vivo* blood transcriptome upon treatment in 42 LTBI participants constitutes a significant novel addition to the literature.

By monitoring blood transcriptomic changes directly *ex vivo* upon treatment, we observed a selective downregulation of several of the IFN associated progression signature genes upon anti-TB therapy in LTBI-Risk but not LTBI-Other participants, highlighting the potential value of these signatures and the IFN pathway to monitor LTBI treatment in individuals with increased risk of progression to ATB. This observation requires further validation in larger studies as we identified that preventive TB treatment induces subtle longitudinal gene expression changes *ex vivo* in blood in LTBI. In particular, our small cohort of paired blood samples collected pre and post treatment in LTBI-Risk (n = 6) and ATB (n = 8) participants precluded us to use “strict” statistical cutoffs, that could be reused in future studies. Additionally, since our study is the first of its kind, we have no previous datasets to include as validation cohorts, compare our findings, or run meta-analyses as was elegantly done in the context of ATB in Chowdhury et al. [50]. Such studies would increase the robustness of the statistical thresholds required to select genes used as biomarkers. Specifically, it would be of interest to compare our results to LTBI samples collected in areas with higher endemicity where it is logistically more challenging to treat the LTBI population, and where increased exposure to *Mtb* might shape differently the immune responses to treatment. Finally, blood transcriptomic changes upon treatment need to be investigated in association with clinical parameters such as occurrence of progression to ATB. A recent prospective longitudinal study in South Africa found no evidence that preventative treatment reduced the rate of incident tuberculosis over 15 months in LTBI participants classified as higher risk of progression to ATB based on the expression of a subset of the Zak16 signature prior to initiation of therapy [42]. This important result suggests that the LTBI-Risk group might be further heterogeneous, and that only assessing blood RNA signatures prior to initiation of treatment is not informative enough to predict progression to ATB upon treatment completion. Additionally, Gupta et al. in their multicohort meta-analysis found that whole blood gene signatures of incipient TB, including Zak16, have a relatively short window of prognostic utility [22]. Close prospective

monitoring of blood transcriptomic changes occurring early after initiation of preventive therapy in such cohorts could shed light on the immune events specifically occurring among individuals who may re-activate, and might ultimately allow the prediction of treatment failure or success in LTBI.

5. Conclusions

In summary, our study clearly demonstrates that individuals with LTBI display a heterogeneous blood transcriptome profile upon anti-TB therapy, which might reflect distinct “*Mtb* infection states” within LTBI. Upon chemoprophylactic treatment, individuals at higher risk of developing ATB (LTBI-Risk) showed similar blood transcriptomic changes to ATB infected individuals undergoing anti-TB therapy in comparison to the remaining LTBI cohort. In particular, both ATB and LTBI-Risk groups showed selective downregulation of genes that are associated with IFN signaling and that were previously identified in progression signatures. This corroborates the hypothesis that LTBI-Risk individuals have incipient disease and that treatment elicits immune responses that lead to elimination of *Mtb* bacilli similar to those occurring upon ATB treatment. It also suggests that IFN associated genes from progression signatures might be a promising target to monitor the course of treatment in these individuals. Overall, this study confirms the value of using blood transcriptomics to monitor immune changes occurring upon preventive therapy in LTBI. Future larger studies will be necessary to further dissect LTBI heterogeneity in immune responses to treatment, in particular in the context of post-treatment clinical outcomes and identify genes that could be used as biomarkers to monitor the course of treatment within each group.

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Author contributions

MKO conceived and designed the study. RT and EP conducted the experiments. JGB, MKO and BP led the data analysis and interpretation with input from all authors. JGB, MKO and BP wrote the manuscript and all authors edited the manuscript.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tube.2021.102127>.

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