A modular transcriptional signature identifies phenotypic heterogeneity of human tuberculosis infection

Akul Singhania[1*], Raman Verma[2*], Christine M. Graham[1], Jo Lee[2], Tran Trang[3], Matthew Richardson[2], Patrick Lecine[3], Philippe Leissner[3], Matthew P.R. Berry[4], Robert J. Wilkinson[5,6,7], Karine Kaiser[8], Marc Rodrigue[8], Gerrit Woltmann[2], Pranabashis Haldar[2,¶], Anne O'Garra[1,9,¶]

[1] Laboratory of Immunoregulation and Infection, The Francis Crick Institute, London, United Kingdom.
[2] Respiratory Biomedical Research Centre, Institute for Lung Health, Department of Infection Immunity and Inflammation, University of Leicester, Leicester, United Kingdom.
[3] BIOASTER Microbiology Technology Institute, Lyon, France.
[4] Department of Respiratory Medicine, Imperial College Healthcare NHS Trust, St Mary's Hospital, London, United Kingdom.
[5] Wellcome Centre for Infectious Diseases Research, Africa; Institute for Infectious Diseases and Molecular Medicine, University of Cape Town, Observatory 7925, South Africa.
[6] Department of Medicine, Imperial College London, London, United Kingdom.
[7] Tuberculosis Laboratory, The Francis Crick Institute, London, United Kingdom.
[8] Medical Diagnostic Discovery Department, bioMérieux SA, Marcy l'Etoile, France.
[9] National Heart and Lung Institute, Imperial College London, London, United Kingdom.

[*] contributed equally.

¶co-supervised this work

Correspondence to AOG. (email: Anne.OGarra@crick.ac.uk)

Abstract

Whole blood transcriptional signatures distinguishing active tuberculosis patients from asymptomatic latently infected individuals exist. Consensus has not been achieved regarding the optimal reduced gene sets as diagnostic biomarkers that also achieve discrimination from other diseases. Here we show a blood transcriptional signature of active tuberculosis using RNA-Seq, confirming microarray results, that discriminates active tuberculosis from latently infected and healthy individuals, validating this signature in an independent cohort. Using an advanced modular approach, we utilise information from the entire transcriptome, which includes over-abundance of type I interferon-inducible genes and under-abundance of *IFNG* and *TBX21*, to develop a signature that discriminates active tuberculosis patients from latently infected individuals, or those with acute viral and bacterial infections. We suggest methods targeting gene selection across multiple discriminant modules can improve development of diagnostic biomarkers with improved performance. Finally, utilising the modular approach we demonstrate dynamic heterogeneity in a longitudinal study of recent tuberculosis contacts.

Introduction

Tuberculosis (TB) is the leading cause of global mortality from an infectious disease. In 2016, there were 10.4 million incident and 6.3 million new cases of TB disease and 1.67 million deaths and its diagnosis is problematic[1]. Active pulmonary TB diagnosis requires culture of *Mycobacterium tuberculosis*, which may take up to 6 weeks[2]. Although the World Health Organisation[1] endorsed GeneXpert MTB/RIF automated molecular test for *M. tuberculosis* results in rapid diagnosis[3], this test still requires sputum which can be difficult to obtain. Difficulties in obtaining sputum lead to approximately 30% of patients in the USA and 50% of South African patients to be treated empirically[1,4]. However, clinical disease represents one end of a spectrum of infection states. An estimated one third of all individuals worldwide have been infected with the causative pathogen, *M. tuberculosis,* but the vast majority remain clinically asymptomatic with no radiological or microbiological evidence for active infection. This state is termed latent TB infection (LTBI) and conceptually denotes that *M. tuberculosis* persists within its host, while maintaining viability with the potential to replicate and cause symptomatic disease. Indeed, LTBI represents the primary reservoir for future incident TB, with 90% of all TB cases estimated to arise from reactivation of existing infection[1,5]. The risk of incident TB arising from existing LTBI is heterogeneous, poorly characterised and modifiable with anti-tuberculous treatment. Modelling studies indicate that effective TB prevention to reduce future TB incidence, requires policies directed at the identification and treatment of LTBI[6]. However, implementation of mass screening programmes for this purpose are severely constrained by the size of the target population. Transformative advances in diagnostic tools that can effectively help to stratify TB risk in the LTBI population are therefore implicit to the realisation of systematic screening.

The basis for LTBI heterogeneity rests with the limited scope of the tools we have available to identify the state. LTBI is inferred solely through evidence that immune sensitization has occurred, by the tuberculin skin test (TST) or the *M. tuberculosis* antigen-specific interferon-gamma (IFN-γ) release assay (IGRA). Although these tests are both sensitive and specific for identifying exposure, that has been associated with establishment of an

adaptive immune response, neither distinguishes active from latent infection. Moreover, T-cell responses to mycobacterial antigens persist for several years after an infection has been treated, implying that these tests may not reliably inform the presence of viable organisms *in vivo*. For 'true' LTBI, in which the pathogen remains viable, it is envisaged that a dynamic equilibrium exists between the host immune response and the pathogen, with a shifting balance in favour of one or the other, influencing the future risk of TB reactivation[7]. A study using highly sensitive radiological imaging with combined Positron Emission Tomography and Computerised Tomography has reported evidence to support this dynamic state and demonstrated phenotypic imaging characteristics associated with the risk of developing TB among subjects with conventionally defined LTBI[8]. A proportion of these LTBI patients were identified with radiological features of subclinical active TB[8], with a subgroup failing to respond to prophylactic LTBI treatment regimens. These observations support the view that injudicious use of LTBI chemoprophylaxis using presently available diagnostic tools for mass screening, risks promoting drug resistance in unrecognised active infection.

We have previously characterised an interferon (IFN) inducible transcriptional signature of 393 gene transcripts in whole blood that discriminates patients with active pulmonary TB (from high and low-incidence TB burden countries) from healthy individuals, patients with other chronic respiratory and systemic conditions, and the majority of patients with LTBI[9,10]. This TB signature revealed an unexpected dominance of type I IFN-inducible genes[9] more frequently associated with viral infections[11]. We[12-14] and others[13-23] have since shown that elevated and sustained levels of type I IFN, result in an enhanced mycobacterial load and disease exacerbation in experimental models of TB. Similar findings of a blood signature in active TB patients have since been reported[24-30], and our meta-analysis of 16 datasets, including many of these studies, identified 380 genes differentially abundant in active TB across all datasets[31]. However, there is a relative lack of concordance across studies that have reported a reduced and optimised diagnostic gene signature, although agreement exists for some of the pathways they represent[24-26,32,33]. While some genes overlap between the different reduced signatures, the overall composition of each reduced signature is unique, both in size and transcript profile. In this respect, we note that a consistent statistical approach to optimising gene selection has not been used across studies and where the approach was consistent, a different optimal reduced signature was reported for discriminating active TB from either LTBI and controls or other diseases[25]. Additionally, recent reports suggest these signatures do not effectively discriminate TB from other diseases such as pneumonia, lowering their value as stand-alone diagnostic tests[29,30].

We have previously observed and reported that 10 – 20% of subjects with IGRA positive LTBI in our studies had a transcriptional signature that overlapped with active TB patients and clustered with this group[9]. By definition, the transcriptional signature in this LTBI outlier group shares important similarities with the signature of active TB that requires further characterisation. Importantly, the biological significance of this statistical observation remains unclear. However, these observations support utilisation of a transcriptional approach to explore LTBI heterogeneity. In keeping with this, Zak et al.[33] have recently reported evidence for a gene signature of TB several months in advance of clinical presentation with disease, among a cohort of South African adolescents, contained within the previously described TB signature[9]. This suggests that transcriptional signatures of TB in subjects with presumed LTBI may indicate either, a high risk of progression to active disease, or existing subclinical disease. However, interpretation of the study was limited

by the confounding risk of new TB exposure in a high TB incidence setting, particularly to determine longitudinal changes in signature expression and dynamic heterogeneity of the host immune response. Analysis focussed on the subgroup with IGRA defines LTBI, despite a proportion of prospective TB cases developing in subjects that were IGRA negative at baseline, suggesting either new TB exposure during prospective observation in a high TB incidence setting and/or that the IGRA test did not reliably inform underlying LTBI. In this context, studies evaluating the diagnostic performance of IGRAs in microbiologically confirmed active TB report an overall sensitivity of approximately 85%[34], implying that a proportion of *M. tuberculosis* infections may be missed using this test alone.

To address some of these questions, here we: (i) validate microarray findings by RNA-sequencing in our published[9] cohorts and a new cohort of TB; (ii) evaluate current TB gene signatures from the literature against TB and other infections; (iii) develop and test a modular TB signature in multiple TB cohorts and other diseases; (iv) develop a reduced TB-specific gene signature from modules of TB and test against viral infections and other diseases; (v) evaluate the transcriptional profile of LTBI-Outliers and compare with active TB; (vi) evaluate the blood transcriptional signature at baseline and longitudinally in TB contacts who develop TB against those TB contacts who remain healthy, in a low TB incidence setting **(Figure 1; Supplementary Figure 1)**. As a proof of principle, our reduced TB-specific gene set developed from the modular signature, not only distinguishes active TB and LTBI but additionally does not detect viral and bacterial infections. We identify immunological heterogeneity of LTBI, with a percentage of individuals showing a transcriptional signature of active TB, which only develop longitudinally in a small proportion of the recent close contacts of TB.

Results

RNA-seq recapitulates the microarray TB gene signature

We validated our microarray-derived blood 393-transcript signature[9] in patients with active TB using RNA-Seq in the Berry London and South Africa cohorts showing identical clustering of active TB and LTBI cases (**Supplementary Figure 2a** and **2b**). A 373-gene signature was then independently re-derived from the Berry London RNA-Seq data **(Supplementary Figure 2c; Supplementary Data 1; Figure 2a)** and validated in the Berry South Africa cohorts **(Figure 2a)** and a new Leicester cohort **(Supplementary Table 1; Figure 2b).** Consistent with our previous microarray signature, the RNA-Seq signature was absent in the majority of individuals with LTBI and healthy controls, and identified with perfect agreement the LTBI subjects that cluster with active TB, henceforth referred to as LTBI outliers, in both Berry cohorts **(Supplementary Figure 2b** and **2d).** A similar proportion of outliers were also observed in the Leicester cohort **(Figure 2b; Supplementary Figure 2e).** There was great similarity in the composition of the microarray and RNA-Seq based signatures, with over-abundance of IFN-inducible genes and under-abundance of B- and T-cell genes as previously reported[9]. This was supported by an *in silico* cellular deconvolution analysis of the RNA-Seq that showed diminished percentages of CD4, CD8 and B cells in the blood of active TB patients, and an increase in monocytes/macrophages and neutrophils (**Supplementary Figure 3**), in keeping with our previous findings using flow cytometry[9]**.**

175 **Published TB gene signatures identify acute viral infections**
176
177 Applying the published 16-gene signature of Zak et al.[33] to the Berry and Leicester TB
178 cohorts, single sample Gene Set Enrichment Analysis[35] (ssGSEA) across all three cohorts,
179 demonstrated high enrichment of the Zak et al. signature[33] in active TB and a low
180 enrichment in healthy controls and the majority of LTBI patients **(Figure 2c)**. We observed
181 higher enrichment scores in the LTBI outlier groups **(Figure 3a** and **3b)** of all three cohorts
182 that overlapped with scores observed in active TB cohorts **(Figure 2c)**. Higher enrichment
183 scores were also noted in a small proportion of IGRA^{-ve} individuals recruited as healthy
184 controls **(Figure 2c)**. There was comparable discrimination in enrichment scores between
185 TB and LTBI in the Zak et al.[33] 16-gene signature, and the Kaforou et al., 27 and 44-gene
186 signatures[25] **(Figure 3a).** Only the Kaforou 44-gene signature was developed to discriminate
187 between active TB and other diseases (including infectious meningitis, pneumonia, gastric
188 diseases and malignancies)[25], rather than LTBI.
189
190 The composition of all three signatures[25,33] is dominated (≥50% of the signature) by IFN-
191 inducible genes **(Supplementary Table 2)**, raising the possibility that they are not TB
192 specific but may also be expressed in acute viral infections. We therefore evaluated
193 enrichment of these signatures in two independent published datasets of influenza
194 infection from Parnell et al.[36] and Zhai et al.[37] **(Supplementary Table 3; Figure 3b)**.
195 Subjects with influenza at baseline showed a high enrichment score for the three TB
196 signatures as compared with healthy controls, which diminished with time, in keeping with
197 recovery **(Figure 3b)**. In contrast, enrichment scores for the three signatures,
198 demonstrated heterogeneity in patients diagnosed with bacterial pneumonia from the
199 Parnell study[36], with little change over 5 days and poor discrimination from controls,
200 consistent with our previous findings for this group[9,10] **(Figure 3b).**
201
202
203 **A modular signature discriminates TB from other diseases**
204
205 A limitation of the gene reduction methodologies[25,33] used to date has been the
206 prioritisation of the most discriminant genes, with little consideration to the correlation
207 between the selected genes in this iterative process. Although non-selective and lacking
208 subjective bias, this approach favours selection of a highly correlated gene set with a
209 narrow immunological focus. In this context, limited diversity risks loss of specificity, with
210 an increased likelihood of overlap between multiple pathologies and responses to different
211 infections for a specific immune pathway. We therefore hypothesised that methodologies
212 which incorporate information from the entire transcriptome may better inform
213 development of a unique biosignature for TB. Weighted gene co-expression network
214 analysis[38] (WGCNA) is a well validated clustering technique for reducing high dimensional
215 data into modules that preserve intrinsic relationships between variables within a network
216 structure. When applied to the blood transcriptome, modules of co-ordinately expressed
217 genes with a coherent functional relationship are generated. The complete transcriptome
218 is thus expressed as a signature defined by the relative perturbation of individual modules.
219
220 We applied WGCNA analysis to the blood transcriptional data from our Berry and Leicester
221 TB cohorts, those TB cohorts published by Zak and Kaforou[25,33], and to several others that
222 included sample sets of other viral and bacterial infections[36,37,39,40], together with our
223 previous cohorts of sarcoidosis and lung cancer[10] as conditions that may mimic TB, all
224 compared against their healthy controls **(Figure 4; Supplementary Table 3** (Information

of published cohorts)**, Supplementary Data 2** (genes in each module) and **Supplementary Table 4** (module annotation)**)**. The modular signature for active TB was qualitatively consistent across all the TB cohorts and absent in LTBI. The IFN-modules (lightgreen and yellow) were over-abundant in TB **(Figure 4a)** as we have previously published[9,10] and also in acute influenza infection, but absent in bacterial infection[9,10] **(Figure 4b)**. However, we observed clear differences between TB and both influenza and other bacterial infections in the pattern of specific perturbation of other modules, including under-abundance of gene expression in the T-cell (blue and cyan) and B-cell (midnightblue) modules **(Figure 4)** for TB. On the other hand, we observed over-abundance of genes in the Cell Proliferation/Metabolism (darkturquoise) module and under-abundance of genes associated with Haematopoeisis (pink) in severe influenza but not in TB **(Figure 4)**. In this context, the classical approaches of gene signature reduction algorithms[41-43] used by Kaforou et al. to distinguish TB from LTBI or TB from other diseases[25], and Zak et al. to distinguish TB from LTBI, risk of progression[33] are notable for formulating gene signatures that we show here map predominantly to the yellow module (Interferon/complement/myeloid), with many of these genes also over-abundant in both influenza cohorts (**Supplementary Figure 4** and **5).**

A reduced TB-specific signature from modular gene expression

Interrogating the whole gene-set of the yellow module in TB, influenza and bacterial infection, we observed a subset of genes expressed specifically in TB **(Figure 5, orange squares)**. Similarly, other genes were specifically expressed in influenza. Thus, although modular expression of the yellow module is comparable between TB and influenza, gene subsets within the module exhibit differential expression between the two conditions. This provides scope to select genes from this dominant module that can be used to develop a TB signature, while retaining discriminant value from viral infection. Using this rationale, as a proof of principle, we identified and extracted 303 unique gene candidates in the Berry London TB dataset that were selectively perturbed in TB, but not in any confounding viral infections, from all modules that contributed to and exhibited consistency across the TB datasets that we analysed (**Figure 4; Supplementary Figure 6a; Supplementary Figure 6b**). Using this gene set, we developed a reduced gene signature to distinguish active TB from LTBI. We applied the Boruta algorithm[42] based on random forest to this set of genes, yielding 61 genes **(Supplementary Figure 6c)** that was further reduced by selecting the top 20 genes, ranked according to GINI score using Random Forest **(Supplementary Figure 6d)**. Our 20-gene signature **(Figure 6a)** included genes from six different modules **(Supplementary Figure 6d)**, representing both over-abundance and under-abundance in TB. Using a modified Disease Risk Score (See Methods), we identified powerful discrimination between active TB and LTBI/controls in Berry London & South Africa and Leicester cohorts **(Figure 6b)**. In contrast, the signature identified no difference between influenza and controls or between bacterial pneumonia and controls at any time-point across five days **(Figure 6c).** Our 20-gene signature also discriminated active TB from LTBI and controls, in three additional published cohorts, similarly to the 44-gene signature described by Kaforou et al.,[25] **(Figure 7a).** Both our 20-gene signature and the 44-gene signature of Kaforou also discriminated active TB from other diseases, albeit to a lower extent **(Figure 7b).** In keeping with this, our 20-gene signature, and the signatures published by Zak et al.[33], Kaforou et al.[25], Roe et al.[28], Sweeney et al.[44], Maertzdorf et al.[45], distinguished active TB and LTBI with high specificity and sensitivity **(Figure 8a)**. Whereas our 20-gene signature did not discriminate influenza from controls, all other

signatures demonstrated excellent discrimination between influenza from controls, comparable with their performance for TB **(Figure 8b)**, indicating that influenza and other types of viral infections may inadvertently be detected and confound the TB diagnosis.

The modular signature of LTBI outliers resembles that of TB

We have previously reported evidence for a small proportion of LTBI subjects that clustered with active TB using our 393-transcript signature[9] that we refer to as an LTBI outlier group. This group was reproduced using RNA-Seq in the Berry cohorts (10.9%) and a similar proportion were also identified in our new Leicester cohort (10%) (**Figure 2)**. To compare and contrast the signature of this group with active TB and the majority of LTBI resembling healthy controls **(Supplementary Figure 2d** and **2e)**, we specifically examined the WGCNA modular signature in LTBI outliers using the combined Berry London and South Africa datasets and Leicester datasets respectively, compared with healthy controls **(Figure 9a).** The modular signature of LTBI outliers in both datasets showed over-abundance of the lightgreen (IFN/Pattern recognition receptors) and yellow (IFN/Complement/myeloid) modules as seen in active TB **(Figure 9a** and **9b)**. This is entirely in keeping with our earlier finding **(Figure 2c and 3a)** that gene enrichment scores using the published signatures [25,33], all of which are comprised primarily of genes from the yellow module **(Supplementary Figures 4 and 5)**, were consistently higher in LTBI outliers. Of note, these gene-sets were not present in the lightgreen (IFN/Pattern recognition receptors). In addition to over-abundance of the IFN modules, the LTBI outlier group of the Leicester dataset showed changes in other modules also perturbed in active TB, suggesting a host response that is evolving towards the phenotype typically observed in active TB **(Figure 9a)**. Of particular interest was the observation of under-abundance in the tan module (Th1 and NK cells) that is associated with IFN-γ expression, a cytokine required for protection against TB[16,46-52]. Under-abundance of this module was a consistent finding across all the TB datasets that we analysed **(Figure 4, Figure 9)**.

We performed differential gene expression analysis between active TB, LTBI outliers, and LTBI with outliers removed, and identified a set of 70 genes that was consistently upregulated in active TB and LTBI outliers compared to LTBI (without outliers) in both the Berry and Leicester datasets **(Figure 9d; Supplementary Data 3),** which were enriched for the IFN signalling pathway and innate immunity.

Dynamic transcriptional heterogeneity in recent TB contacts

Longitudinal RNA-Seq was performed in a subset of our Leicester cohort **(Methods; Figure 10a)** that included 15 IGRA^{-ve} contacts, 16 IGRA^{+ve} contacts, both of whom remained healthy, and 9 subjects recruited as contacts that were subsequently diagnosed with microbiologically confirmed TB during prospective observation (**Figure 10a; Supplementary Table 5)**. Five contacts (4 IGRA^{+ve} and 1 IGRA^{-ve}) identified as outliers at baseline sequencing **(Figure 2b)** were included.

In contrast with other studies, the control population of our Leicester cohort comprised subjects that were IGRA^{-ve} contacts of TB. This is a group in which recent exposure to active TB is documented, placing them at higher risk of recently acquired infection. Our rationale for this approach was to evaluate whether blood transcriptional data may identify LTBI

that is not detected using IGRA. The observations that: firstly, the Leicester control group had greater overlap in enrichment scores with the IGRA^{+ve} LTBI group using the Zak and Kaforou signatures, compared with the Berry London cohort **(Figure 2c** and **3a)**; and secondly, one subject from this group was identified as an outlier, together suggest that IGRA testing alone may miss some *M. tuberculosis* infection. We therefore elected to define our TB contacts henceforth as IGRA^{+ve} or IGRA^{-ve} with no deterministic reference to LTBI.

The modular signatures of both IGRA^{-ve} and IGRA^{+ve} contacts qualitatively demonstrated considerable between-subject heterogeneity and some within-subject variability; a comparison between the groups suggested more transcriptional activity, in the form of a higher frequency and greater breadth of modules exhibiting overabundance and under-abundance within the IGRA^{+ve} group **(Supplementary Figure 7)**. For the cohort that developed TB after recruitment to the study **(Supplementary Figure 7),** we stratified subjects on the basis of their longitudinal clinical course as true progressors (no evidence of TB at baseline, with features developing during observation); subclinical TB (objective evidence of pathology, usually as radiological change, in the absence of reported symptoms); and active TB (symptoms at baseline with either radiological or microbiological evidence for TB subsequently identified) **(Supplementary Table 5)**. This stratification was performed to better understand the dynamic relationship between the modular signature and onset of TB.

To quantitatively evaluate the modular signatures in each group for their proximity to TB, we applied the modified disease risk score for our 20-gene signature **(Figure 10b)**. Higher risk scores were generally observed in the IGRA^{+ve}, compared with the IGRA^{-ve} cohort, although there was considerable variability and overlap. Longitudinal observations suggest relative stability of the risk score in the majority of both IGRA^{+ve} and IGRA^{-ve} subjects that were examined. In contrast, 6 of the 9 subjects that were diagnosed with TB demonstrated high baseline modified disease risk scores that tended to increase further, prior to diagnosis of active TB. In the other 3 contacts (Subjects 245, 348 and 278) the modified disease risk score remained low at all time-points, before and at the time of TB diagnosis **(Figure 10b)**.

Baseline scores demonstrated clustering near baseline for IGRA^{-ve} subjects **(Supplementary Figure 8; Figure 10b)**. In contrast, both the IGRA^{+ve} group, and the group that developed TB exhibited a higher risk score **(Figure 10b; Supplementary Figure 8)**. Subjects identified as outliers **(Figure 2b),** marked with an asterisk* **(Figure 10b),** had higher TB scores than the majority of LTBI subjects that were not outliers **(Figure 10b)**. However, some discordance between clustering outcomes **(Figure 2b)** and the TB score was observed **(Figure 10b)**, with two subjects that were not outliers having TB scores within the outlier range (subject 185 and 040). Furthermore, the IGRA^{-ve} subject categorised as an outlier (Subject 209) had a very low TB score **(Figure 10b)**. Overall, the longitudinal within-subject expression of the 20-gene TB signature in both the IGRA^{+ve} and IGRA^{-ve} cohorts could be categorised into three groups: i. Subjects that did not express the signature at any time-point (10 of 15 IGRA^{-ve} subjects and 6 of 16 IGRA^{+ve} subjects); ii. Subjects that transiently expressed the signature, albeit generally to a low extent, in the first three to four months (4 of 15 IGRA^{-ve} subjects and 8 of 16 IGRA^{+ve} subjects; iii) Subjects that had or developed a persistent TB signature at and beyond 4 months (1 of 15 IGRA^{-ve} subjects and 2 of 16 IGRA^{+ve} subjects)**(Figure 10b)**. We did not observe subjects developing the signature *de novo* after 3 months. Using the 16-gene signature of Zak et al.[33], we report similar findings **(Supplementary Figure 9)**. However, this 16-gene signature showed scores for additional

IGRA^{-ve} and IGRA^{+ve} individuals **(Supplementary Figure 9, marked with red arrow**), possibly resulting from intercurrent infections.

In the cohort that developed TB, 5 of the 9 subjects demonstrated high baseline modified disease risk scores **(Figure 10b)**. In 6 of the 9 subjects a moderate to high TB score was observed at the visit prior to TB diagnosis. For the remaining 3 subjects (Subject 245, Subject 278 and Subject 348), a 20-gene signature of TB was not expressed. For Subject 245 an explanation may be that this patient received antibiotics for bacterial pneumonia which are known to have immunosuppressive effects that may have affected expression of the immune signature. For Subjects 278 and 348 we have not identified potential confounding factors for this observation. Subjects categorised as true progressors exhibited a dynamic modular signature, with increasing TB scores at all visit time-points within 2 months of diagnosis.

Discussion

We have recapitulated a blood transcriptional signature of active TB using RNA-Seq, previously reported by microarray[9,25,26,31,53] that discriminates active TB from LTBI and healthy individuals, and is largely characterised by an over-abundance of IFN-inducible genes and an under-abundance of B and T cell genes. We show that an advanced modular approach, rather than a traditionally derived reduced gene set, is robust in discriminating active TB patients from individuals with LTBI whilst additionally not detecting acute viral and bacterial infections. Our findings highlight the need to consider additional approaches to develop transcriptional biomarkers of the highest sensitivity and specificity for distinguishing active TB from LTBI and other diseases. Using this modular approach and our reduced gene set, we also demonstrate heterogeneity of LTBI in a prospective study of contacts of patients with active TB.

RNA-Seq[33] has now replaced microarray[9,10,24-27,31,32,53-55] for transcriptional studies and the existing literature is limited by uncertainty regarding the equivalence of RNA-Seq and microarray. In this study, we repeated analysis of our previous Berry et al.[9] cohorts using RNA-Seq and provide reassurance that RNA-Seq recapitulates outcomes derived using microarray. The vast majority of genes in our RNA-Seq derived 373-gene signature also comprised our original 393-gene transcript signature. Furthermore, there was equivalence in allocation of subjects to clusters, including those with LTBI that clustered with active TB and thus referred to as outliers. This is of considerable translational significance as no field applicable test is likely to be based on RNA-seq or microarray analysis.

In transcriptomic studies of disease there has been focus on deriving reduced gene signatures to develop clinical diagnostics, with inconsistencies in both deriving and defining the optimal reduced gene signature. Studies defining signatures distinguishing active TB and LTBI[25,26,28,33,44,56] are illustrative of this issue. We evaluated the diagnostic performance of some of the published reduced gene signatures[25,28,33,44,45] on our independent TB cohorts and confirm excellent specificity and sensitivity to distinguish active TB patients from those with LTBI. However, we identified dominance of IFN-inducible genes in these signatures and demonstrated enrichment of these signatures in published datasets of acute influenza infection[36,37], which represents the immune response globally observed in viral infections. However, this signature was not apparent in bacterial pneumonia[36], highlighting the

423 apparent lack of IFN-inducible genes in the immunological response to bacterial
424 pneumonia. However, it is clear that the immune response in TB has a dominant IFN-
425 inducible gene signature resembling that of viral infections. It follows that such IFN-
426 inducible signatures, whilst optimised for discriminating active TB patients and healthy
427 individuals, with and without LTBI, with high sensitivity, may also detect other pathologies
428 and/or infectious diseases that may exhibit a similar clinical presentation. It is clear that
429 IFN-inducible genes are dominant discriminators of active TB from healthy LTBI, leading to
430 preferential selection of this gene set to define an optimal signature. However, this
431 dominance precludes consideration of most other gene sets and may also detect other
432 diseases, such as viral infections. This view is supported by differences in the reported
433 signatures of Kaforou et al.[25] that were independently derived to discriminate active TB
434 from LTBI, or active TB from other diseases. The 44-gene signature, derived using the latter
435 approach, included more genes and exhibited greater diversity, when compared with the
436 27-gene signature for discriminating LTBI from TB. However, both signatures[25] in addition
437 to others[28,33,44,45] still showed high specificity and sensitivity for influenza. These
438 observations suggest that a trade-off exists between these two objectives, of achieving
439 high sensitivity and high specificity, and that a single signature may not be optimal for
440 both. The development of biomarkers for clinical practice is defined and optimised
441 according to the clinical context for use. In a clinical context, the two objectives of a TB
442 signature fulfil distinct requirements. A signature that discriminates active TB from LTBI is
443 a useful screening tool for testing in healthy populations. Identification of an active TB
444 signature when screening for LTBI can inform the need for further investigation. In
445 contrast, a signature that discriminates active TB from other diseases would be applied for
446 the investigation of unwell patients presenting with symptoms that suggest the possibility
447 of TB, but may also be other infections including viral infections. While discrimination of
448 TB from LTBI in screening programmes, and TB from other diseases in patients that are
449 unwell, represent distinct clinical settings, the potential requirement of two different
450 biomarkers adds complexity to models of implementation, particularly in a field setting.
451 Furthermore, there is overlap between screening and clinical diagnostics as people
452 attending for screening may present with an intercurrent illness (either symptomatic or
453 asymptomatic) that is unrelated to TB. A single biomarker that is able to achieve reliable
454 discrimination of TB from both LTBI and other conditions will have greater utility in clinical
455 practice. A trade-off in sensitivity to distinguish active TB from LTBI may result from losing
456 the IFN-inducible genes that are highly induced by viral infections. However, it is possible
457 that this knowledge will allow the retention of such genes which distinguish active TB from
458 LTBI with high sensitivity, by also including additional genes that are detected in response
459 to viral infections and not TB, as an additional discriminatory approach to maintaining high
460 sensitivity and specificity for TB against LTBI.
461
462 We developed the WGCNA derived modular signature for active TB across our cohorts and
463 determined consistency of the signature in our cohorts and those from other published
464 datasets. When taking into consideration all 23 modules, the signature in active TB was
465 distinct from both viral and bacterial infections. In keeping with our earlier findings of an
466 IFN-inducible signature of active TB[9], we here also demonstrate an IFN-inducible gene
467 signature, in both the active TB and LTBI outliers. The IFN-inducible signature, however,
468 is now distributed across three different modules; two over-abundant modules; the yellow
469 module that includes *BATF2, AIM2, FCGR1A and B*, and a number of *GBPs*; the Lightgreen
470 module, which we show is also strongly over-abundant in influenza infection, includes many
471 *IFITs, ISGs and OASs*, very reminiscent of type-I IFN inducible genes induced during viral
472 infections. In contrast, the Tan module, which includes *IFNG* and *TBX21*, is significantly

473 under-abundant in TB and some LTBI outliers, in keeping with the reported down-regulation
474 of *IFNG* expression and signalling by high levels of type I IFN, contributing to the
475 pathogenesis of TB[15]. This could also represent the reduced number of $CD4^+$ and $CD8^+$ T
476 cells that we observed in the blood of active TB patients as we have previously discussed[9].
477 We also observed that this IFNG module was under-abundant in those contacts who
478 progressed to TB (7 out of 9), whereas few of the $IGRA^{+ve}$ (3 out of 16) and $IGRA^{-ve}$ (2 out
479 of 15), showed an under-abundance of this module. This supports the hypothesis that the
480 ratio of type I IFN versus IFNG inducible genes, may be critical in determining protection
481 or progression to TB disease.
482
483 To tackle the challenge of developing a high performing TB signature, we explored the
484 modular tool for systematic gene reduction into biologically meaningful modules that
485 together represent the entire transcriptome. Furthermore, we additionally identified gene
486 clusters that were differentially expressed in TB but not influenza from within the modules
487 of IFN signalling. This was an important observation as the opportunity to select specific
488 genes from these dominant modules offered scope to improve the sensitivity of the
489 signature and discrimination of TB from LTBI and other diseases. Based on these findings
490 we developed and evaluated a two-step approach for targeted gene selection to derive a
491 TB signature. Modules perturbed in TB were first interrogated to establish a gene set
492 comprising genes that are differentially expressed in TB compared with other diseases. *A*
493 *priori* gene selection in this way provided a gene set with high TB specificity against other
494 diseases. In the second step, traditional gene reduction methodology was applied to
495 separate TB from LTBI using this gene set. As a proof of principle, we developed a 20-gene
496 signature using this approach that was diverse in its modular representation, incorporating
497 genes from 6 modules. We demonstrate here that this 20-gene signature has robust
498 sensitivity and specificity for discriminating active TB from LTBI in our cohorts, in addition
499 to across a number of different published cohorts[10,25,28]. Our 20-gene signature also showed
500 discrimination of TB from other diseases, similarly to the 44-gene signature of Kaforou et
501 al.[25]. However, our 20-gene signature did not detect influenza from healthy controls, in
502 contrast to all the other reported TB signatures[25,28,33,44,45], which not only detected TB at
503 high specific and sensitivity against LTBI, but additionally showed a high specificity and
504 sensitivity for influenza versus controls. Therefore, the development of our 20-gene
505 signature provides a novel approach to discriminate TB from LTBI, whilst not detecting viral
506 infections, here exemplified by influenza, offering scope for further refinement in further
507 translational clinical studies.
508
509 Heterogeneity of LTBI was suggested in our previous study[9] with the identification of an
510 outlier group after clustering. In the present study, we identified a similar proportion of
511 LTBI outliers in the new Leicester cohort. We demonstrated enrichment scores using the
512 published signatures of Zak et al.[33] and Kaforou et al.[25], dominated by IFN-inducible genes,
513 that were higher in outliers compared with other LTBI in both the Berry and Leicester
514 cohorts, and overlapped with scores obtained in active TB. These observations suggested
515 LTBI outliers are characterised by an overabundance of IFN-inducible genes, a view that
516 was corroborated in their modular signatures, together with identification of seventy
517 selectively upregulated genes, common to both the Berry and Leicester LTBI outliers, which
518 mapped to IFN signalling pathways. The clinical significance of these observations remains
519 unclear, however the recent study of Zak et al.[33] suggests expression of a TB-like signature,
520 characterised by enrichment of IFN-inducible genes which we show from our analysis, may
521 indicate either subclinical disease or increased risk of progression to TB within a few
522 months, although this may be confounded by viral infections.

We utilised the modular signature for deeper characterisation of heterogeneity in recent TB contacts and identified instances of similarity with TB in a few of the IGRA^{-ve} (2) and IGRA^{+ve} (4) individuals, representing IFN and other signalling pathways. There were higher perturbations in the modular signature in IGRA^{+ve} individuals. Our observations of low modular activity for the IGRA^{-ve} cohort is consistent with the absence of LTBI and likely to reflect a robust finding. In contrast, enrichment scores of the published signatures we tested indicated considerably more overlap of IGRA^{-ve} subjects with the IGRA^{+ve} group, again indicating impaired specificity of these signatures. Other modular changes, discordant with TB, appeared to be driven by differences in the pattern of perturbation in modules than those representing IFN signalling pathways. The majority of contacts who developed TB had a modular signature (6 out of 9) comparable to that of active TB patients, observable before a diagnosis was made.

In keeping with the global modular activity, we observed evidence of dynamic change in the reduced 20-gene signature derived from the modules, of some TB contacts that can be categorised into three patterns of longitudinal expression that may reflect early immunological events following TB exposure. We suggest the absence of a signature at any time point may indicate the absence of infection being acquired. This pattern was seen in 67% of our IGRA^{-ve} cohort and 38% of our IGRA^{+ve} cohort. A transient signature may indicate an infection that was acquired but has either been controlled or cleared. In this context, the observation that 26% of our IGRA^{-ve} cohort and in 50% of our IGRA^{+ve} cohort demonstrated this pattern suggests that the blood transcriptional signature represents immune responses that may precede priming and activation of IFN-γ producing CD4 T-cells. Finally, subjects with an evolving and persistent modular TB signature may represent subjects that have acquired an infection requiring active control to maintain latency. This pattern was seen in 7% of IGRA^{-ve} subjects and 12% of IGRA^{+ve}. These observations require validation in larger longitudinal cohorts, but do suggest that the blood transcriptome may offer a more sensitive approach to characterising the state of latent infection following TB exposure, with implications for better stratification of prospective TB risk.

For our cohort of 9 subjects identified with TB during prospective observation, a high or rising 20-gene signature score was observed in most. This was most apparent in the subjects defined as true progressors, who had no signature at baseline. Our study was limited by small numbers and the identification of TB within a short period of prospective observation, suggesting that incipient TB is likely to have been present at the time of baseline assessment in a proportion of cases. We are therefore presently unable to comment on the dynamic properties of this response or determine accurately the interval between the signature becoming detectable and manifestation of active TB. It is notable also that three subjects did not express a signature at any time point and yet went on to be diagnosed with TB. One of these was on anti-bacterial drugs which have known immunosuppressive properties, which could have diminished the signature. Additionally, interrogating the modules for these subjects indicates a weak transcriptional response that may suggest pathogen induced host immunomodulation, which is well recognised in active TB[16,46]. We are unable to determine whether a delayed transcriptional response would have developed over the natural time-course of infection as our rigorous protocol of frequent surveillance identified active disease at the earliest stage, however it is apparent that heterogeneity of the host immune response and its association with the state of *M.tuberculosis* requires further investigation.

573 A robust, objective definition of LTBI is unavailable. Patients with IGRA positivity have a
574 heterogeneous risk of developing TB and secondly, a proportion of patients that are IGRA
575 negative at screening proceed to develop TB in the future. It therefore follows that an IGRA
576 is not a reliable gold standard to determine the validity of new biomarkers. In this respect,
577 our observations of a TB like modular signature being expressed in both IGRA positive and
578 IGRA negative contacts of TB is not surprising. The difference between the groups in the
579 proportion of subjects expressing the signature (19% in $IGRA^{+ve}$ vs 7% in $IGRA^{-ve}$ is
580 comparable with the relative risk of TB according to IGRA status (Incident rate ratio for TB
581 2.11,[57]). This provides support for the validity of our observations and supports developing
582 a transcriptional biomarker for defining LTBI.
583
584 Here in summary, we have validated whole blood transcriptomic findings, previously
585 identified by microarray by RNA-sequencing of our previously published TB cohorts and a
586 new cohort from a low-TB-incidence setting. We further developed an advanced modular
587 signature of active TB, and validated it in our new cohort and a number of TB cohorts
588 published by other groups. Using this modular signature, we obtained a reduced TB-specific
589 20-gene signature which showed very high specificity and sensitivity in individuals with
590 active TB against those with LTBI and other diseases. Moreover, this signature did not
591 detect influenza, representative of many viral infections that share a strong IFN-inducible
592 signature, providing a proof-of-principle for the development of transcriptional biomarkers
593 for TB as diagnostics, with the aim of obtaining the highest sensitivity, whilst maintaining
594 specificity against LTBI and other diseases. Our findings highlight the need to consider
595 additional approaches to develop transcriptional biomarkers of the highest sensitivity and
596 specificity for distinguishing active TB from LTBI and other diseases. The reduced gene
597 signatures for discriminating active TB from LTBI and other infections, also demonstrated
598 important clinical outcomes and heterogeneity in LTBI. Our improved approach for the
599 development of diagnostic biomarkers consisting of reduced gene-sets, is broadly
600 applicable across diverse infectious and inflammatory diseases.

601 # Methods
602
603 **Study cohorts for analysis**
604
605 Cohorts analysed in Berry et al. 2010[9] using microarrays were subjected to RNA-Seq and
606 analysed as part of this study. Test and validation sets, termed Berry London and Berry
607 South Africa sets, respectively, based on the geographical location of patient recruitment,
608 were retained for RNA-seq analysis in this study (**Supplementary Figure 1a**).
609
610 An independent cohort was recruited (between 09/2015 and 09/2016) at the Glenfield
611 Hospital, University Hospitals of Leicester NHS Trust, Leicester, UK. The cohort consisted
612 of active TB patients (n=53) and recent close contacts (n=108). Patients who were
613 pregnant, immunosuppressed, had previous TB or previous treatment for LTBI were
614 excluded from this study. All participants had routine HIV testing and patients with a
615 positive result were excluded. Patients with active TB were confirmed by laboratory
616 isolation of *M. tuberculosis* on culture of a respiratory specimen (sputum or
617 bronchoalveloar lavage) with sensitivity testing performed by the Public Health Laboratory
618 Birmingham, Heart of England NHS Foundation Trust, Birmingham, UK. All recent close
619 contacts were IGRA tested using the QuantiFERON Gold In-Tube Assay (Qiagen) and were
620 subsequently categorised as either IGRA negative (n=50) or IGRA positive (n=49). All
621 participants were prospectively enrolled and sampled before the initiation of any anti-
622 mycobacterial treatment. A subset of subjects recruited initially as close contacts were
623 identified with active TB during longitudinal assessment (n=9), based on microbiological
624 confirmation of *M. tuberculosis* by culture or positive Xpert MTB/RIF (Cepheid).
625 (**Supplementary Tables 1** and **5**; **Figure 7a**). The Research Ethics Committee (REC) for East
626 Midlands – Nottingham 1, Nottingham, UK (REC 15/EM/0109) approved the study. All
627 participants were older than 16 years and gave written informed consent.
628
629
630 **RNA extraction and cDNA library preparation for RNA-Seq**
631
632 3 ml whole blood were collected by venepuncture into Tempus™ blood RNA tubes (Fisher
633 Scientific UK Ltd), tubes were mixed vigorously immediately after collection, and then
634 stored in a −80°C freezer prior to use. Total RNA was isolated from 1 ml whole blood using
635 the MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Applied Biosystems/Thermo
636 Fisher Scientific) according to the manufacturer's instructions. Globin RNA was depleted
637 from total RNA (1.5–2 µg) using the human GLOBINclear kit (Thermo Fisher Scientific)
638 according to manufacturer's instructions. RNA yield of total and globin-reduced RNA was
639 assessed using a NanoDrop™ 8000 spectrophotometer (Thermo Fisher Scientific). Quality
640 and integrity of total and globin-reduced RNA were assessed with the HT RNA Assay reagent
641 kit (Perkin Elmer) using a LabChip GX bioanalyser (Caliper Life Sciences/Perkin Elmer) and
642 assigned an RNA Quality Score (RQS). Samples (200 ng) with an RQS > 6 were used to prepare
643 a cDNA library using the TruSeq Stranded mRNA HT Library Preparation Kit (Illumina). The
644 tagged libraries were sized and quantitated in duplicate (Agilent TapeStation
645 system), using D1000 ScreenTape and reagents (Agilent), normalised, pooled and then
646 clustered using the HiSeq® 3000/4000 PE Cluster Kit (Illumina). The libraries were imaged
647 and sequenced on an Illumina HiSeq 4000 sequencer using the HiSeq® 3000/4000 SBS kit
648 (Illumina) at a minimum of 25 million paired end reads (75 bp) per sample.
649

RNA-seq data analysis

Raw paired-end RNA-seq data obtained for Berry London, Berry South Africa and Leicester cohorts was processed separately and subjected to quality control using FastQC (Babraham Bioinformatics) and MultiQC[58]. Trimmomatic[59] v0.36 was used to remove adapters and filter raw reads below the 36 bases long and leading and trailing bases below quality 25. Filtered reads were aligned to the *Homo sapiens* genome Ensembl GRCh38 (release 86) using HISAT2[60] v2.0.4 with default settings and RF rna-strandedness including unpaired read reads resulting from Trimmomatic. Mapped and aligned reads were quantified to obtain gene-level counts using HtSeq[61] v0.6.1 with default settings and reverse strandedness. Raw counts were processed using the *bioconductor* package *edgeR*[62] v3.14.0 in R. Genes expressed with counts per million (CPM) >2 in at least 5 samples were considered and normalised using trimmed mean of M- values (TMM) to remove library-specific artefacts. Only protein coding genes were considered for subsequent analyses. Differentially abundant genes were calculated using likelihood ratio tests in edgeR by fitting generalized linear models to the non-normally distributed RNA-seq data. Genes with $\log_2$ fold change >1 or <-1 and false discovery rate (FDR) p-value < 0.05 corrected for multiple testing using the Benjamini-Hochberg (BH) method[63] were considered significant. For subsequent analysis, voom transformation was applied to RNA-seq count data to obtain normalized expression values on the $\log_2$ scale. For Berry Combined dataset, raw counts from Berry London and South Africa cohorts were combined as one dataset and processed in edgeR as described above and batch effects were removed from $\log_2$ expression values using surrogate variable analysis (sva) using the *bioconductor* package *sva*[64] in R. RNA-seq data obtained from Zak et al. 2016[33] in the SRA format were converted to fastq files using the SRA toolkit and processed as above.

Microarray data analysis

External microarray datasets retrieved from GEO as non-normalized matrices were processed in GeneSpring GX v14.8 (Agilent Technologies). Flags were used to filter out probe sets that did not result in a 'present' call in at least 10% of the samples, with the 'present' lower cut-off of 0.8. Signal values were then set to a threshold level of 10, $\log_2$ transformed, and per-chip normalised using 75th percentile shift algorithm. Next per-gene normalisation was applied by dividing each messenger RNA transcript by the median intensity of all the samples. The training, test and validation sets in Bloom et al. 2013[10] were combined and batch effects were removed using *sva*[64]. In Kaforou et al. 2013[25], HIV+/- groups were combined and analysed as one dataset. In all datasets, multiple probes mapping to the same gene were removed and the probe with the highest inter-quartile range across all samples was retained, to match with the RNA-seq data. Differentially expressed genes were identified using the *bioconductor* package *limma*[65] in R and only genes with FDR p-value < 0.05 corrected for multiple testing using the BH method[63] were considered significant.

Gene signature enrichment analysis

Enrichment of TB gene signatures was carried out on a per sample basis using single sample Gene Set Enrichment Analysis (ssGSEA)[35] using the *bioconductor* package *gsva*[66] in R.

Enrichment scores were obtained similar to those from Gene Set Enrichment Analysis (GSEA) but based on absolute expression rather than differential expression[35], to quantify the degree to which a gene set is over-represented in a particular sample.

Weighted gene co-expression network analysis

Modular analysis was performed using the WGCNA package in R. Modules were constructed using the Berry Combined dataset (combined Berry London and South Africa sets) using 5,000 genes with highest covariance across all samples using $\log_2$ RNA-seq expression values. A signed weighted correlation matrix containing pairwise Pearson correlations between all genes across all samples was computed using a soft threshold of $\beta = 14$ to reach a scale-free topology. Using this adjacency matrix, the Topological Overlap Measure (TOM) was calculated, which measures the network interconnectedness and used as input to group highly correlated genes together using average linkage hierarchical clustering. The WGCNA dynamic hybrid tree-cut algorithm[67] was used to detect network modules of co-expressed genes, with a minimum module size of 20. All modules were assigned a colour arbitrarily and annotated using Ingenuity Pathway Analysis (IPA) (QIAGEN Bioinformatics) and Literature Lab (Acumenta Biotech, Massachusetts, USA). Literature Lab mines the PubMed literature and identifies significant associations in 20 MeSH (Medical Subject Headings) domains, including pathways, diseases and cell biology. Significantly enriched canonical pathways from IPA (p-value<0.05), and strongly associated terms from Literature Lab were obtained. Modules were assigned annotation terms based on pathways and processes that showed corroboration between both tools (**Supplementary Table 4**). Representative terms were then selected and assigned to modules (**Figure 4**). For each module, module eigengene (ME) values were calculated, which represent the first principal component of a given module and summarize the gene abundance profile in that module. For each module, top 50 hub genes with high intramodular connectivity and a minimum correlation of 0.75 were calculated and exported into Cytoscape v3.4.0 to create interaction networks.

WGCNA module enrichment analysis

Fold enrichment for the WGCNA modules was calculated using quantitative set analysis for gene expression (QuSAGE)[68] using the *bioconductor* package *qusage* in R, to identify the modules of genes over- or under-expressed in a dataset compared to a control group. Linear mixed models were incorporated in the analysis using QGen algorithm in QuSAGE, and patients in datasets with repeated measures were modelled as random effects. Only modules with FDR p-value < 0.05 were considered significant. To test the modules in microarray datasets, only those modules with a >70% match in genes symbols was present in the microarray dataset. To obtain a modular profile of a disease group, single sample enrichment scores were calculated using ssGSEA and the average enrichment score of the control group was subtracted from the average enrichment score of the disease group. To obtain a modular profile on a single sample basis, average enrichment score of the control group was subtracted from the enrichment score of the sample.

Class prediction

749 In order to develop a TB-specific gene signature, only genes significantly differentially
750 expressed in Berry London set and not in other flu cohorts were considered, from only those
751 modules that were perturbed in TB (a module was considered perturbed in TB if it followed
752 a similar profile (up or down compared to control) in at least 4 of the 5 TB datasets (Berry
753 London, Berry South Africa, Leicester cohort, Kaforou et al. 2013[25] and Zak et al. 2016[33]),
754 and given that for the 5th dataset the module did not reach significance when compared to
755 control). These genes were then reduced using the *Boruta*[42] package in R. Boruta is a
756 feature selection wrapper algorithm based on random forest and is particularly useful in
757 biomedical applications as it captures features by incorporating the outcome variable.
758 Next, the features identified as predictive using Boruta were ranked using the GINI score
759 in random forest and the top 20 genes were selected. For classifying patients as active TB
760 or latent TB, the random forest algorithm was used in *caret*[69] package in R, using LOOCV
761 over 1,000 iterations. Each of the TB datasets was randomly split into training (70%) and
762 test (30%) sets to classify patients as active TB or latent TB. For the Zhai et al. 2015[37], the
763 Influenza A group at Day 0 was randomly split into training (70%) and test (30%) sets to
764 classify patients as infected with Influenza A or healthy controls.
765
766
767 **Modified Disease Risk Score**
768
769 To test the TB-specific 20-gene signature, a modified version of the Disease Risk Score
770 (DRS) established by Kaforou et al. 2013[25] was used. Briefly, the DRS is obtained from
771 normalized data in a non-log space, by adding the total intensity of up-regulated transcripts
772 and subtracting the total intensity of down-regulated transcripts from a gene signature. In
773 this study, normalized CPM values were used for the RNA-seq data and non-log normalized
774 expression values were used for microarray data. As part of the modification of the DRS,
775 the absolute values of the total intensity of up-regulated transcripts and total intensity of
776 down-regulated transcripts were added to obtain a composite score.
777
778
779 **Deconvolution analysis**
780
781 Deconvolution analysis for quantification of relative levels of distinct cell types on a per
782 sample basis was carried out using CIBERSORT[70]. CIBERSORT estimates relative subsets of
783 RNA transcripts using linear support vector regression. Cell signatures for 22 cell types were
784 obtained using the LM22 database from CIBERSORT and grouped into 11 representative cell
785 types. Fractions of cell types were compared across different groups using One-way ANOVA,
786 and p-value < 0.05 was considered significant.
787
788
789 **Data availability**
790
791 **Novel data newly generated in this study:**
792 Sequence data generated for our study that support the findings have been deposited in
793 NCBI GEO database with the primary accession code GSE107995, and in BioProject with the
794 primary accession code PRJNA422124. Additional data which we have generated that
795 support the findings of our study are available upon request.
796
797 **Data we accessed from the literature and GEO for our study:**

798 The TB datasets from the literature that have been used in this study as comparators are
799 available in GEO with the primary accession codes GSE37250, GSE79362 also in BioProject
800 with the primary accession code PRJNA315611, and in SRA with the primary accession code
801 SRP071965, GSE20346, GSE68310, GSE42026, GSE60244 and GSE42834.

Acknowledgements

We acknowledge the Francis Crick Advanced Sequencing Facility, and Bioinformatics and Biostatistics Science Technology Platforms for their contribution to our sequencing processing. We acknowledge the NIHR Leicester Biomedical Research Centre for their support of the study at Leicester. The views expressed are those of the author(s) and not necessarily those of the NHS the NIHR or the Department of Health. We thank the patients for their participation. We thank Asmaà Fritah-Lafont for help in co-ordinating the meetings regarding the study. We thank Dr. Lúcia Moreira-Teixeira for reviewing the manuscript and for valuable discussion. AOG, CMG and AS were funded by The Francis Crick Institute, (Crick 10126; Crick 10468), which receives its core funding from Cancer Research UK, the U.K. Medical Research Council, and the Wellcome Trust; and the sequencing project by the BIOASTER Microbiology Technology Institute, Lyon, France; Medical Diagnostic Discovery Department, bioMérieux SA, Marcy l'Etoile, France; and funded in part by Illumina Inc., San Diego, CA, USA. RV and JL, University of Leicester, were funded by BIOASTER Microbiology Technology Institute, Lyon, France. This work has received, through BIOASTER investment, funding from the French Government through the Investissement d'Avenir program (Grant NO. ANR-10-AIRT-03). RJW was supported by The Francis Crick Institute, (Crick 10128), which receives its core funding from Cancer Research UK, the U.K. Medical Research Council, and Wellcome; by Wellcome (104803; 203135); MRC South Africa under strategic health innovation partnerships; and NIH 019 AI 111276.

Author contributions

AOG and PH co-led the whole study; AOG, MPRB, PH, RV, GW, MRo designed the study; RV and JL recruited TB, LTBI and contacts to the study for the Leicester cohort; CMG led and performed the RNA-Seq sample and raw data generation. RV and CMG helped to co-ordinate logistics of the study; RJW, PLei, PLec, KK gave feedback and concrete discussion during the study; TT and MRi contributed towards feedback on bioinformatics analysis; AS led and performed all the bioinformatics analysis;. AOG, AS, RV and PH wrote the manuscript and RJW gave substantial input; all co-authors have read, reviewed and approved the paper.

ORCID IDs: 0000-0001-9845-6134 (AOG); 0000-0002-6941-3618 (AS)

Competing interests

The authors declare no competing interests and note that previous patents held by Anne O'Garra on the use of the blood transcriptomic for diagnosis of tuberculosis have lapsed and discontinued. Neither bioMérieux nor BIOASTER have filed patents related to this study.

843 # References
844

845 1 World Health Organisation. Global TB Report. (2015).
846 2 Pfyffer, G. E., Cieslak, C., Welscher, H. M., Kissling, P. & Rusch-Gerdes, S. Rapid
847 detection of mycobacteria in clinical specimens by using the automated BACTEC
848 9000 MB system and comparison with radiometric and solid-culture systems. J Clin
849 Microbiol 35, 2229-2234 (1997).
850 3 Boehme, C. C. *et al*. Rapid molecular detection of tuberculosis and rifampin
851 resistance. N Engl J Med 363, 1005-1015 (2010).
852 4 CCDC. Center for Communicable Disease Control and Prevention. Reported
853 Tuberculosis in the United States, 2007. C. U.S. Department of Health and Human
854 Services. Atlanta, GA. (2007).
855 5 Vynnycky, E. & Fine, P. E. Lifetime risks, incubation period, and serial interval of
856 tuberculosis. Am J Epidemiol 152, 247-263 (2000).
857 6 Abu-Raddad, L. J. *et al*. Epidemiological benefits of more-effective tuberculosis
858 vaccines, drugs, and diagnostics. Proc Natl Acad Sci U S A 106, 13980-13985 (2009).
859 7 Barry, C. E., 3rd *et al*. The spectrum of latent tuberculosis: rethinking the biology
860 and intervention strategies. Nat Rev Microbiol 7, 845-855 (2009).
861 8 Esmail, H. *et al*. Characterization of progressive HIV-associated tuberculosis using 2-
862 deoxy-2-[18F]fluoro-D-glucose positron emission and computed tomography. Nat
863 Med 22, 1090-1093 (2016).
864 9 Berry, M. P. *et al*. An interferon-inducible neutrophil-driven blood transcriptional
865 signature in human tuberculosis. Nature 466, 973-977 (2010).
866 10 Bloom, C. I. *et al*. Transcriptional blood signatures distinguish pulmonary
867 tuberculosis, pulmonary sarcoidosis, pneumonias and lung cancers. PLoS One 8,
868 e70630 (2013).
869 11 Yan, N. & Chen, Z. J. Intrinsic antiviral immunity. Nat Immunol 13, 214-222 (2012).
870 12 McNab, F. *et al*. Type I IFN induces IL-10 production in an IL-27-independent manner
871 and blocks responsiveness to IFN-γ for production of IL-12 and bacterial killing in
872 Mycobacterium tuberculosis-infected macrophages. J Immunol (2014).
873 13 McNab, F. W. *et al*. TPL-2-ERK1/2 signaling promotes host resistance against
874 intracellular bacterial infection by negative regulation of type I IFN production. J
875 Immunol 191, 1732-1743 (2013).
876 14 Redford, P. S. *et al*. Influenza A virus impairs control of Mycobacterium tuberculosis
877 coinfection through a type I interferon receptor-dependent pathway. J Infect Dis
878 209, 270-274 (2014).
879 15 McNab, F., Mayer-Barber, K., Sher, A., Wack, A. & O'Garra, A. Type I interferons in
880 infectious disease. Nat Rev Immunol 15, 87-103 (2015).
881 16 O'Garra, A. *et al*. The immune response in tuberculosis. Annu Rev Immunol 31, 475-
882 527 (2013).
883 17 Antonelli, L. R. *et al*. Intranasal Poly-IC treatment exacerbates tuberculosis in mice
884 through the pulmonary recruitment of a pathogen-permissive
885 monocyte/macrophage population. J Clin Invest 120, 1674-1682 (2010).
886 18 Dorhoi, A. *et al*. Type I IFN signaling triggers immunopathology in tuberculosis-
887 susceptible mice by modulating lung phagocyte dynamics. Eur J Immunol 44, 2380-
888 2393 (2014).
889 19 Manca, C. *et al*. Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice
890 is determined by failure to induce Th1 type immunity and is associated with
891 induction of IFN-a/b. Proc Natl Acad Sci U S A 98, 5752-5757 (2001).

892 20 Manca, C. *et al*. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I
893 IFNs and increase expression of negative regulators of the Jak-Stat pathway. J
894 Interferon Cytokine Res 25, 694-701 (2005).
895 21 Mayer-Barber, K. D. *et al*. Host-directed therapy of tuberculosis based on
896 interleukin-1 and type I interferon crosstalk. Nature 511, 99-103 (2014).
897 22 McNab, F. W. *et al*. Type I IFN induces IL-10 production in an IL-27-independent
898 manner and blocks responsiveness to IFN-gamma for production of IL-12 and
899 bacterial killing in Mycobacterium tuberculosis-infected macrophages. J Immunol
900 193, 3600-3612 (2014).
901 23 Ordway, D. *et al*. The hypervirulent Mycobacterium tuberculosis strain HN878
902 induces a potent TH1 response followed by rapid down-regulation. J Immunol 179,
903 522-531 (2007).
904 24 Joosten, S. A., Fletcher, H. A. & Ottenhoff, T. H. A helicopter perspective on TB
905 biomarkers: pathway and process based analysis of gene expression data provides
906 new insight into TB pathogenesis. PLoS One 8, e73230 (2013).
907 25 Kaforou, M. *et al*. Detection of tuberculosis in HIV-infected and-uninfected African
908 adults using whole blood RNA expression signatures: a case-control study. PLoS
909 medicine 10, e1001538 (2013).
910 26 Maertzdorf, J. *et al*. Human gene expression profiles of susceptibility and resistance
911 in tuberculosis. Genes and immunity 12, 15-22 (2011).
912 27 Ottenhoff, T. H. *et al*. Genome-wide expression profiling identifies type 1 interferon
913 response pathways in active tuberculosis. PLoS One 7, e45839 (2012).
914 28 Roe, J. K. *et al*. Blood transcriptomic diagnosis of pulmonary and extrapulmonary
915 tuberculosis. JCI Insight 1, e87238 (2016).
916 29 Walter, N. D. *et al*. Blood Transcriptional Biomarkers for Active Tuberculosis among
917 Patients in the United States: a Case-Control Study with Systematic Cross-Classifier
918 Evaluation. J Clin Microbiol 54, 274-282 (2016).
919 30 Walter, N. D., Reves, R. & Davis, J. L. Blood transcriptional signatures for
920 tuberculosis diagnosis: a glass half-empty perspective. Lancet Respir Med 4, e28
921 (2016).
922 31 Blankley, S. *et al*. A 380-gene meta-signature of active tuberculosis compared with
923 healthy controls. Eur Respir J 47, 1873-1876 (2016).
924 32 Blankley, S. *et al*. The Transcriptional Signature of Active Tuberculosis Reflects
925 Symptom Status in Extra-Pulmonary and Pulmonary Tuberculosis. PLoS One 11,
926 e0162220 (2016).
927 33 Zak, D. E. *et al*. A blood RNA signature for tuberculosis disease risk: a prospective
928 cohort study. The Lancet 387, 2312-2322 (2016).
929 34 Diel, R., Loddenkemper, R. & Nienhaus, A. Evidence-based comparison of
930 commercial interferon-gamma release assays for detecting active TB: a
931 metaanalysis. Chest 137, 952-968 (2010).
932 35 Barbie, D. A. *et al*. Systematic RNA interference reveals that oncogenic KRAS-driven
933 cancers require TBK1. Nature 462, 108 (2009).
934 36 Parnell, G. *et al*. Aberrant cell cycle and apoptotic changes characterise severe
935 influenza A infection–a meta-analysis of genomic signatures in circulating
936 leukocytes. Plos one 6, e17186 (2011).
937 37 Zhai, Y. *et al*. Host transcriptional response to influenza and other acute respiratory
938 viral infections–a prospective cohort study. PLoS pathogens 11, e1004869 (2015).
939 38 Langfelder, P. & Horvath, S. WGCNA: an R package for weighted correlation network
940 analysis. BMC Bioinformatics 9, 559 (2008).

941 39 Herberg, J. A. *et al*. Transcriptomic profiling in childhood H1N1/09 influenza reveals
942 reduced expression of protein synthesis genes. The Journal of infectious diseases
943 208, 1664-1668 (2013).
944 40 Suarez, N. M. *et al*. Superiority of transcriptional profiling over procalcitonin for
945 distinguishing bacterial from viral lower respiratory tract infections in hospitalized
946 adults. The Journal of infectious diseases 212, 213-222 (2015).
947 41 Friedman, J. H. Stochastic gradient boosting. Computational Statistics & Data
948 Analysis 38, 367-378 (2002).
949 42 Kursa, M. B. & Rudnicki, W. R. Feature selection with the Boruta package. J Stat
950 Softw 36, 1-13 (2010).
951 43 Zou, H. & Hastie, T. Regularization and variable selection via the elastic net. Journal
952 of the Royal Statistical Society: Series B (Statistical Methodology) 67, 301-320
953 (2005).
954 44 Sweeney, T. E., Braviak, L., Tato, C. M. & Khatri, P. Genome-wide expression for
955 diagnosis of pulmonary tuberculosis: a multicohort analysis. Lancet Respir Med 4,
956 213-224 (2016).
957 45 Maertzdorf, J. *et al*. Concise gene signature for point-of-care classification of
958 tuberculosis. EMBO Mol Med 8, 86-95 (2016).
959 46 Cooper, A. M. Cell-mediated immune responses in tuberculosis. Annu Rev Immunol
960 27, 393-422 (2009).
961 47 Altare, F. *et al*. Impairment of mycobacterial immunity in human interleukin-12
962 receptor deficiency. Science (New York, N.Y.) 280, 1432-1435 (1998).
963 48 Casanova, J. L. & Abel, L. Genetic dissection of immunity to mycobacteria: the
964 human model. Annu Rev Immunol 20, 581-620 (2002).
965 49 de Jong, R. *et al*. Severe mycobacterial and Salmonella infections in interleukin-12
966 receptor-deficient patients. Science (New York, N.Y.) 280, 1435-1438 (1998).
967 50 Fortin, A., Abel, L., Casanova, J. L. & Gros, P. Host genetics of mycobacterial
968 diseases in mice and men: forward genetic studies of BCG-osis and tuberculosis.
969 Annu Rev Genomics Hum Genet 8, 163-192 (2007).
970 51 Jouanguy, E. *et al*. A human IFNGR1 small deletion hotspot associated with dominant
971 susceptibility to mycobacterial infection. Nature genetics 21, 370-378 (1999).
972 52 Newport, M. J. *et al*. A mutation in the interferon-gamma-receptor gene and
973 susceptibility to mycobacterial infection. The New England journal of medicine 335,
974 1941-1949 (1996).
975 53 Cliff, J. M., Kaufmann, S. H., McShane, H., van Helden, P. & O'Garra, A. The human
976 immune response to tuberculosis and its treatment: a view from the blood. Immunol
977 Rev 264, 88-102 (2015).
978 54 Bloom, C. I. *et al*. Detectable changes in the blood transcriptome are present after
979 two weeks of antituberculosis therapy. PLoS One 7, e46191 (2012).
980 55 Cliff, J. M. *et al*. Distinct phases of blood gene expression pattern through
981 tuberculosis treatment reflect modulation of the humoral immune response. J Infect
982 Dis 207, 18-29 (2013).
983 56 Joosten, S. A. *et al*. Identification of biomarkers for tuberculosis disease using a
984 novel dual-color RT-MLPA assay. Genes and immunity 13, 71-82 (2012).
985 57 Rangaka, M. X. *et al*. Predictive value of interferon-gamma release assays for
986 incident active tuberculosis: a systematic review and meta-analysis. Lancet Infect
987 Dis 12, 45-55 (2012).
988 58 Ewels, P., Magnusson, M., Lundin, S. & Kaller, M. MultiQC: summarize analysis results
989 for multiple tools and samples in a single report. Bioinformatics 32, 3047-3048
990 (2016).

991 59 Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina
992 sequence data. Bioinformatics 30, 2114-2120 (2014).
993 60 Kim, D., Langmead, B. & Salzberg, S. L. HISAT: a fast spliced aligner with low
994 memory requirements. Nat Methods 12, 357-360 (2015).
995 61 Anders, S., Pyl, P. T. & Huber, W. HTSeq--a Python framework to work with high-
996 throughput sequencing data. Bioinformatics 31, 166-169 (2015).
997 62 Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for
998 differential expression analysis of digital gene expression data. Bioinformatics 26,
999 139-140 (2010).
1000 63 Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and
1001 powerful approach to multiple testing. Journal of the royal statistical society. Series
1002 B (Methodological), 289-300 (1995).
1003 64 Leek, J. T., Johnson, W. E., Parker, H. S., Jaffe, A. E. & Storey, J. D. SVA: surrogate
1004 variable analysis. R package version 3 (2013).
1005 65 Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-
1006 sequencing and microarray studies. Nucleic Acids Res 43, e47 (2015).
1007 66 Hanzelmann, S., Castelo, R. & Guinney, J. GSVA: gene set variation analysis for
1008 microarray and RNA-seq data. BMC Bioinformatics 14, 7 (2013).
1009 67 Langfelder, P., Zhang, B. & Horvath, S. Defining clusters from a hierarchical cluster
1010 tree: the Dynamic Tree Cut package for R. Bioinformatics 24, 719-720 (2008).
1011 68 Yaari, G., Bolen, C. R., Thakar, J. & Kleinstein, S. H. Quantitative set analysis for
1012 gene expression: a method to quantify gene set differential expression including
1013 gene-gene correlations. Nucleic acids research 41, e170-e170 (2013).
1014 69 Kuhn, M. Caret: classification and regression training. Astrophysics Source Code
1015 Library (2015).
1016 70 Newman, A. M. *et al.* Robust enumeration of cell subsets from tissue expression
1017 profiles. Nat Methods 12, 453-457 (2015).

1018

1019

Figure 1. The objectives of this study. An overview of the analysis undertaken in the study. Figures associated with each objective are stated below the box.

Figure 2. Whole-blood transcriptional gene signatures in TB. a Heatmaps depicting unsupervised hierarchical clustering of active TB (red), LTBI (black) and control samples (purple) using a 373-gene signature derived using the Berry London cohort, tested in the Berry South Africa cohort, and **(b)** validated in an independent cohort from Leicester. Gene expression values were averaged and scaled across the row to indicate the number of standard deviations above (red) or below (blue) the mean, denoted as row Z-score. **c** Bar graphs depicting enrichment scores derived on a single sample basis using ssGSEA in the Berry London, Berry South Africa and Leicester cohorts using the 16-gene signature from Zak et al. 2016[33]. Purple, black and red bars represent control, LTBI and active TB samples, respectively, and * (control outliers), # (LTBI outliers) and § (active TB outliers) represent outlier samples identified by hierarchical clustering.

Figure 3. Enrichment of published reduced TB gene signatures in TB, and other viral and bacterial infections. a Box plots depicting enrichment scores derived on a single sample basis using ssGSEA, using the 16-gene signature from Zak et al. 2016[33], and the 27-gene (TB vs. LTBI) and 44-gene (TB vs. other diseases (OD)) signatures from Kaforou et al. 2013[25] in tuberculosis datasets (Berry London, Berry South Africa and Leicester), and **(b)** in datasets of other infections – severe influenza from Parnell et al.[36], Influenza A from Zhai et al.[37], and bacterial pneumonia from Parnell et al.[36]. The box represents 25th to 75th percentile, with a line inside the box indicating the median, and the whiskers represent the minimum to the maximum points in the data.

Figure 4. Modular transcriptional signatures of TB and other diseases. a Twenty-three modules of co-expressed genes derived using WGCNA from Combined Berry dataset (London & South Africa) and tested in other TB datasets, and **(b)** datasets with additional diseases. Fold enrichment scores derived using QuSAGE are depicted, with red and blue indicating modules over- or under-expressed compared to controls. Colour intensity and size represent the degree of enrichment compared to controls. Only modules with fold enrichment scores with FDR p-value < 0.05 were considered significant and depicted here. §, fold enrichment scores in the lightgreen module greater than the maximum score depicted on the scale (i.e. >1.3) in severe influenza (Parnell et al.[36], score: 1.55) and influenza A (Zhai et al.[37], score: 1.97).

Figure 5. Gene expression in the yellow module in TB compared to other viral and bacterial infections. a Log_2 fold changes for genes in the yellow module from Berry London cohort (active TB vs. controls; *y-axis*) compared to log_2 fold changes in other datasets (respective cases vs. controls; *x-axis*) in TB, and **(b)** other infections (Herberg et al. 2013[39], Suarez et al. 2015[40], time-course data from Zhai et al. 2015[37] (influenza A) and Parnell et al. 2011[36] (severe influenza and bacterial pneumonia)). Shapes and colours represent significantly differentially expressed genes (FDR p-value < 0.05) in either Berry London only (orange squares), respective dataset only (cyan diamonds), both dataset (yellow circles) or significant in neither (black triangles).

Figure 6. Whole-blood TB-specific 20-gene signature tested in TB and other infections. a A reduced 20-gene signature of TB derived from the TB modular signature using genes significantly differentially expressed in Berry London cohort only, and not in other flu datasets **(Supplementary Figure 6 5)**. **b** Box plots depicting the modified Disease Risk Scores derived using the TB-specific 20-gene signature in TB datasets, and in **(c)** datasets of other infections. The box represents 25th to 75th percentile, with a line inside the box indicating the median, and the whiskers represent the minimum to the maximum points in the data.

Figure 7. Comparison of our TB-specific 20-gene signature with Kafourou's in distinguishing TB and other diseases. a Receiver operating characteristic (ROC) curves depicting the predictive potential of the TB-specific 20-gene signature and the 44-gene (TB vs. other diseases (OD)) signature from Kaforou et al. 2013[25] in classifying a sample as TB or LTBI/Control, or **(b)** in classifying a sample as TB or other disease, in datasets from Kaforou et al. 2013[25], Roe et al. 2016[28] and Bloom et al. 2013[10]. Area under the curve (AUC) is shown for each ROC curve.

Figure 8. Comparison of our TB-specific 20-gene signature and others in distinguishing TB and influenza. a Receiver operating characteristic curves (ROC) depicting the predictive potential of the TB-specific 20-gene signature and the other published gene signatures in classifying a sample as TB or LTBI in the Berry London, Berry South Africa and Leicester cohorts, and **(b)** in classifying a sample as influenza A or control in the Zhai et al. 2015[37] dataset. Area under the curve (AUC) is shown for each ROC curve.

Figure 9. Blood transcriptional profile of LTBI outliers compared with active TB. a Modules of co-expressed genes tested in LTBI-Outliers from the Combined Berry and Leicester cohorts. Fold enrichment scores derived using QuSAGE are depicted, with red and blue indicating modules over- or under-expressed compared to controls. Colour intensity and size represent the degree of enrichment compared to controls. Only modules with fold enrichment scores with FDR p-value < 0.05 were considered significant and depicted here. **b** Gene networks depicting the top 50 'hub' genes, i.e. genes with high intramodular connectivity, for the yellow, lightgreen and **(c)** tan modules. Each gene is represented as a square node with edges representing correlation between the gene expression profiles of the two respective genes (minimum Pearson correlation of 0.75). A key describing the four different partitions within each square node is shown, with each partition representing $\log_2$ fold changes for active TB (without outliers) and LTBI–Outliers from the Berry Combined and Leicester cohorts, compared to respective controls (without outliers). Red and blue represent up- and down-regulated genes, respectively. In the tan module, the expression for IFNG is also shown, although it was not one of the top 50 hub genes within that module. Boxplots depicting the module eigengene expression, i.e. the first principal component for all genes within the module, are shown below each gene network. **d** Volcano plots depicting differentially expressed genes for active TB (without outliers) and LTBI–Outliers in the Berry Combined and Leicester cohorts, compared to respective LTBI (without outliers). Significantly differentially expressed genes ($\log_2$ fold change >1 or <-1, and FDR p-value < 0.05) are represented as red (upregulated) or blue (downregulated) dots, along with a Venn diagram and table summarising overlaps between these different comparisons.

1120
1121
1122 **Figure 10. Blood transcriptional profile of TB contacts followed over time. a** Schematic
1123 diagram representing active TB patients from the Leicester cohort, and their contacts
1124 followed over time. Purple, black and red represent $IGRA^{-ve}$ (controls), $IGRA^{+ve}$ (LTBI) and
1125 active TB patients, respectively. **b** Bar plots depicting the modified Disease Risk Scores
1126 using the TB-specific 20-gene signature, in TB contacts who remained $IGRA^{-ve}$ and did not
1127 develop TB (n=15), TB contacts who remained $IGRA^{+ve}$ and did not develop TB (n=16), and
1128 TB contacts who developed TB during the study (n=9). For TB contacts who developed TB
1129 during the study, the time point when the contact was diagnosed with active TB in the
1130 clinic is represented by a red bar. Baseline in the barplot is set at 766.64, average of all
1131 Baseline time-point modified Disease Risk Scores from all $IGRA^{-ve}$ contacts (n=15).

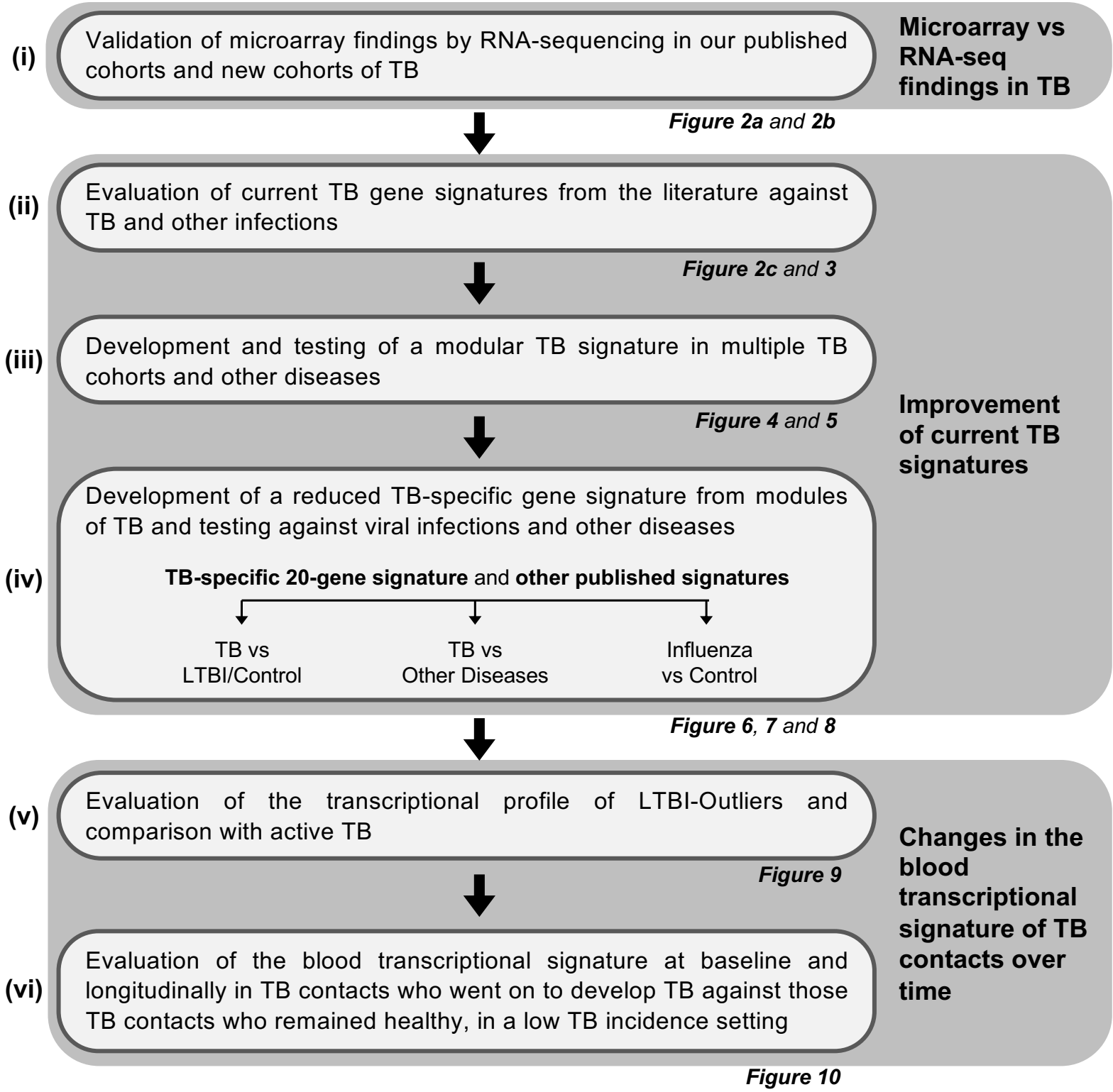

(i)
Validation of microarray findings by RNA-sequencing in our published cohorts and new cohorts of TB
Microarray vs RNA-seq findings in TB
Figure 2a and 2b
(ii)
Evaluation of current TB gene signatures from the literature against TB and other infections
Figure 2c and 3
(iii)
Development and testing of a modular TB signature in multiple TB cohorts and other diseases
Figure 4 and 5
(iv)
Development of a reduced TB-specific gene signature from modules of TB and testing against viral infections and other diseases
TB-specific 20-gene signature and other published signatures
TB vs LTBI/Control
TB vs Other Diseases
Influenza vs Control
Improvement of current TB signatures
Figure 6, 7 and 8
(v)
Evaluation of the transcriptional profile of LTBI-Outliers and comparison with active TB
Figure 9
(vi)
Evaluation of the blood transcriptional signature at baseline and longitudinally in TB contacts who went on to develop TB against those TB contacts who remained healthy, in a low TB incidence setting
Changes in the blood transcriptional signature of TB contacts over time
Figure 10

Figure 1

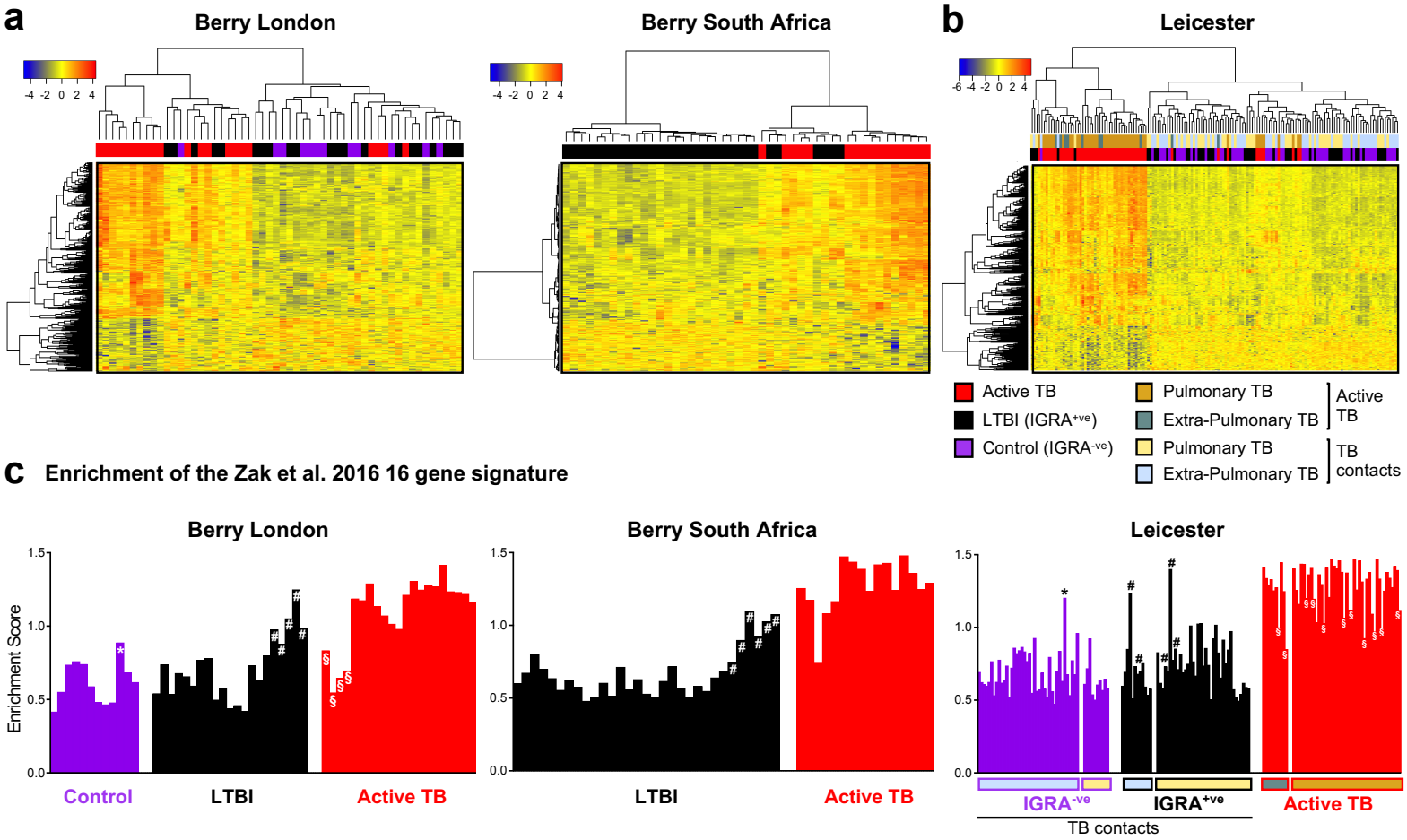

a
Berry London
Berry South Africa
b
Leicester
Active TB
LTBI (IGRA+ve)
Control (IGRA-ve)
Pulmonary TB
Extra-Pulmonary TB
Active TB
Pulmonary TB
Extra-Pulmonary TB
TB contacts
c
Enrichment of the Zak et al. 2016 16 gene signature
Berry London
Berry South Africa
Leicester
Enrichment Score
Control
LTBI
Active TB
LTBI
Active TB
IGRA-ve
IGRA+ve
Active TB
TB contacts

Figure 2

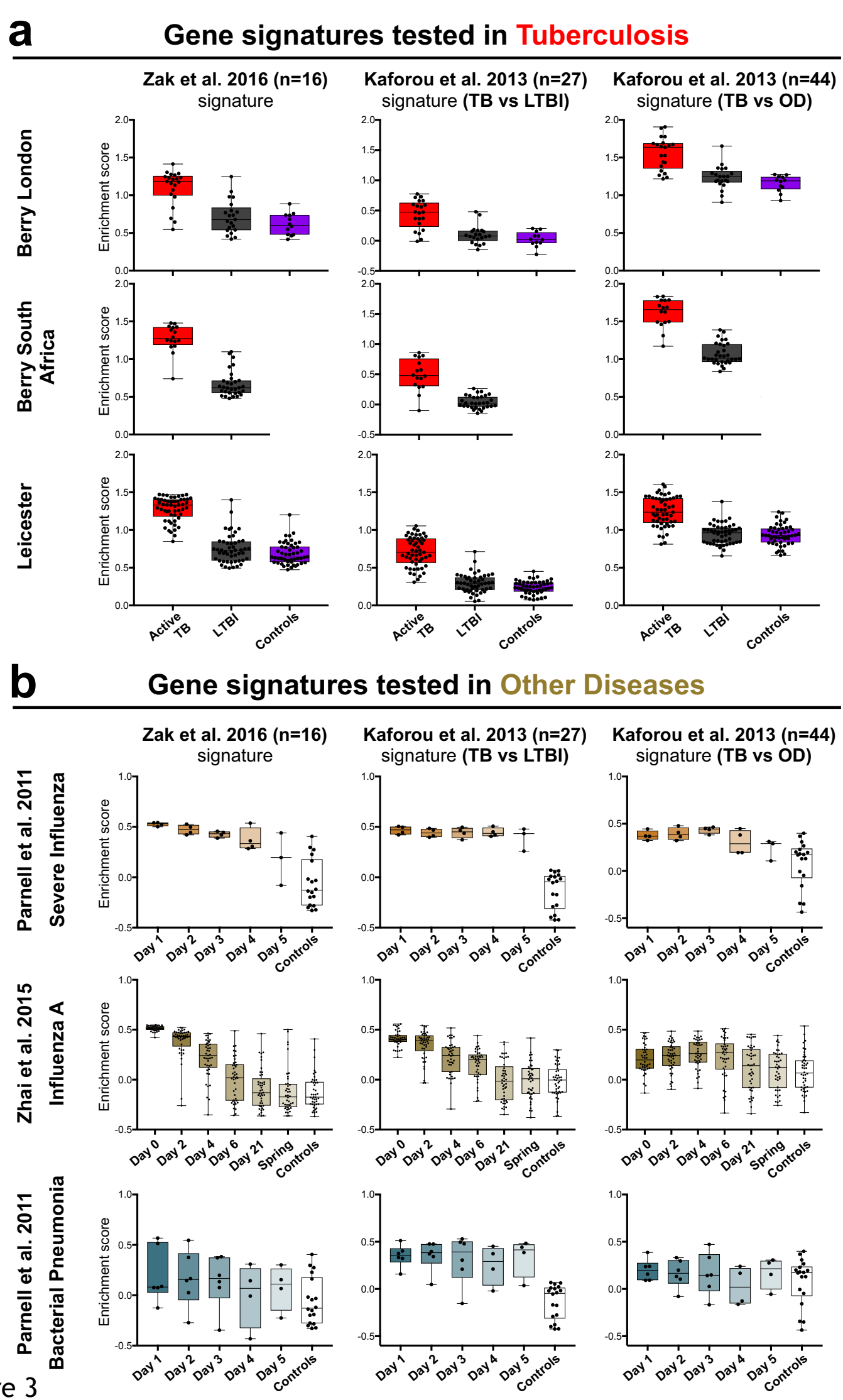

a
Gene signatures tested in Tuberculosis
Zak et al. 2016 (n=16) signature
Kaforou et al. 2013 (n=27) signature (TB vs LTBI)
Kaforou et al. 2013 (n=44) signature (TB vs OD)
Berry London
Berry South Africa
Leicester
Enrichment score
Active TB
LTBI
Controls
b
Gene signatures tested in Other Diseases
Zak et al. 2016 (n=16) signature
Kaforou et al. 2013 (n=27) signature (TB vs LTBI)
Kaforou et al. 2013 (n=44) signature (TB vs OD)
Parnell et al. 2011 Severe Influenza
Zhai et al. 2015 Influenza A
Parnell et al. 2011 Bacterial Pneumonia
Enrichment score
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 21
Spring
Controls

Figure 3

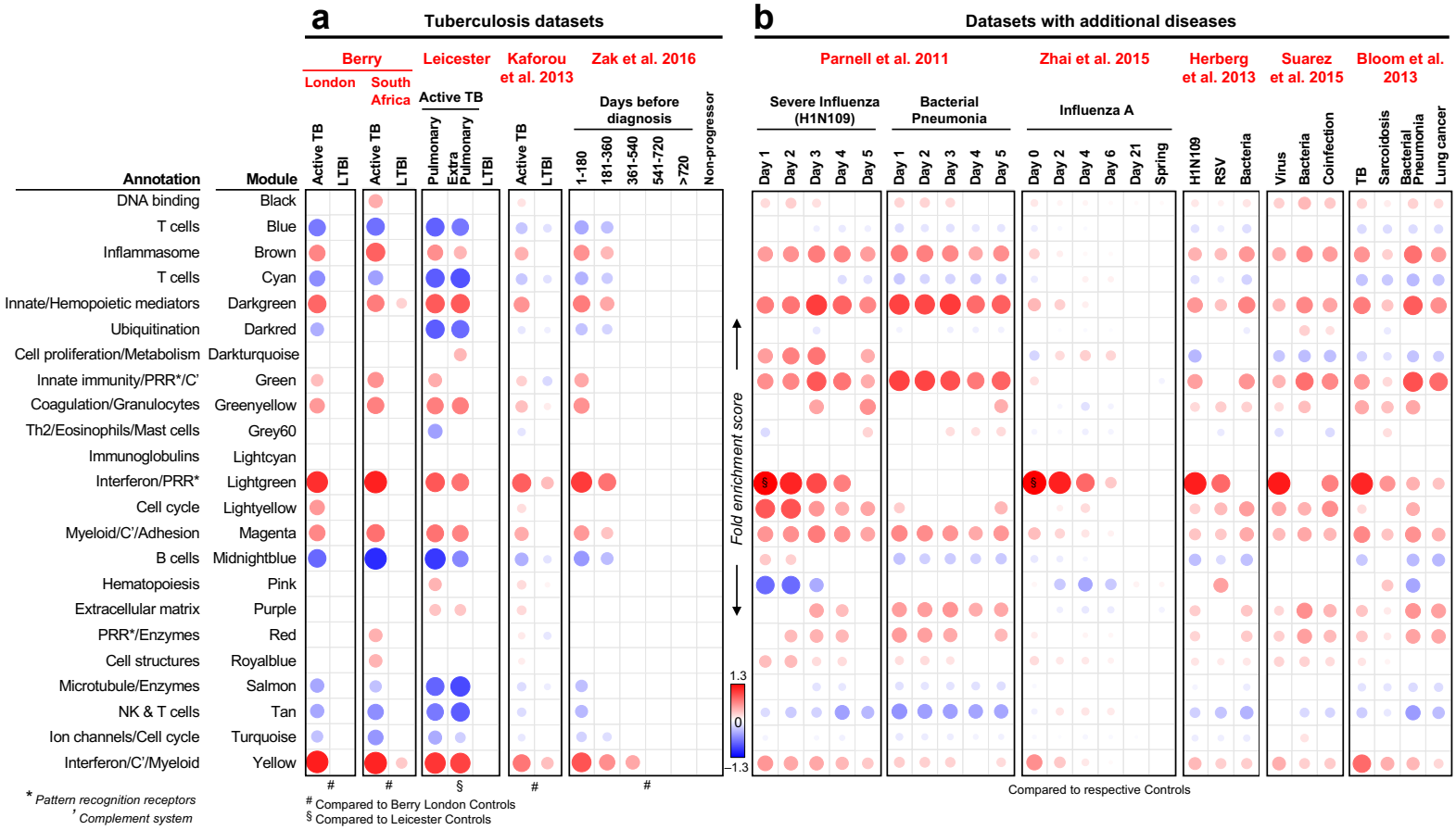

a
Tuberculosis datasets
b
Datasets with additional diseases
Berry
London
South Africa
Leicester
Active TB
Kaforou et al. 2013
Zak et al. 2016
Days before diagnosis
Parnell et al. 2011
Severe Influenza (H1N109)
Bacterial Pneumonia
Zhai et al. 2015
Influenza A
Herberg et al. 2013
Suarez et al. 2015
Bloom et al. 2013
Active TB
LTBI
Active TB
LTBI
Pulmonary
Extra Pulmonary
LTBI
Active TB
LTBI
1-180
181-360
361-540
541-720
>720
Non-progressor
Day 1
Day 2
Day 3
Day 4
Day 5
Day 1
Day 2
Day 3
Day 4
Day 5
Day 0
Day 2
Day 4
Day 6
Day 21
Spring
H1N109
RSV
Bacteria
Virus
Bacteria
Coinfection
TB
Sarcoidosis
Bacterial Pneumonia
Lung cancer
Annotation
Module
DNA binding Black
T cells Blue
Inflammasome Brown
T cells Cyan
Innate/Hemopoietic mediators Darkgreen
Ubiquitination Darkred
Cell proliferation/Metabolism Darkturquoise
Innate immunity/PRR*/C' Green
Coagulation/Granulocytes Greenyellow
Th2/Eosinophils/Mast cells Grey60
Immunoglobulins Lightcyan
Interferon/PRR* Lightgreen
Cell cycle Lightyellow
Myeloid/C'/Adhesion Magenta
B cells Midnightblue
Hematopoiesis Pink
Extracellular matrix Purple
PRR*/Enzymes Red
Cell structures Royalblue
Microtubule/Enzymes Salmon
NK & T cells Tan
Ion channels/Cell cycle Turquoise
Interferon/C'/Myeloid Yellow
Fold enrichment score
1.3
0
−1.3
Compared to Berry London Controls
§ Compared to Leicester Controls
Compared to respective Controls
* Pattern recognition receptors
' Complement system

Figure 4

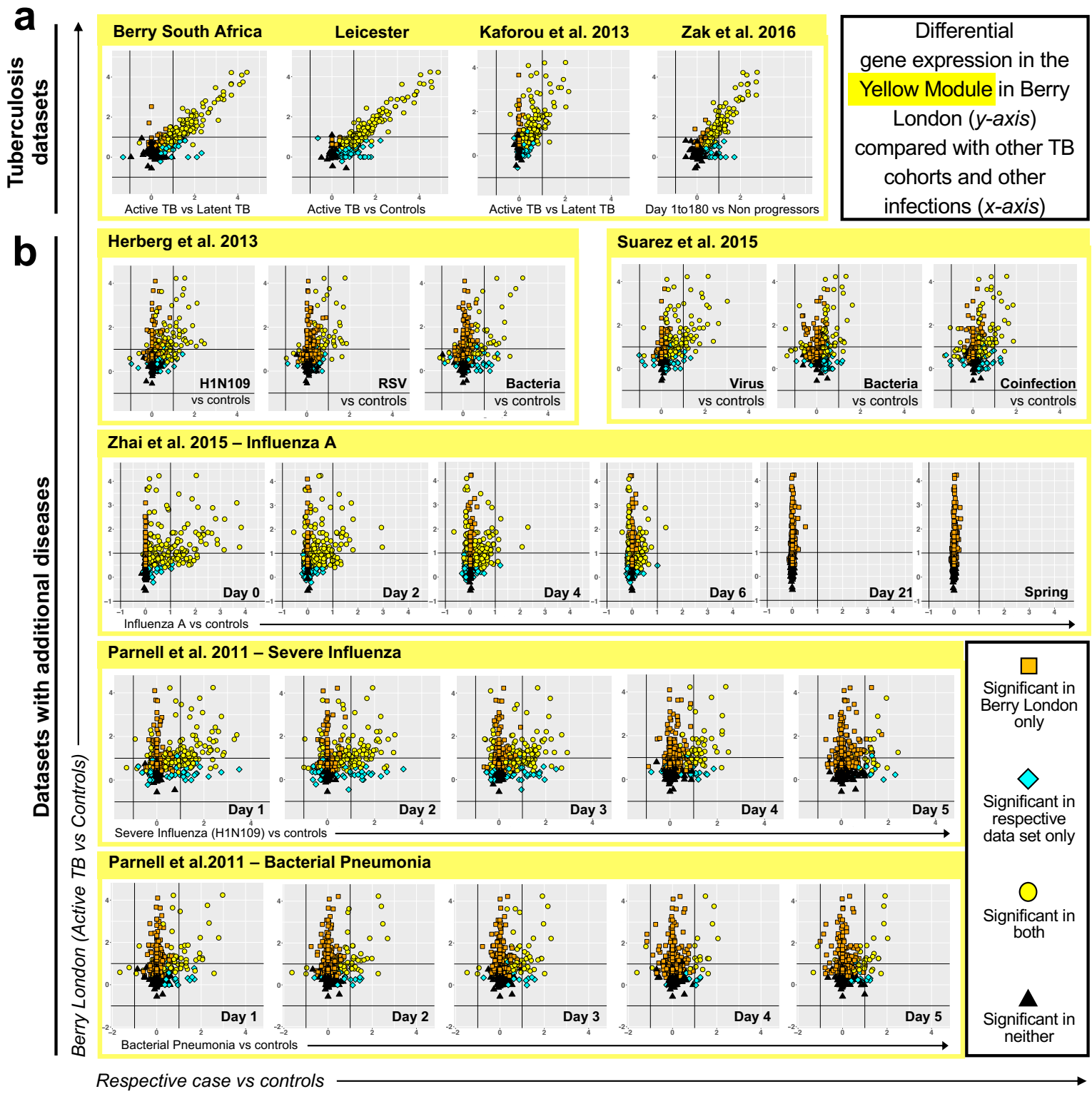
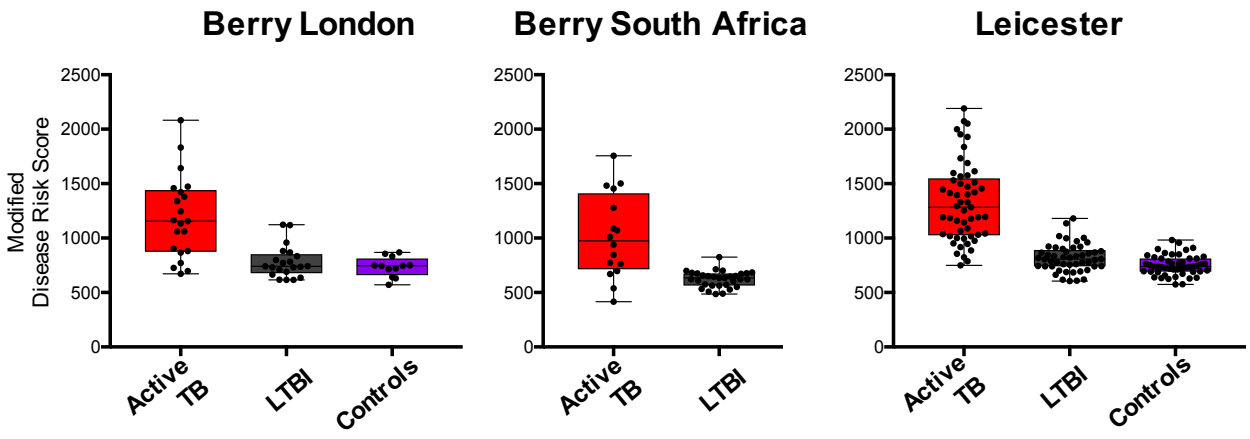

a
Tuberculosis datasets
Berry South Africa
Leicester
Kaforou et al. 2013
Zak et al. 2016
Active TB vs Latent TB
Active TB vs Controls
Active TB vs Latent TB
Day 1to180 vs Non progressors
Differential gene expression in the Yellow Module in Berry London (y-axis) compared with other TB cohorts and other infections (x-axis)
b
Datasets with additional diseases
Herberg et al. 2013
H1N109 vs controls
RSV vs controls
Bacteria vs controls
Suarez et al. 2015
Virus vs controls
Bacteria vs controls
Coinfection vs controls
Zhai et al. 2015 – Influenza A
Day 0
Day 2
Day 4
Day 6
Day 21
Spring
Influenza A vs controls
Parnell et al. 2011 – Severe Influenza
Day 1
Day 2
Day 3
Day 4
Day 5
Severe Influenza (H1N109) vs controls
Parnell et al.2011 – Bacterial Pneumonia
Day 1
Day 2
Day 3
Day 4
Day 5
Bacterial Pneumonia vs controls
Significant in Berry London only
Significant in respective data set only
Significant in both
Significant in neither
Berry London (Active TB vs Controls)
Respective case vs controls

Figure 5

a TB-specific 20-gene signature

- *APOL4*
- *ARHGEF9*
- *ARNTL2*
- *BACH2*
- *BDH1*
- *CCDC120*
- *COL4A4*
- *CTSB*
- *ICAM1*
- *KCNC4*
- *LIMK1*
- *PAIP2B*
- *SCARF1*
- *SMYD5*
- *SPHK1*
- *TMEM25*
- *TRAF4*
- *TRIM47*
- *USP54*
- *VEGFB*

b TB-specific 20-gene signature tested in Tuberculosis



c TB-specific 20-gene signature tested in Other diseases

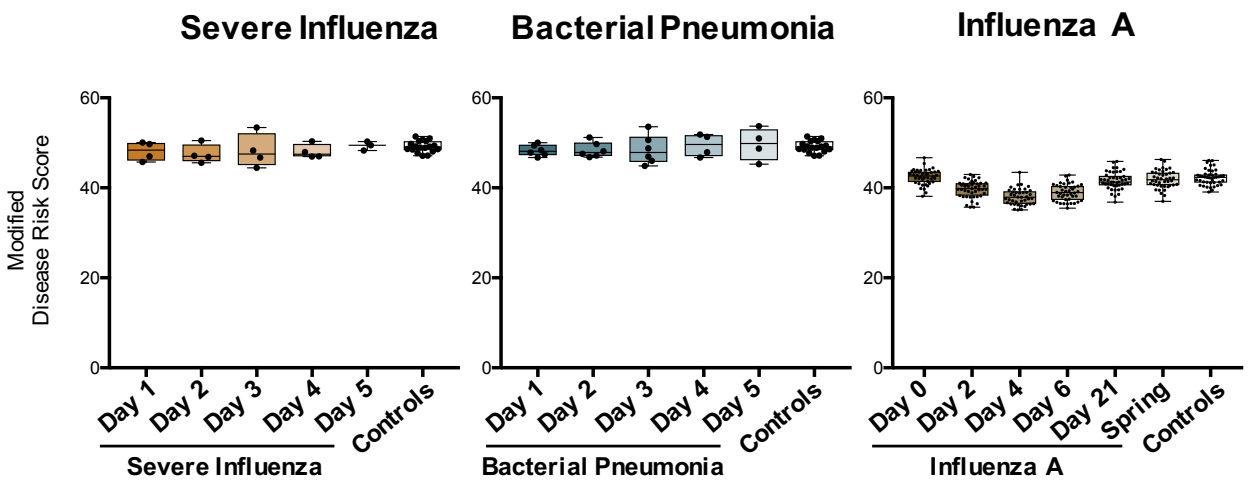


Figure 6

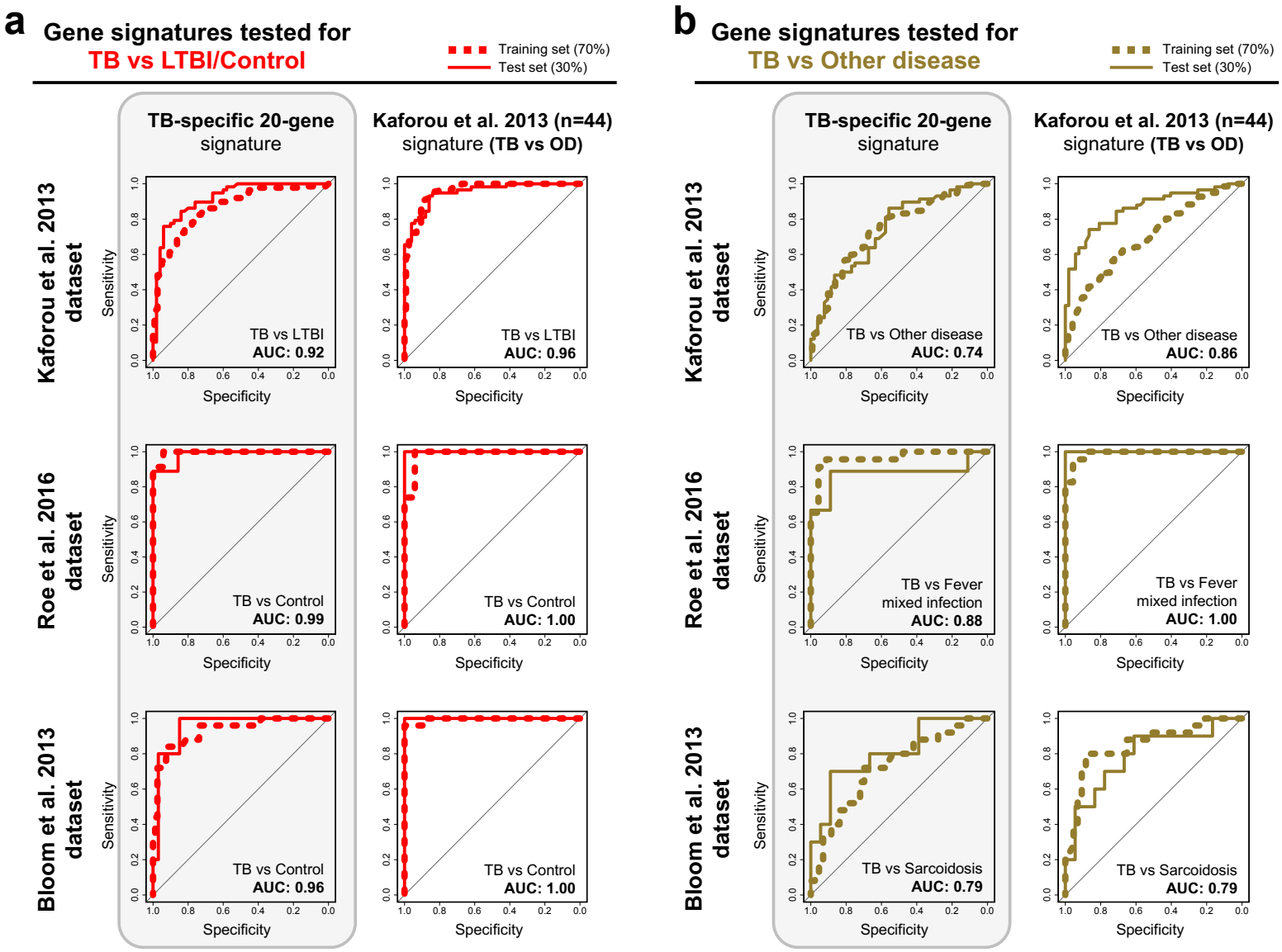
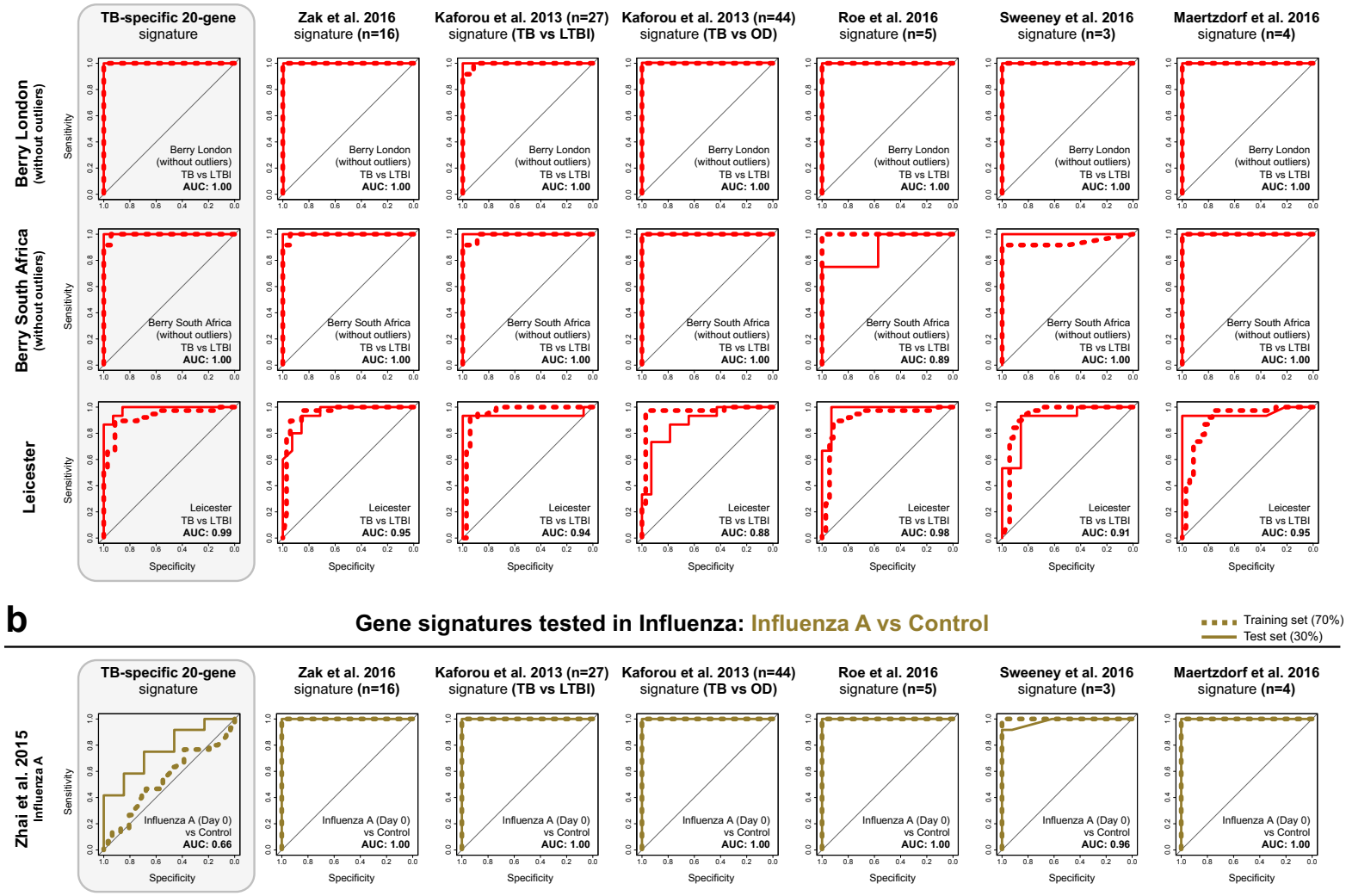

a
Gene signatures tested for
TB vs LTBI/Control
Training set (70%)
Test set (30%)
TB-specific 20-gene signature
Kaforou et al. 2013 (n=44) signature (TB vs OD)
Kaforou et al. 2013 dataset
Roe et al. 2016 dataset
Bloom et al. 2013 dataset
Sensitivity
Specificity
TB vs LTBI
AUC: 0.92
TB vs LTBI
AUC: 0.96
TB vs Control
AUC: 0.99
TB vs Control
AUC: 1.00
TB vs Control
AUC: 0.96
TB vs Control
AUC: 1.00
b
Gene signatures tested for
TB vs Other disease
Training set (70%)
Test set (30%)
TB-specific 20-gene signature
Kaforou et al. 2013 (n=44) signature (TB vs OD)
Kaforou et al. 2013 dataset
Roe et al. 2016 dataset
Bloom et al. 2013 dataset
Sensitivity
Specificity
TB vs Other disease
AUC: 0.74
TB vs Other disease
AUC: 0.86
TB vs Fever mixed infection
AUC: 0.88
TB vs Fever mixed infection
AUC: 1.00
TB vs Sarcoidosis
AUC: 0.79
TB vs Sarcoidosis
AUC: 0.79

Figure 7


a
Gene signatures tested in Tuberculosis: TB vs LTBI
Training set (70%)
Test set (30%)
TB-specific 20-gene signature
Zak et al. 2016 signature (n=16)
Kaforou et al. 2013 (n=27) signature (TB vs LTBI)
Kaforou et al. 2013 (n=44) signature (TB vs OD)
Roe et al. 2016 signature (n=5)
Sweeney et al. 2016 signature (n=3)
Maertzdorf et al. 2016 signature (n=4)
Berry London (without outliers)
Berry South Africa (without outliers)
Leicester
Sensitivity
Specificity
Berry London (without outliers) TB vs LTBI AUC: 1.00
Berry South Africa (without outliers) TB vs LTBI AUC: 1.00
Berry South Africa (without outliers) TB vs LTBI AUC: 0.89
Leicester TB vs LTBI AUC: 0.99
Leicester TB vs LTBI AUC: 0.95
Leicester TB vs LTBI AUC: 0.94
Leicester TB vs LTBI AUC: 0.88
Leicester TB vs LTBI AUC: 0.98
Leicester TB vs LTBI AUC: 0.91
Leicester TB vs LTBI AUC: 0.95

b
Gene signatures tested in Influenza: Influenza A vs Control
Training set (70%)
Test set (30%)
TB-specific 20-gene signature
Zak et al. 2016 signature (n=16)
Kaforou et al. 2013 (n=27) signature (TB vs LTBI)
Kaforou et al. 2013 (n=44) signature (TB vs OD)
Roe et al. 2016 signature (n=5)
Sweeney et al. 2016 signature (n=3)
Maertzdorf et al. 2016 signature (n=4)
Zhai et al. 2015 Influenza A
Sensitivity
Specificity
Influenza A (Day 0) vs Control AUC: 0.66
Influenza A (Day 0) vs Control AUC: 1.00
Influenza A (Day 0) vs Control AUC: 1.00
Influenza A (Day 0) vs Control AUC: 1.00
Influenza A (Day 0) vs Control AUC: 1.00
Influenza A (Day 0) vs Control AUC: 0.96
Influenza A (Day 0) vs Control AUC: 1.00

Figure 8

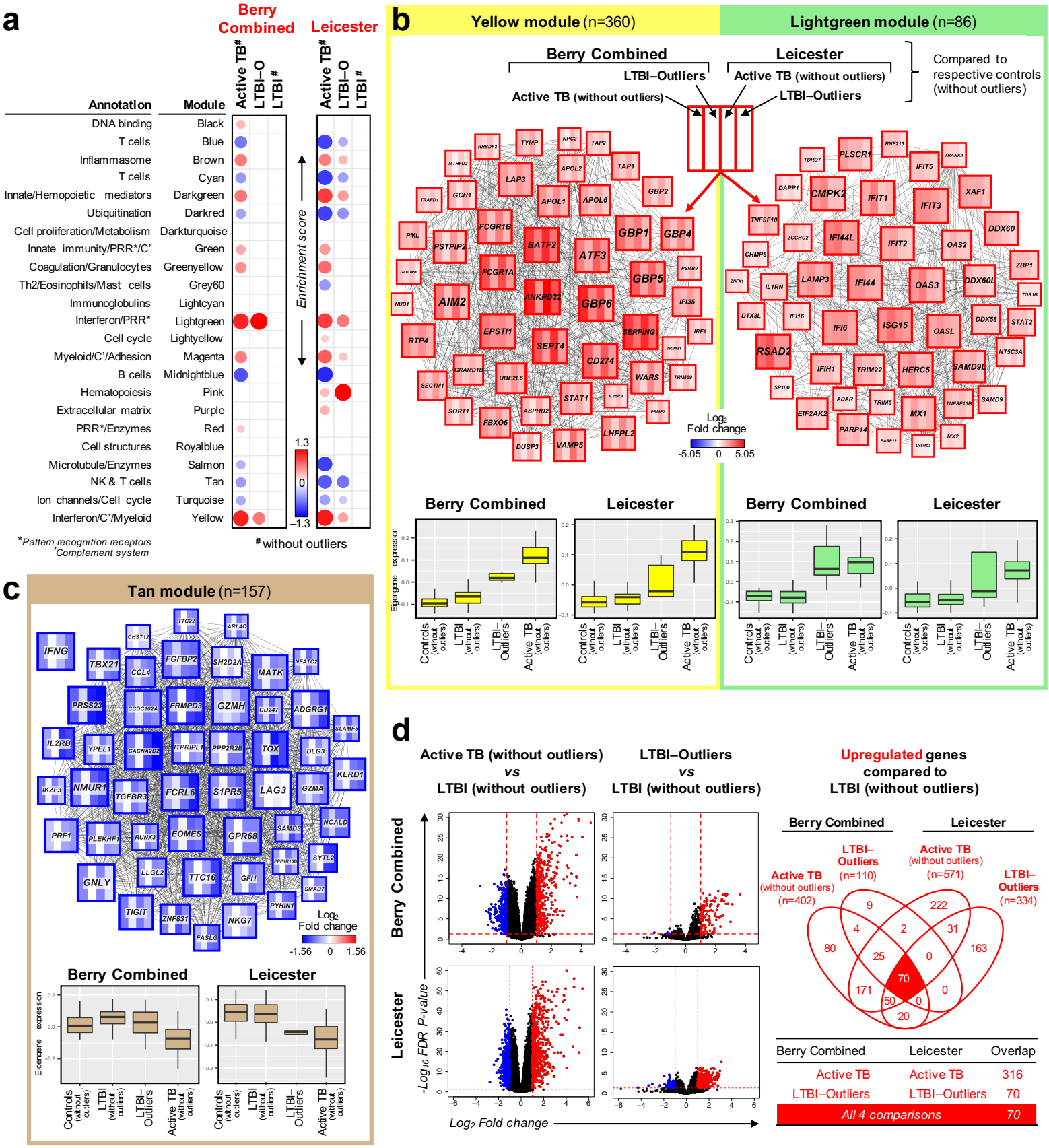
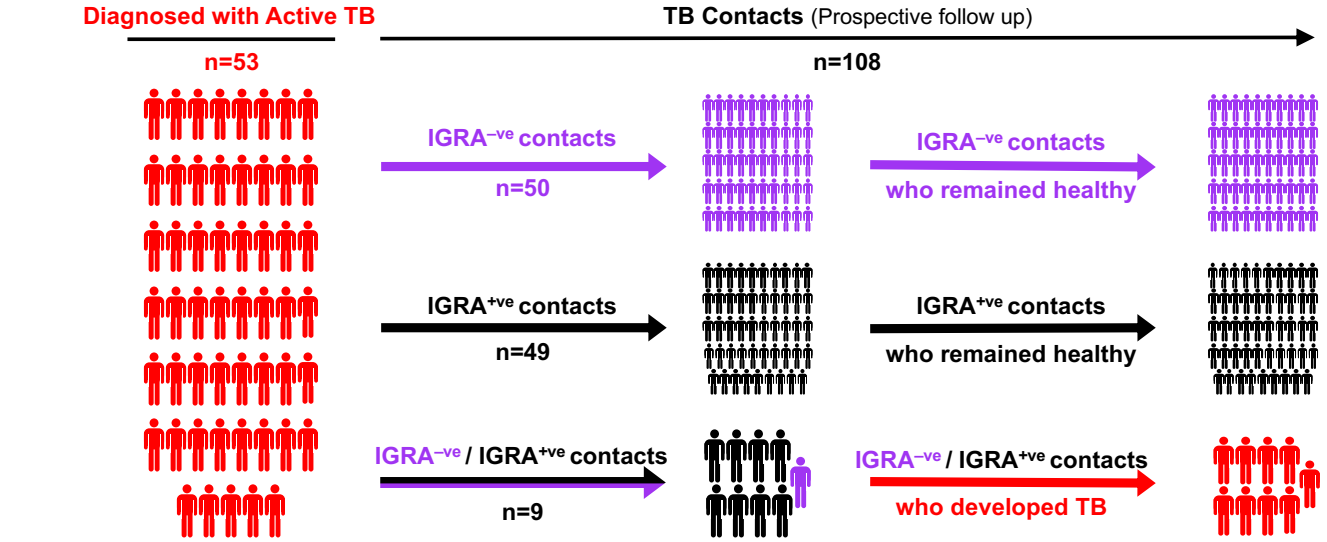

a
Berry Combined
Leicester
Active TB#
LTBI–O
LTBI#
Annotation
Module
DNA binding
T cells
Inflammasome
T cells
Innate/Hemopoietic mediators
Ubiquitination
Cell proliferation/Metabolism
Innate immunity/PRR*/C'
Coagulation/Granulocytes
Th2/Eosinophils/Mast cells
Immunoglobulins
Interferon/PRR*
Cell cycle
Myeloid/C'/Adhesion
B cells
Hematopoiesis
Extracellular matrix
PRR*/Enzymes
Cell structures
Microtubule/Enzymes
NK & T cells
Ion channels/Cell cycle
Interferon/C'/Myeloid
Black
Blue
Brown
Cyan
Darkgreen
Darkred
Darkturquoise
Green
Greenyellow
Grey60
Lightcyan
Lightgreen
Lightyellow
Magenta
Midnightblue
Pink
Purple
Red
Royalblue
Salmon
Tan
Turquoise
Yellow
Enrichment score
1.3
0
–1.3
*Pattern recognition receptors
'Complement system
without outliers
b
Yellow module (n=360)
Lightgreen module (n=86)
Berry Combined
Leicester
LTBI–Outliers
Active TB (without outliers)
Active TB (without outliers)
LTBI–Outliers
Compared to respective controls (without outliers)
Log2 Fold change
-5.05
0
5.05
Berry Combined
Leicester
Berry Combined
Leicester
Eigengene expression
Controls (without outliers)
LTBI (without outliers)
LTBI–Outliers
Active TB (without outliers)
c
Tan module (n=157)
Log2 Fold change
-1.56
0
1.56
Berry Combined
Leicester
Eigengene expression
Controls (without outliers)
LTBI (without outliers)
LTBI–Outliers
Active TB (without outliers)
d
Active TB (without outliers) vs LTBI (without outliers)
LTBI–Outliers vs LTBI (without outliers)
Upregulated genes compared to LTBI (without outliers)
Berry Combined
Leicester
-Log10 FDR P-value
Log2 Fold change
Berry Combined
Leicester
Active TB (without outliers) (n=402)
LTBI–Outliers (n=110)
Active TB (without outliers) (n=571)
LTBI–Outliers (n=334)
9
222
4
2
31
80
25
0
163
70
171
0
50
0
20
Berry Combined | Leicester | Overlap
Active TB | Active TB | 316
LTBI–Outliers | LTBI–Outliers | 70
All 4 comparisons | 70

Figure 9

a **Contacts of active TB patients followed over time in Leicester**



b **TB-specific 20-gene signature in TB contacts followed over time**

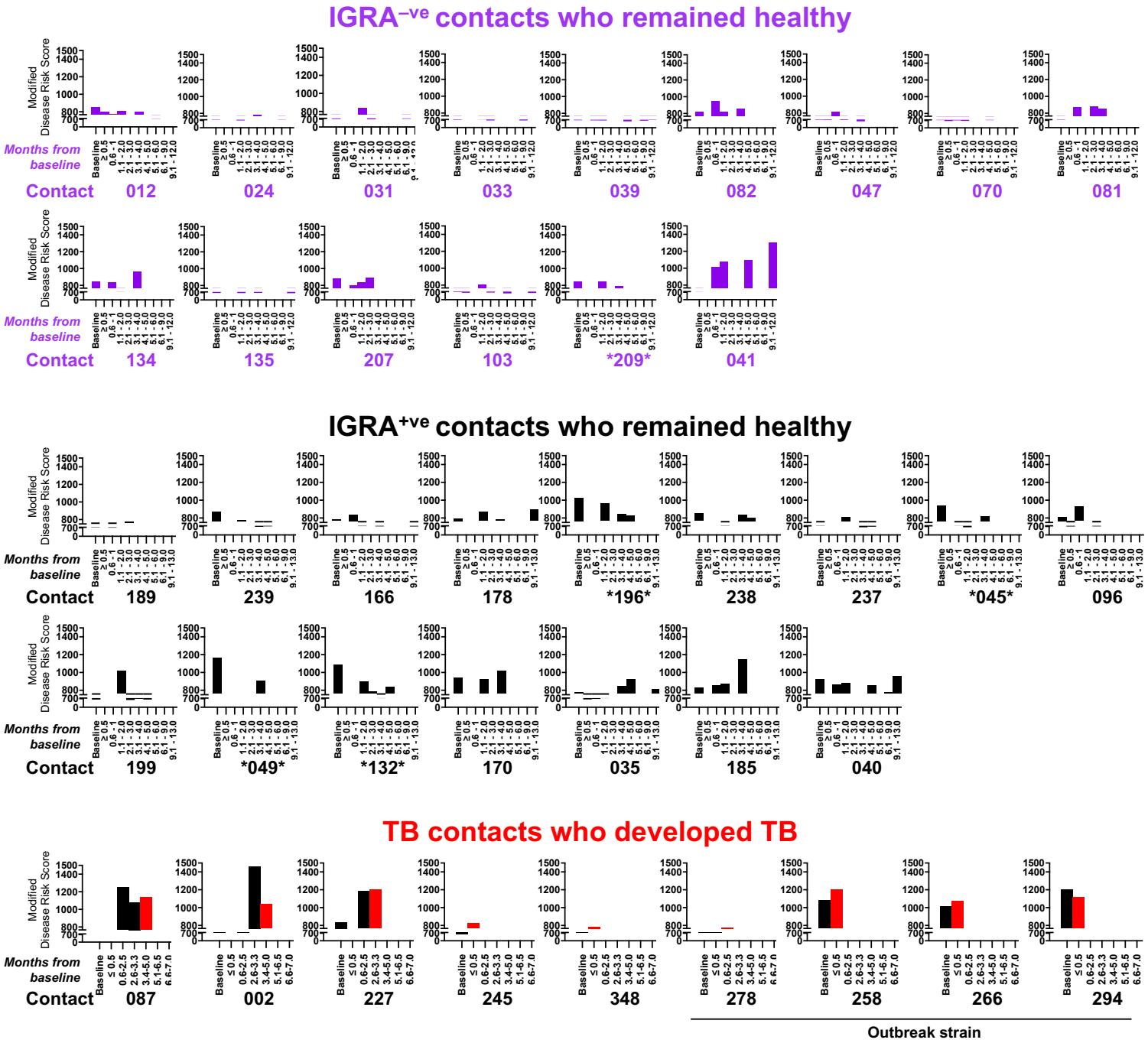


Figure 10