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Tuberculosis and diabetes: biomarkers and drug candidates from a host perspective

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Chapter 6

Summarizing Discussion

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Introduction

Bacterial virulence, host genetic and host non-genetic immune factors underly and regulate tuberculosis (TB) pathogenesis. A rapid and successful immune response is required to eliminate *Mtb*, but on the other hand disease-associated immune responses characterize, and can be used as correlates of, TB disease activity. In addition, host immunity can be targeted by compounds (host-directed therapy, HDT) to improve mycobacterial control. The challenges of developing better diagnostic tools, vaccines and therapies for TB are formidable despite significant progress in each of these fields (1, 2). Comorbidities like HIV and type 2 diabetes (DM) significantly affect TB disease susceptibility and severity. TB-DM comorbidity complicates TB diagnosis and treatment, as indicated by the lower diagnostic accuracy of active TB in patients with concomitant DM (3) and by the impaired treatment success in TB-DM patients compared to TB-only patients (4), respectively. The studies described in this thesis aimed to identify TB-DM associated host-biomarkers as well as to discover and evaluate novel drug candidates for HDT that target intracellular pathogens such as *Mtb* as well as Salmonella bacteria, another leading human pathogen. First, we used transcriptomic-based biomarkers in TB-DM patients and patients with TB-related hyperglycaemia, and described the potential of such TB-DM biomarkers in TB-DM-endemic areas. Second, we conducted *in vitro* studies using primary human macrophages to identify potential drug candidates and host targets for the development of HDT. In this final chapter, the findings described in this thesis and potential implications for future research will be discussed.

Improving TB diagnostics: identification of host immune biomarkers

TB appears to be the main driver of transcriptomic differences between TB-DM patients and healthy controls (3, 5). Nevertheless, the TB transcriptome is modulated by concurrent DM, in that TB-DM patients display increased inflammatory and decreased adaptive immune responses (T-cell activation, differentiation, and B cells) compared to patients with uncomplicated TB (3). Before our work, it was unknown whether TB-DM patients also might display a kinetically different transcriptomic response throughout anti-TB treatment compared to TB patients without DM.

In **Chapter 2 and 3**, we describe the transcriptomic response to anti-TB treatment in patients with uncomplicated TB or with TB and DM-comorbidity. TB patients with concomitant hyperglycaemia or DM displayed increased gene expression perturbation at diagnosis compared to TB-only patients, which remained throughout anti-TB treatment (**Chapter 2**). In contrast with TB-only patients, TB-DM patients exhibited increased

expression of T cell subset markers (*e.g.*, *CD8A*, *PTPRCv1*, *CD3E*) and Th2 associated genes (*e.g.*, *GATA3*, *IL-13*) throughout anti-TB treatment. This was caused by lower expression of the aforementioned genes at diagnosis in TB-DM patients versus TB-only patients, and expression of these genes normalized towards TB-only patients throughout anti-TB treatment. Considering these genes, TB-only patients were more similar to healthy controls compared to TB-DM patients. This is particularly interesting because DM patients did not exhibit lower expression of these genes versus healthy controls, in our South African cohort and in a published USA cohort (6), and thus transcriptomic interference by DM does not explain the lower expression of these genes at diagnosis in TB-DM patients. Interestingly, some of these genes were associated with treatment outcome, and three genes (*i.e.*, *CD3E*, *PTPRCv1*, and *GATA3*) were even part of our treatment outcome biomarker signature (**Chapter 3**). Low *CD3E* expression at diagnosis was associated with a poor versus a good treatment outcome, and *CD3E* expression was increased throughout anti-TB treatment in both patient groups. *PTPRCv1* expression was similar between patients with a poor versus a good treatment outcome at diagnosis, but was more increased throughout anti-TB treatment in the poor treatment outcome group. *GATA3* and *IL-13* expression levels were lower in the patients with a poor versus good treatment outcome, and remained lower throughout anti-TB treatment. Interestingly, *IL-13* expression was, together with *AIRE* expression, part of a biomarker signature for TB progression, and *IL-13* expression levels were higher in TB-HIV patients that progressed to TB compared to HIV-patients that did not develop TB (7). Together, our results suggest that genes with differential longitudinal expression in TB-DM versus TB-only patients also showed different kinetics in response to anti-TB treatment when stratified by treatment outcome. The correlation between TB-DM-associated genes and treatment outcome was likely not caused by a bias by DM in the treatment outcome analysis, since the proportion of TB-DM patients was only slightly higher in the poor versus good treatment outcome group (38% versus 32%).

Despite differences in the transcriptomic response to anti-TB treatment between TB-only and TB-DM patients, these patients displayed qualitative and kinetic similarities regarding the regulation of TB-associated key genes (**Chapter 2**). The blood transcriptome of TB patients is characterized by a neutrophil-driven interferon (IFN) response (8-11), and the expression of IFN-inducible genes was downregulated through anti-TB treatment in TB-only patients as well as TB-DM patients. In addition, levels of *GNLY* and *MMP9* - genes that are associated with active TB (12, 13) - normalized throughout anti-TB treatment in TB-DM patients.

The presence of an anti-TB treatment response in TB-DM patients motivated us to identify a universal gene signature for TB treatment response, that can be applied

irrespective of glycemic index (**Chapter 2**). Such signatures can contribute to the development of TB diagnostic assays in TB-DM-endemic areas. We identified a 15-gene signature characterizing the TB treatment response with high classification power between gene expression at diagnosis versus six months of anti-TB treatment (AUCs = 0.88 for TB-only, AUC = 0.85 for TB-DM). Almost all genes in our 15-gene signature (except *CCL13, TNF,* and *ZNF532*) appear in multiple published gene signatures for active TB (8, 13-18), TB treatment (14, 15, 19-21) and TB progression (22) (Figure 1). As expected, many genes among the 15-gene signature are involved in IFN signaling (*e.g.*, *FCGR1A*, *GBP5*, *IFIT5*, *TAP1*). In contrast with the majority of IFN-signaling genes, which are downregulated throughout anti-TB treatment, *IFIT5* was significantly upregulated during anti-TB treatment in patients with hyperglycaemia, pre-DM or DM, but not in patients with TB-only. This was particularly unexpected because *IFIT5* was previously associated with active TB (8), and this gene was expected to be downregulated upon anti-TB treatment. Why *IFIT5* fails to be downregulated during anti-TB treatment in DM patients and how this is related to TB and/or DM pathogenesis remains to be investigated. Remaining genes in our gene signature are associated with specific immune cell subsets: B cells (*i.e.*, *CD19*), T helper 1 cells (*i.e.*, *TNF*), regulatory T cells (*i.e.*, *CCL4*), T cell subsets (*i.e.*, *CD3E, CD4, PTPRCv1*), and myeloid cells (*i.e.*, *FPR1*).

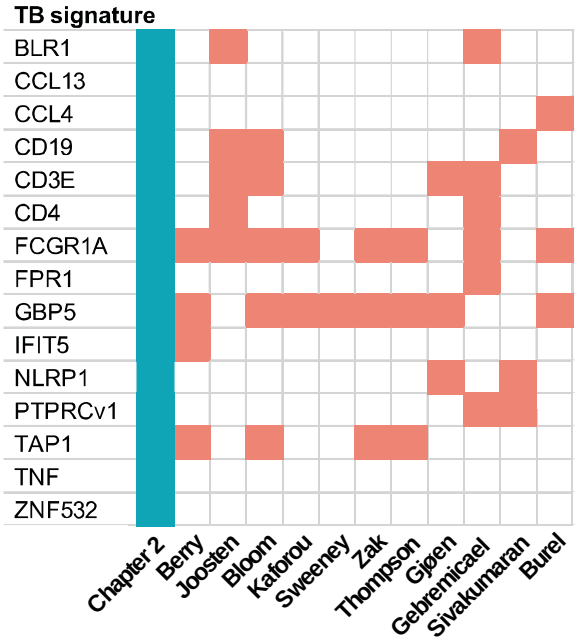

FIGURE 1 | TB treatment response signature in comparison with published TB signatures.

To our knowledge, three genes (*i.e.*, *CCL13*, *TNF*, and *ZNF532*) of our treatment response signature were, although associated with TB, not part of previously published TB signatures. CCL13 (C-C Motif Chemokine Ligand 3) is a chemokine that is mainly responsible for macrophage and lymphocyte homing to tissues and is involved in granuloma formation (23), and is increased in the bronchoalveolar lavage fluid of TB patients (24). The appearance of TNF (tumor necrosis factor) in our TB treatment response signature might result from the inclusion of DM patients in our cohort. It has been reported that TNF-α was increased in TB-DM patients compared to patients with uncomplicated TB (25, 26). Moreover, TNF-α is secreted by adipocytes in obese and diabetic mice, and TNF-α was directly linked with insulin-resistance in mice (27). Finally, TNF-α plays a role in granuloma formation and macrophage activation, and asymptomatic TB-infected patients on anti-TNF therapy have a higher risk of developing TB if not receiving preventive TB treatment (28). Little is known about ZNF532 (zinc finger protein 532), apart from its potential transcriptional regulation of adipogenesis (29). *ZNF532* expression was marginally upregulated among statin users who developed type 2 DM (30), and thus the high relative number of DM patients in our cohort might explain the appearance of this gene in our treatment response signature.

In **Chapter 3**, we aimed to identify gene signatures that *predicted* anti-TB treatment outcome in TB and TB-DM patients. Our data showed that patients with a poor treatment outcome displayed a transcriptomic response to anti-TB treatment, both at the level of individual key genes associated with active TB and of global gene expression (*i.e.*, gene expression was perturbed throughout anti-TB treatment). This may explain why several genes from our treatment response signature (*e.g.*, *FCGR1A*, *CCL4, GBP5,* and *CD3E*) appeared in our treatment outcome signature. However, the magnitude of the treatment response was different: although levels of *FCGR1A* and *GBP5* were decreased throughout anti-TB treatment in patients with a poor treatment outcome, the decrease was delayed (two months versus two weeks) and levels remained higher after six months of treatment compared with patients with a good treatment outcome. *CCL4* expression levels were upregulated in both patient groups upon treatment, but the response was delayed to two months versus two weeks in patients with a poor versus good treatment outcome. Furthermore, the magnitude of the *global* gene expression was different between patients with a poor versus good treatment outcome: whereas patients with a good treatment outcome normalized towards healthy controls regarding molecular degree of perturbation (MDP) scores, patients with a poor treatment outcome did not. Patients with a poor treatment outcome displayed a different transcriptome before the onset of treatment and may therefore require longer or additional treatment to reach a similar clinical endpoint as patients with a good treatment outcome.

Many genes that appeared in our treatment outcome signatures were already described in a published treatment outcome signature (*i.e.*, *CD3E, PTPRCv1, NOD2*) (20), and in published TB risk signatures (*i.e.*, *GBP1, GBP2, GBP5, FCGR1A, STAT1, TAP1*) (22, 31). Interestingly, four genes (*i.e.*, *BCL2*, *CCL4*, *PTPRCv1*, and *CD3E*) that appeared in our treatment outcome signatures at diagnosis and week two were recently described to be differentially expressed between deceased and surviving HIV-TB-coinfected patients, and *PTPRCv1* and *CD3E* were among the seven genes that could accurately predict mortality in HIV-TB patients (AUC = 0.86 for each single gene) (32). The occurrence of genes in multiple published gene signatures underscores the validity of our approach in identifying novel biomarkers predicting treatment outcome and suggests that these biomarkers can be applied to TB patients with concomitant DM.

From gene signatures to clinical application: future prospects

To date, numerous transcriptomic signatures for active TB have been reported, but none of them has been developed into a diagnostic point-of care test. Transcriptomic-based diagnostic signatures requires highly specialized and skillful personnel and analytical techniques and is yet not applicable in resource-limited settings. Published gene signatures often contain a large number of transcripts, which is useful for biomarkers discovery, but a TB biomarker signature preferably contains only a few genes to allow application in low-resource settings. Thus, further research is required to develop transcriptomic-based diagnostic assays that can be used at point-of-care facilities.

Several studies have demonstrated the potential of minimal TB signatures. After biomarker identification, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) can be applied to perform absolute quantification of gene expression, leading to minimal gene signatures. Jacobsen and colleagues performed RT-qPCR following microarray analysis to obtain a three-gene signature (*i.e.*, *Rab33A*, *CD64*, and *LTF*) that discriminated well between TB patients and non-*Mtb*-infected healthy controls or *Mtb*-infected healthy controls with a prediction accuracy of 85% and 80% in an independent validation dataset, respectively (33). Maertzdorf and colleagues used a similar approach to identify minimal gene signatures: they selected genes that were differentially expressed between TB patients and healthy controls in published microarray datasets and they measured the expression of those genes by RT-PCR (34). The identified four-gene signature (*i.e.*, *GBP1*, *ID3*, *P2RY14* and *IFITM3*) showed excellent classification performance of TB by internal cross-validation (AUC = 0.98) and high performance in external datasets (AUC = 0.82-0.89).In addition to the reduction of gene signatures by using RT-qPCR, computational methods can be used to obtain minimal biomarker sets. Roe and colleagues applied machine learning with feature selection on microarray data to

diagnose active pulmonary and extrapulmonary TB based on the transcript levels of a single biomarker (*i.e.*, *BATF2*), with high performance in multiple published cohorts (AUC = 0.93-0.99), and slightly reduced performance in HIV-infected individuals (AUC = 0.85) (35). Gliddon and colleagues also applied feature selection to reduce the number of transcripts of the Kaforou signature (36). In that study, a four-gene signature (*i.e.*, *GBP6*, *TMCC1*, *PRDM1*, and *ARG1*) distinguished TB from other diseases (*i.e.*, pneumonia, lung cancer, Stills disease, Systemic Lupus Erythematosus, *Staphylococcus,* and *Streptococcus*) and a three-gene signature (*i.e.*, *FCGR1A*, *ZNF296*, and *C1QB*) distinguished TB from LTBI with a high performance within their cohort (AUCs = 0.94 and 0.95, respectively) in cross-validation datasets (AUCs = 0.88-0.98 and > 0.91, respectively). Further, data from different cohorts can be combined to generalize gene signatures among heterogenous populations varying in age, ethnicity, or comorbidities. Sweeney and colleagues performed a multicohort analysis to identify a three-gene signature (*i.e.*, *GBP5*, *DUSP3*, and *KLF2*) that showed robust performance across multiple validation datasets (AUC active TB versus healthy = 0.90; AUC active TB versus LTBI = 0.88) (17). This three-gene signature could distinguish TB progressors from non-progressors (LTBI) at six months prior to positive sputum test (AUC = 0.86) (31). Thus, further studies can employ minimal gene signatures to allow application in TB-endemic areas and facilitate diagnostic test development.

In our cohort studies, we did not study the classification capacity of gene signatures to distinguish TB or TB/DM from other diseases. Transcriptional signatures may represent general host responses, which can be shared among other infectious diseases, thereby reducing the disease specificity of host biomarkers. For example, the blood transcriptome of TB patients was similar to the transcriptome of active sarcoidosis (a granulomatous lung disease with unknown etiology but with many features shared with TB) patients and both transcriptomes were dominated by overexpression of IFN-inducible genes (11, 37). Indeed, many genes that frequently appear in gene signatures for active TB (*e.g.*, *FCGR1A, IFI6, IFIT3, IFITM3, GBP1, GBP5, INDO, STAT1*), including our signatures, are also overexpressed in patients with active sarcoidosis (11). TB, however, could be distinguished from sarcoidosis by classification based on discriminatory genes. Thus, gene signatures need to be finetuned to distinguish TB from sarcoidosis (11, 37). Together, these studies suggest that gene signatures could distinguish TB from other diseases, when models are trained on datasets that include data from patients with other diseases.

Despite the large number of identified transcriptomic TB biomarkers in the last decades, gene signatures have not been translated to clinical practice yet, and the diagnostic test performance of published signatures across different populations has only recently been evaluated in independent populations. The WHO proposed minimal and optimal target product profiles (TPP) for a biomarker-based non-sputum-based test to

diagnose TB (38). Minimal TPP includes a sensitivity of at least 65% and a 98% specificity for a confirmatory test (in combination with smear microscopy) and a 95% sensitivity and 80% specificity (depending on TB prevalence) for a point-of-care triage test to identify people who need further testing. Warsinke and colleagues evaluated the potential of 16 reported transcriptomic signatures to diagnose active TB in a pooled dataset, and found that two signatures achieved the minimal WHO TPP for a triage test (39). Furthermore, seven gene signatures could predict progression from LTBI to active TB six months prior to sputum conversion. Gupta and colleagues evaluated the performance of 17 reported transcriptomic signature to diagnose incipient (*i.e.*, the phase between LTBI and active disease) TB in a pooled dataset with samples from four countries (South Africa, Ethiopia, The Gambia, and the UK) (40). Eight signatures met or approximated the minimum TPP for predicting the short-term (0-3 months) risk of TB. In a follow-up meta-analysis, Turner and colleagues extended the inclusion criteria to validate the performance of 27 reported transcriptomic signatures to identify active TB in South African adults (41). Here, four signatures met or approximated the minimal requirements for a triage test, but not for an optimal triage test or a minimal confirmatory test. Finally, Mulenga and colleagues evaluated the performance of 24 signatures for TB diagnosis, and found that four signatures had the potential as diagnostic test (42). Among the aforementioned studies, the Sweeney 3-gene (17), Zak 16-gene (22), Kaforou 25-gene (16), Roe 3-gene (43) and BATF2 (35) gene signatures met the WHO TPP criteria in multiple studies, demonstrating the robustness of these signatures.

Taken together, published host transcriptomic-based biomarker signatures achieve promising sensitivity and specificity rates for a clinical triage test, but current biomarker signatures require further optimization to reach minimal WHO TPP criteria for application as diagnostic TB test. Also, further steps are required to develop low-cost point-of-care assays, that can be used in resource-limited settings. In conclusion, current meta-analysis studies support the research pipeline of transcriptomic-based biomarker discovery using genome-wide approaches for the development of TB diagnostic assays, and suggest that meta-analyses are useful to assess the potential of biomarker signatures as standalone diagnostic tests.

Drug discovery: identification of drug candidates

The second objective of this thesis was to identify novel drug candidates with translational potential for HDT against TB or related intracellular infections such as Salmonella bacteria. Drug discovery can follow two pipelines: the drug-to-target or the target-to-drug approach (44). The drug-to-target approach requires screening assays to identify drugs with antimicrobial activity, followed by drug target identification. This approach has been

successfully applied by our laboratory to discover drug candidates with HDT activity against *Mtb* and *Stm* in human cell lines (45, 46). However, considering the polypharmacology of many compounds, identification of the target responsible for the antimicrobial effect is challenging. Alternatively, the target-to-drug approach can be applied, using promising host or pathogenic targets as starting point. The 'target' in the target-to-drug approach was originally designed based on a protein, enzyme, or other pathway essential for *Mycobacterium tuberculosis* (*Mtb*) growth or survival *in vitro* or *in vivo*, which was identified by genetic studies using mutant *Mtb* strains, leading to rationally designed inhibitors of the identified *Mtb* essential molecules. In the context of HDT, often a drug-repurposing strategy is followed in which a drug-to-target approach prevails: in this case 'host effectors' – which can be host proteins, enzymes, or pathways, are the target. Once a potential drug presumably acting on a target has been functionally identified and the target validated, additional drugs directed at the same target can be identified relatively rapidly.

Genetic inhibition screens can identify promising host targets against *Mtb*. Jayaswal and colleagues screened a commercial siRNA library for *in vitro* activity against 744 kinases and 288 phosphatases in a *Mtb* infection model in the murine J774.1 macrophage line (47). In our laboratory, Korbee and colleagues screened a commercial siKinome library for *in vitro* activity against *Mtb* in a human melanoma cell line (MelJuSo cells), also identifying potential host targets (45). Both studies validated that targets identified by siRNA screens, including the transforming growth factor beta (TGF-β) type-1 receptor (47), and tyrosine-protein host kinases ABL1, BLK, and NTRK1 (45), were druggable by chemical compounds, leading to improved *Mtb* control. Despite the power of such technological approaches, there nevertheless may be discrepancies between transcriptional and protein expression or targeting data. For example, pyruvate dehydrogenase kinase (PDK) 2 was identified as host target during *Mtb* by knockdown of *PDK2* at the genetic level resulting in reduced *Mtb* outgrowth (45), while chemical inhibition of this host target by DCA failed to reduce intracellular *Mtb* in human macrophages (**Chapter 5**). Although there are numerous possible explanations for this discrepancy, including DCA's affinity for PDK2 versus other PDK isoforms (*i.e.*, PDK1, PDK3 and PDK4) or intrinsic cell-specific effects (*e.g.*, in basal *PDK* expression/activity or downstream cellular functions) in cell lines versus primary macrophages, the precise reasons for this currently remains elusive. However, in our and others' experience (48), primary macrophage cultures are challenging to transfect and do not yield high numbers of cells for larger genetic screens, thereby confining the use of larger genetic screens to model human cell lines.

Due to the limitation of genetic screens as mentioned above, we studied the effect of candidate drugs with reported functional abilities to interfere with DM-associated

pathways which, given the reported TB-DM interactions in macrophages (49-53), are likely to interfere with host immunity against *Mtb* at the cellular level. This approach could provide an opportunity to target both TB and DM simultaneously. Since DM is likely to increase glucose uptake and metabolism in macrophages (54), we particularly studied the effect of modulating key metabolic pathways on intracellular *Mtb.* Using this approach, we identified novel host targets involved in the interaction between macrophages and *Mtb*. Also, we compared the response of macrophages to *Salmonella enterica* serovar Typhimurium (*Stm*) versus *Mtb* infection, improving our understanding of the biology of host-pathogen interactions. In the next sections, pathways described in this thesis in the context of previous studies will be summarized and reflected upon, to conclude with promising host targets for HDT.

Murine versus human macrophages in *in vitro* (infection) models

Most studies on macrophage activation and metabolism have been performed using mouse bone-marrow-derived macrophages (BMDMs), but it remains incompletely understood to what extent these findings translate to human macrophages. Several studies have shown that murine and human macrophages respond differently to immunological stimuli and infections with pathogens in the context of metabolism. Stimulation with interleukin (IL)-4 caused only moderate changes of oxidative phosphorylation (OxPhos) and fatty acid oxidation (FAO) in human alternatively activated macrophages (M2), in contrast to mouse BMDMs (55). Importantly, human M2s did not display increased oxidative metabolism compared to human classically activated macrophages (M1s). IL-4 stimulation slightly increased oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in human M2 without changing the OCR/ECAR ratio (55). Importantly, these data suggest that macrophage activation increases the metabolic flux in human M2 but is not accompanied by a switch from glycolytic to oxidative metabolism. In another study, human M2 did not alter OxPhos in response to lipopolysaccharide (LPS) and slightly reduced glycolysis (56). In contrast, mouse BMDMs polarized to M2 shifted their metabolism from OxPhos to glycolysis in response to LPS. Further, LPS-stimulation induced high lactate levels in mouse BMDMs, but not in human monocyte-derived macrophages (MDMs). In this thesis, human M1 had a higher metabolic flux compared to human M2, but both macrophage subsets increased the ECAR/OCR ratio in response to activation with *Stm* or *Mtb* lysate, again showing an absence of a shift from glycolysis to OxPhos in M2 (**Chapter 5**). Together, these data highlight fundamental differences in the metabolism of polarized macrophages of murine versus human origin, which is summarized in Figure 2. These differences argue for performing studies on the potential of metabolic compounds for HDT in human macrophages.

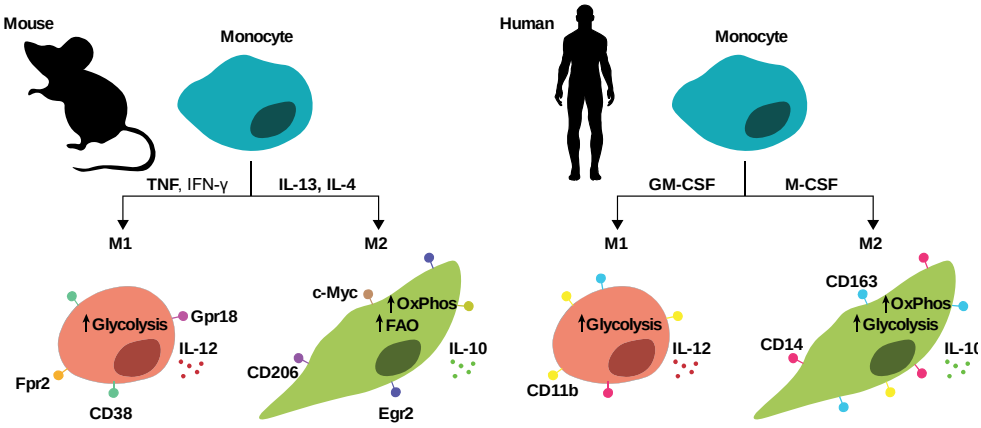

FIGURE 2 | *In vitro* macrophage polarization in human models versus mouse models. Mouse M1 or M2 can be derived from monocytes by TNF- or IFN-γ-induced differentiation or by IL-13- or IL-4-induced differentiation, respectively. Murine M1 and M2 phenotypes are characterized by IL-12 and IL-10 production, and by expression of surface markers (M1: CD38, Fpr2, Gpr19; M2: CD206, c-Myc, Egr2), respectively (48, 57-59). Upon stimulation with bacterial stimuli, *e.g.*, LPS, murine M1 macrophages induce glycolysis, whereas murine M2 macrophages induce oxidative phosphorylation (OxPhos) and fatty acid oxidation (FAO) (48, 60-65). In contrast, human M1 or M2 cells can be derived from monocytes by GM-CSF- or M-CSF-induced differentiation, respectively. Human M1 and M2 phenotypes are characterized by surface markers expression (M1: $CD11b^{high}$, $CD14^{low}$, $CD163^{low}$; M2: $CD11b^{low}$, $CD14^{high}$, $CD163^{high}$) (46, 66). Human M1 and M2 produce IL-12 and IL-10 upon stimulation with bacterial stimuli, respectively, and both macrophage subsets induce glycolysis (**Chapter 5**).

Central host metabolism

Consistent with induced glycolysis upon M1 activation, transcripts and proteins associated with the Warburg effect were upregulated in *Mtb*-infected mouse and rabbit lungs and in lung granulomas of patients with active TB (67, 68). Glycolytic adenosine triphosphate (ATP) synthesis is less efficient than OxPhos, but it can be induced more rapidly. The Warburg effect's extent may depend on the location inside the granuloma, as reflected by spatial differential expression of Warburg-effect markers in macrophages, with a lower Warburg effect in the center of granulomas compared to the periphery (68).

In this thesis, human macrophages shifted their metabolism towards glycolysis upon stimulation with *Mtb* lysate, in agreement with published data using *Mtb* lysates (69, 70), but not upon infection with live *Mtb* bacteria, also consistent with published data (71, 72) (**Chapter 5**). This suggests that live but not killed *Mtb* actively suppresses the Warburg effect in host cells. Importantly, human M2s were able to shift their metabolism to glycolysis in the presence of live bacteria. It is speculated that the increase in glycolysis functions rapidly provides energy and biosynthesis of inflammatory molecules during the initial infection stage (73). Gleeson and colleagues indeed showed that the immune

response of human MDMs to *Mtb* was dependent on glycolysis, and inhibition of glycolysis by substitution of glucose for galactose and 2-DG impaired mycobacterial killing by human MDMs and AMs (74). In addition, inhibition of glycolysis by 2-DG inhibited the production of pro-inflammatory cytokines (*i.e.*, TNF-α and IL-12p40) by mouse BMDMs (75). This may explain why human M1s failed to produce IL-12 in response to live *Mtb* infection (**Chapter 4** and **Chapter 5**). Generally, M1s had an attenuated cytokine response to live *Mtb* infection compared to M2s (Figure 3). In contrast, cytokine production was not impaired in M1s upon *Stm* infection (**Chapter 5**). This suggests that the low cytokine production in *Mtb*-infected M1s is not caused by experimental issues, and is in agreement with the observation that glycolysis was not impaired in *Stm*-infected M1. The hypothesis that glycolysis is required for immunity against *Mtb* is further supported by our observation that inhibition of glycolysis by glucose substitution for galactose tended to provide a more beneficial niche for *Mtb* (**Chapter 5**), which has also been detected in human MDMs (74) and mouse BMDMs (76). Remarkably, in contrast to the situation for *Mtb*, glucose substitution for galactose significantly reduced *Stm* levels in human M1 (**Chapter 5**), possibly because *Stm* can utilize glucose as a carbon source (77). This suggests that even when using a similar host niche for persistence and proliferation, *Stm* and *Mtb* may prefer different host metabolic pathways to balance immunometabolism and supply of host nutrients.

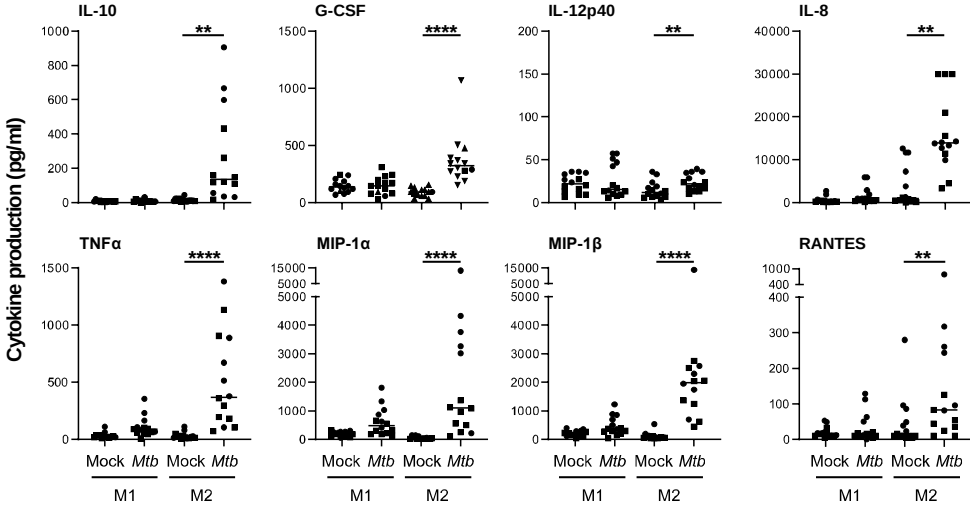

FIGURE 3 | Cytokine and chemokine response of human M1 and M2 upon *Mtb* infection. Cytokines and chemokines measured in the supernatants of *Mtb*-infected macrophages. Differences were significant by non-parametric Friedman's test followed by Dunn's multiple test correction. Adapted from **Chapter 4** and **Chapter 5**.

Together, these studies provide increasing evidence that *Mtb* perturbs the Warburg as a survival strategy (68), potentially interfering with macrophage activation and function (Figure 4). This suggests that compounds that enhance the Warburg effect in host immune cells might be used as HDT against *Mtb* infections (68).

Lactate metabolism

Stimulation of the Warburg effect results in the conversion of pyruvate, the end product of glycolysis, into lactate instead of acetyl-CoA which feeds the tricarboxylic acid (TCA) cycle. This conversion is facilitated by lactate dehydrogenase (LDH), which is expressed upon hypoxia-inducible factor 1-alpha (HIF-1α) activation (78). The resulting lactate accumulation is a hallmark of the Warburg effect. Additionally, lactate levels are elevated in patients with type 2 DM and potentially reflect chronic inflammation (79, 80). Lactate enhanced Nuclear factor kappa B (NF-κB) activity and LPS-induced cytokine expression in U937 macrophage-like cells and expression of inflammatory genes in human monocyte-derived macrophages (81, 82).

In addition, lactate has been associated with TB. Lactate accumulation has been detected in *Mtb*-infected guinea pig lung tissue (83) and LDH was upregulated during *Mtb* infection in mouse lungs (67, 84). Upregulation of the Warburg effect in granulomas of TB patients suggests that increased lactate levels may also be present in TB patients (68).

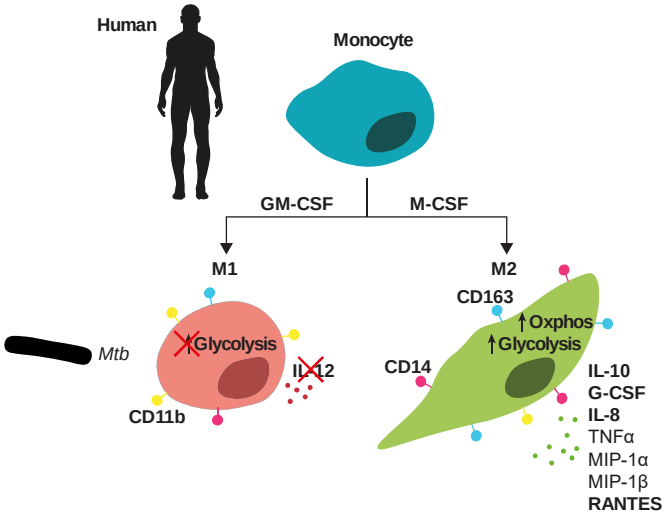

FIGURE 4 | Macrophage response to *Mtb* infection. M1s failed to induce glycolysis (71) (and **Chapter 5**) and to produce IL-12 in response to *Mtb* (66) (and **Chapter 4 and 5**), whereas M2s induced glycolysis (**Chapter 5**) and a cytokine/chemokine response upon *Mtb*-infection (**Chapter 4 and 5**).

Interestingly, lactate can be consumed by various intracellular bacteria during infection, including *Listeria monocytogenes* (85), *Salmonella enterica* serovar Typhi (86), and *Mtb* (87). High concentrations (10 mM) of lactate promoted *Mtb* growth by functioning as a carbon substrate, while very high concentrations (25-50 mM) of lactate were toxic for *Mtb*, suggesting that the effect of lactate on *Mtb* is concentration-dependent (87). Strikingly, *in vitro* proliferation of *Mtb* mutants lacking lldD2 (encoding lactate dehydrogenase) was impaired compared to wildtype *Mtb* in human monocyte-derived macrophages. These data suggest that *Mtb* may benefit from increased glycolytic pathways by extracting carbon substrates for biomass formation, especially in the early phase of infection and in *in vitro* infection models.

In **Chapter 5**, we detected increased lactate levels in *Mtb*-infected M2, but surprisingly not in *Mtb*-infected M1, again in agreement with inhibition of glycolysis, as discussed above. In *Mtb*-infected M1 and M2, lactate levels did not exceed 10 mM, suggesting that lactate may support *Mtb* growth (87). In support of this hypothesis, the LDH-inhibitor FX-11 reduced the percentage of *Mtb*-infected macrophages (**Chapter 4**). These data suggest that inhibition of lactate production may be used as HDT against *Mtb* infections. Further studies are required to establish lactate levels in TB granulomas and the potential of LDH inhibitors for HDT in the late phase of *Mtb* infections.

Pyruvate metabolism

The glycolytic end product pyruvate can be converted into Acetyl-CoA by pyruvate dehydrogenase (PDH). Pyruvate dehydrogenase kinase (PDK) is an inhibitor of PDH and is a direct target of HIF-1α to inhibit TCA cycle supply and promote pyruvate conversion into lactate instead (88, 89). PDK levels are elevated in DM patients, and PDK inhibition has been proposed as a therapeutic target for DM (90, 91).

Chemical inhibition of PDKs by DCA treatment resulted in major *Stm*-reducing effects in M1 and M2 but had no decreasing effect on intracellular *Mtb* (**Chapter 5**). Interestingly, mitochondrial AKT has been shown to be an upstream target of PDK1 and is able to phosphorylate PDK1 on threonine (Thr) 346 to inactivate PDH in a kinase assay in hypoxic conditions (92). H-89, a chemical compound that regulates *Stm* outgrowth in a host-directed manner, is a described AKT1 inhibitor (93). Here, we confirm that, like DCA, H-89 is a suitable inhibitor of *Stm*, but not *Mtb*, implying that H-89-induced *Stm* control may be regulated via the AKT1/PDK pathway. It remains to be elucidated whether H-89 treatment results in activation of PDH, like DCA, in our macrophage model.

In our studies, we showed that DCA activated PDH in human M1 and M2, but downstream OxPhos was only detected in human M1, suggesting that the effect of DCA on *Stm* was *not* caused by a shift from glycolysis to OxPhos. We propose that the effect of

DCA on *Stm* is caused by induction of pathways upstream of OxPhos, for example by effects of Acetyl-CoA or TCA cycle intermediates (Figure 5). DCA treatment increased acetyl-CoA levels and TCA cycle intermediates (*i.e.*, citrate, succinate, fumarate, malate, and oxaloacetate) in lung cancer cells (94). Acetyl-CoA can be recycled to pyruvate through ATP-citrate lyase (ACLY)-catalyzed citrate metabolism, which is accompanied by the production of NO, ROS, and NADPH (73). ACLY converts citrate into oxaloacetate (OAA) and acetyl-CoA in the cytosol, which promotes lipid biosynthesis (73). This may explain why we found significantly increased levels of *Mtb* CFUs in human M2 upon DCA treatment (**Chapter 5**) since fatty acids are implicated as the major energy and nutrient source for *Mtb* (77, 95, 96). Alternatively, acetyl-CoA can be converted into ketone body 3-hydroxybutyric acid (3HB) to drive foamy macrophage differentiation (97), which has been linked with *Mtb* virulence in human THP-1 cells by providing a secure host niche and nutrients (97, 98). This suggests that pyruvate metabolism supports *Mtb* growth in contrast to *Stm*. This is consistent with the published finding that suppression of mitochondrial Acetyl-CoA by the mitochondrial pyruvate carrier (MPC) inhibitor UK5099 restricted *Mtb* growth in streptozotocin-induced diabetic mice (98).

Further, citrate may be converted into UDP-acetylglucosamine (UDP-GlcNAc), a component of hyaluronic acid (HA), which has been found to inhibit *Stm* infection in human epithelial cells and reduce colonization of *Stm* in the intestine of mice (99). Interestingly, DCA induced O-GlcNAcylation as well as HA synthesis in a human oesophageal cancer cells line (100), raising the question of whether the protective effects of DCA are regulated via HA, which remains further investigation.

In conclusion, targeting pathways that reduce mitochondrial Acetyl-CoA levels may have the potential as a novel HDT strategy against *Mtb* infections. In contrast, our data suggest that DCA-induced activation of PDH is a promising strategy for HDT against *Stm* infections. The downstream mechanism of action of DCA that is responsible for *Stm* control remains to be determined (Figure 5). In previous studies, others have suggested the presence of additional targets of DCA. DCA was demonstrated to inhibit the PPP in MDA-MB-cancer cells (101) and it has recently been hypothesized that DCA is an antimetabolite that antagonizes the biochemical effects of acetate (102). Thus, the contribution of alternative DCA-induced (metabolic) pathways to protection against *Stm* should be considered.

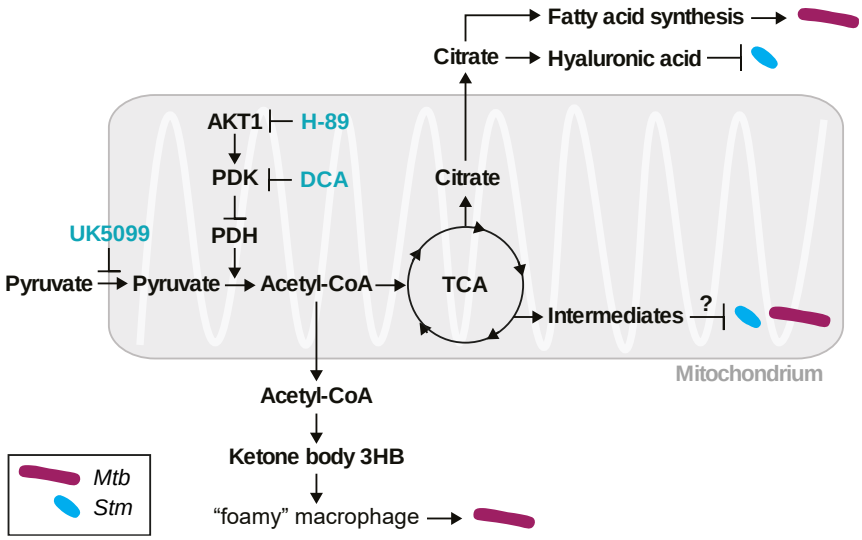

FIGURE 5 | The link between pyruvate metabolism and *Stm* and *Mtb* survival and/or proliferation in human macrophages. Pyruvate metabolism supports intracellular *Mtb* and inhibition of mitochondrial pyruvate and Acetyl-CoA may provide an opportunity for HDT against *Mtb* infections. In contrast, induction of pyruvate metabolism inhibits intracellular *Stm* and may provide an opportunity for HDT against *Stm* infections. Acetyl-CoA can be converted into ketone body 3-hydroxybutyric acid (3HB) to stimulate "foamy" macrophage differentiation (97, 98). TCA cycle intermediate citrate may be exported out of the mitochondria to induce fatty acid synthesis (73), or can be converted into hyaluronic acid (100).

Poly (ADP-ribose) Polymerase (PARP)

Poly (ADP-ribose) Polymerase (PARP) enzymes are involved in DNA repair and programmed cell death. PARP activation has been associated with DM, and mice lacking PARP were resistant to streptozotocin-induced diabetes (103). Excessive oxidative stress leads to PARP overactivation, leading to consequent depletion of its substrate nicotinamide adenine dinucleotide (NAD+) and impaired metabolism (glycolysis and oxidative phosphorylation), eventually resulting in ATP depletion (104). In **Chapter 4**, we found that PARP inhibitors induce *Mtb* control in primary human macrophages. NAD+ is required for many cellular processes and is required to maintain a pro-inflammatory immune response in macrophages (105). Our data support this hypothesis, as we observed a modified macrophage response to *Mtb* upon treatment with PARP inhibitors, as indicated by altered cytokine and chemokine secretion. Although not studied, maintained NAD+ levels could be the mechanism of action of PARP-induced host control against intracellular *Mtb*, which warrants further studies.

Other metabolites

Notably, particular host metabolites are associated with macrophage activation and their immunological responses to LPS and bacterial intracellular pathogens. TCA cycle intermediate citrate can be converted in itaconate, and murine M1-BMDM produces high itaconate levels in response to LPS (in contrast to M2-BMDM) (64, 106). Itaconate-induced anti-inflammatory infects by inhibiting succinate dehydrogenase activity, leading to succinate accumulation. Exogenous itaconate showed direct microbicidal effectivity against *Stm* and *Mtb* (107). Of note, intracellular itaconate levels were much lower in human MDMs compared to RAW264.7 cells (8 mM versus 60 µM), and it is questionable whether such low concentrations show activity against *Stm* and *Mtb* (107). In addition to itaconate, LPS-induced activation of BMDMs leads to accumulation of succinate, which is a pro-inflammatory TCA cycle intermediate that induces HIF-1α expression and IL-1β production (65, 108). Thus, TCA cycle metabolites may regulate macrophage activation and key pro-inflammatory pathways. Hence, other, less well characterized metabolites deserve consideration in the development of HDT against intracellular bacterial infections.

Targeting host metabolic pathways for HDT

In this thesis, we have demonstrated the potential of host metabolic pathways as targets for HDT against intracellular bacterial infections. A schematic overview of macrophage metabolism representing the associations of metabolites with stimulation or inhibition of intracellular *Stm* and *Mtb* survival is shown in Figure 6. Various components of macrophage metabolism have the potential for HDT against *Stm* and *Mtb*, which is summarized in Table 1.

TABLE 1 | Proposed HDT strategies to target host pathways for the treatment of intracellular *Stm* and *Mtb* infections.

HOST TARGET	HDT STRATEGY AGAINST *STM*	HDT STRATEGY AGAINST *MTB*
GLYCOLYSIS	Inhibit glycolysis	Enhance the Warburg effect
PYRUVATE METABOLISM	Increase mitochondrial pyruvate and acetyl-CoA levels	Inhibit mitochondrial pyruvate and acetyl-CoA levels
LACTATE PRODUCTION	-	Inhibit pyruvate conversion into lactate; inhibit lactate dehydrogenase
TCA CYCLE	Induce itaconate	Induce itaconate
PARP	-	Inhibit PARP activation

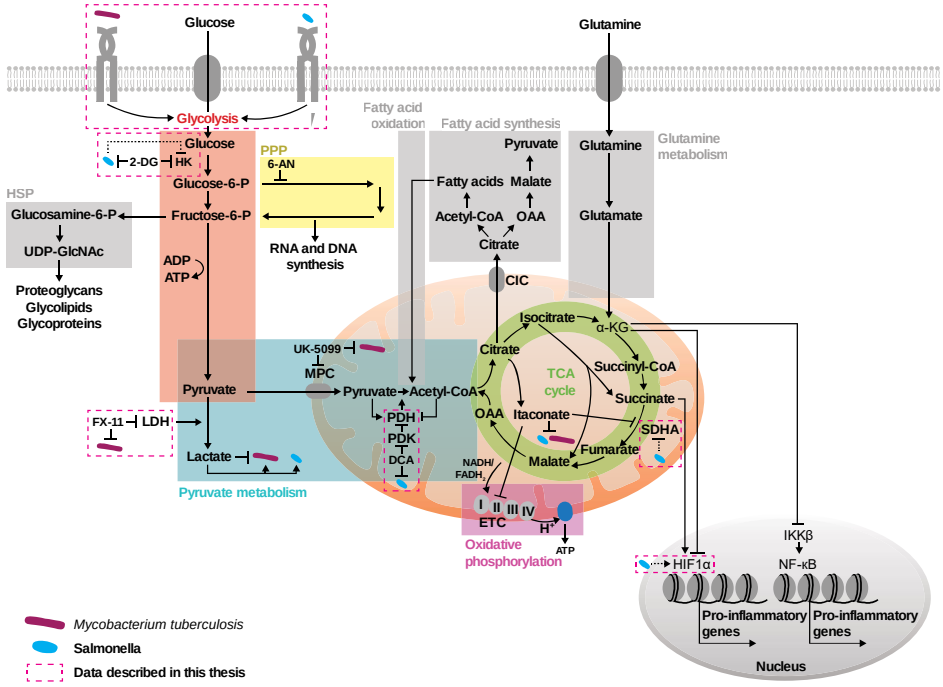

FIGURE 6 | The potential of targeting macrophage metabolic pathways in the context of HDT against Salmonella and *Mtb*. Colors indicate main metabolic pathways that are reflected upon in this thesis (glycolysis, PPP, pyruvate metabolism, TCA cycle and oxidative phosphorylation). Associations of (parts of) metabolic pathways with intracellular survival or control of *Stm* or *Mtb* that are supported by data described in this thesis are annotated with dashed squares. Solid arrows indicate protein associations and dashed arrows indicate gene associations. Protein associations are adapted from following publications: (64, 73). Metabolite associations with Salmonella and *Mtb* are adapted from following publications: lactate and Salmonella (86) or *Mtb* (87); itaconate and *Mtb* (107); UK5099 and *Mtb* (98).

Identification of novel drug candidates for host-directed therapy: future prospects

In this thesis, we have identified host targets and drug candidates that showed activity against intracellular bacteria. Human cell-based screens represent our main approach for drug discovery, and provide knowledge on host-pathogen interactions at least under *in vitro* conditions. However, a significant number of steps need to be taken to translate *in vitro*-identified drug candidates to clinical applications.

First, next to high potency, new anti-TB drugs should have a good safety profile. We have tested the effect of HDT compounds on cellular toxicity, but we did not investigate toxicity effects *in vivo*. Most compounds described in this thesis are repurposed drugs, which are molecules that were clinically used for the treatment of other infections or

diseases. Information on human safety and pharmacokinetics is known for these drugs, which should accelerate their potential translation for other applications, including anti-TB treatment. Whether these drugs are safe in TB patients remains to be investigated.

Second, by definition, *in vitro* conditions differ from *in vivo* conditions, and therefore identified hit compounds may or may not act *in vivo*. For example, novel pyrimidine–imidazoles were identified as potent inhibitors against *Mtb in vitro* but lacked activity *in vivo* (109). Discrepancies between *in vitro* and *in vivo* experiments may be caused by culture environments that are not relevant *in vivo* (*e.g.*, culture medium composition) or by *in vivo* pharmacokinetics.

Third, the HDT compounds identified in this thesis showed varying activity against *Stm* and *Mtb* (Table 2). Generally, compounds were more potent against *Stm* compared to *Mtb*, and the efficacy against *Mtb* was relatively low but in the same range as published anti-mycobacterial HDT compounds (Table 3). The limited activity of HDT compounds may be explained by the experimental design. We used a stringent drug discovery model: mycobacteria are exposed to the compounds after overnight infection, which reflects only one generation time. It is expected that a prolonged exposure time may improve the activity of some, but not all, compounds, as exemplified by metformin which did not improve efficacy with 72h stimulation compared to 24h stimulation in human M2 (110). In contrast, several compounds targeting RNA sensor gene expression (*i.e.*, RM5061, NTZ, TIZ) showed no activity with an exposure time of 24 hours, while the antimycobacterial effect was significant after 2, 3, and 4 days of incubation in PBMCs (111). Whether the efficacy of HDT compounds is sufficient for clinical use remains to be investigated.

Finally, since HDT compounds are expected to synergize with antibiotics, we propose the use of HDT compounds as combinatorial therapy with anti-TB antibiotics. Unfortunately, the potential “dual-hit based” synergy of HDT compounds with anti-TB antibiotics is often overlooked in publications. The potential of HDT compounds, however, may be limited by their interaction with antibiotics, as exemplified by rapamycin. Rapamycin is the best studied autophagy inducer, which reduces *Mtb* in mouse BMDMs (112). Unfortunately, rapamycin is metabolized by CYP3A4, a cytochrome P450 enzyme, which is induced by rifampicin treatment (113). Hence, it is essential to consider the interaction of HDT compounds with anti-TB antibiotics.

TABLE 2 | *In vitro* anti-bacterial activity of HDT compounds identified in this thesis

COMPOUND	TARGET	PATHOGEN	CELLULAR MODEL	EXPO-SURE (H)	REDUCTION (%)	CHAPTER
H-89	AKT1	*Mtb*	M1-M2	24	20-20	4
A-966492	PARP	*Mtb*	M1-M2	24	10-40	4
NIRAPARIB	PARP	*Mtb*	M1-M2	24	20-10	4
PAMIPARIB	PARP	*Mtb*	M1-M2	24	20-10	4
RUCAPARIB	PARP	*Mtb*	M1-M2	24	30-20	4
FX-11	LDH	*Mtb*	M1-M2	24	50-20	4
2-DG	G6P	*Mtb*	M1-M2	24	20-20	5
H-89	AKT1	*Stm*	M1-M2	24	90-99	4
NIRAPARIB	PARP	*Stm*	M1-M2	24	60-70	4
DCA	PARP	*Stm*	M1-M2	24	90-80	5
2-DG	G6P	*Stm*	M1-M2	24	40-30	5

Abbreviations: 2-DG, 2-deoxy-D-glucose; G6P, glucose-6-phosphate; PARP, poly (ADP-ribose) polymerase

Concluding remarks

In this thesis, we identified host transcriptomic biomarkers to detect and predict response and outcome to anti-TB treatment in TB patients with or without concomitant DM and hyperglycaemia. Furthermore, we identified pharmacological compounds with activity against *Mtb* and *Stm* in a host-directed manner and also identified promising host targets that are druggable by HDT, including host metabolic markers that relate to DM.

The next challenges will be to translate the potential of host-based biomarkers to clinical point-of-care tests and to translate the potential of HDT compounds identified in *in vitro* macrophage assays to *in vivo* studies and, ultimately, to clinical studies.

TABLE 3 | *In vitro* anti-mycobacterial activity of compounds in murine and human macrophages

COMPOUND	TARGET	CELLULAR MODEL	EXPO-SURE (H)	CFU REDUC-TION (%)	REF
C40.T4	CD40 and TLR4	BMDM	24	28	(114)
IMATINIB	Abl PTK	AM	24	50	(115)
AT9283	RTKs	M1-M2	24	75-45	(45)
ENMD-2076	RTKs	M1-M2	24	75-50	(45)
DOVITINIB	RTKs	M1-M2	24	75-60	(45)
H-89	AKT1	M1-M2	24	25-20	(45)
RM5061	RNA sensor gene expression	PBMC	24	0	(111)
NTZ	RNA sensor gene expression	PBMC	24	0	(111)
TIZ	RNA sensor gene expression	PBMC	24	0	(111)
IBRUTINIB	Autophagy	THP-1	24	50	(116)
METFORMIN	AMPK-activating antidiabetic drug	M2	24	50	(110)
LOPERAMIDE	Autophagy	AM	24	50	(117)
VERAPAMIL	Autophagy	AM	24	88	(117)
EZETIMIBE	2-azetidinone cholesterol absorption	M1	24	40	(118)
EZETIMIBE	2-azetidinone cholesterol absorption	THP-1	24	80	(118)
SIMVASTATIN	Cholesterol accumulation	BMDM	24	33	(119)
H-89	AKT1	M1-M2	24	10-20	(46)
FLUSPIRILENE	Autophagy	M1-M2	24	25-30	(46)
PIMOZIDE	Autophagy	M1-M2	24	30-30	(46)
RAPAMYCIN	mTORC1	M2	24	10	(120)
C4.T4	CLEC4E and TLR4	BMDM	48	83	(121)
N-ACETYL CYSTEINE	ROS scavenger	THP-1	48	30	(122)
TMP195	HDACs	M1-M2	48	5-15	(123)
TMP269	HDACs	M1-M2	48	5-20	(123)
TSA	HDACs	M1-M2	48	25-25	(123)
1,25D3	Vitamin D3 receptor	THP-1	72	20	(124)
4-PHENYL-BUTYRATE	Multiple activities	M1	72	29	(125)
4-PHENYL-BUTYRATE	Multiple activities	M2	72	60	(126)
LL-37	Multiple, including vitamin D-induced autophagy	M2	72	50	(126)
1,25D3	Vitamin D3 receptor	M2	72	60	(126)
RAPAMYCIN	Autophagy	M2	72	75	(126)
ALL-*TRANS* RETINOIC ACID	Vitamin A metabolite	MDM	72	50	(127)
BAZEDOXI-FENE	Selective estrogen receptor	THP-1	72	70	(128)

Abbreviations: Abl, Abelson; CLEC4E, C-Type Lectin Domain Family 4 Member E; HDACs, Histone Deacetylases; mTORC1, mammalian target of rapamycin complex 1; PTK, protein tyrosine kinases; RTK, Receptor tyrosine kinases; TLR4, Toll-like receptor 4.

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