# Advancing host-directed therapy for Mycobacterium avium infection: identification of drug candidates and potential host targets

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Chapter
7

# Summary, general discussion and future directions

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## Introduction

*Mycobacterium avium* (*Mav*) infections are on the rise globally and their treatment faces important challenges, including extensive and intense antibiotic regimens, severe side effects, resistance to first-line antibiotics, and unsatisfactory treatment success rates. Hence, new treatment strategies to improve treatment outcomes and decrease the risk of drug resistance development are required. Host-directed therapy (HDT), differing from conventional antibiotics in that it targets host immune mechanisms rather than the bacteria, is a promising approach to treat (intracellular) mycobacterial infections. The goal is to dampen destructive inflammation or improve host-mediated control of infection, especially by targeting mechanisms that are counteracted or modulated by the pathogen. This thesis started by providing a review of the current stage of developments in HDT for mycobacteria that notably highlights a gap in the development of HDT for *Mav* compared to *Mycobacterium tuberculosis* (*Mtb*). This lag in HDT development was concluded to reflect the limited efforts as well as the limited knowledge of the host-pathogen interactions during *Mav* infection as opposed to *Mtb*. To fill this gap in *Mav* research, this thesis had two main aims: to identify drug candidates for HDT; and to identify novel host targets to promote the development of these and other HDTs. To address these aims, we performed *in vitro* studies using a well-established primary human macrophage model to repurpose drugs as potential HDT candidates for enhanced host control of *Mav* infection (**Figure 1**). Furthermore, we investigated the intracellular host-pathogen interactions during *Mav* infection by conducting transcriptomic analysis of *Mav*-infected primary human macrophages to reveal host genes that may be involved in host pathways and therefore might represent new host targets for HDT to treat *Mav* infection.

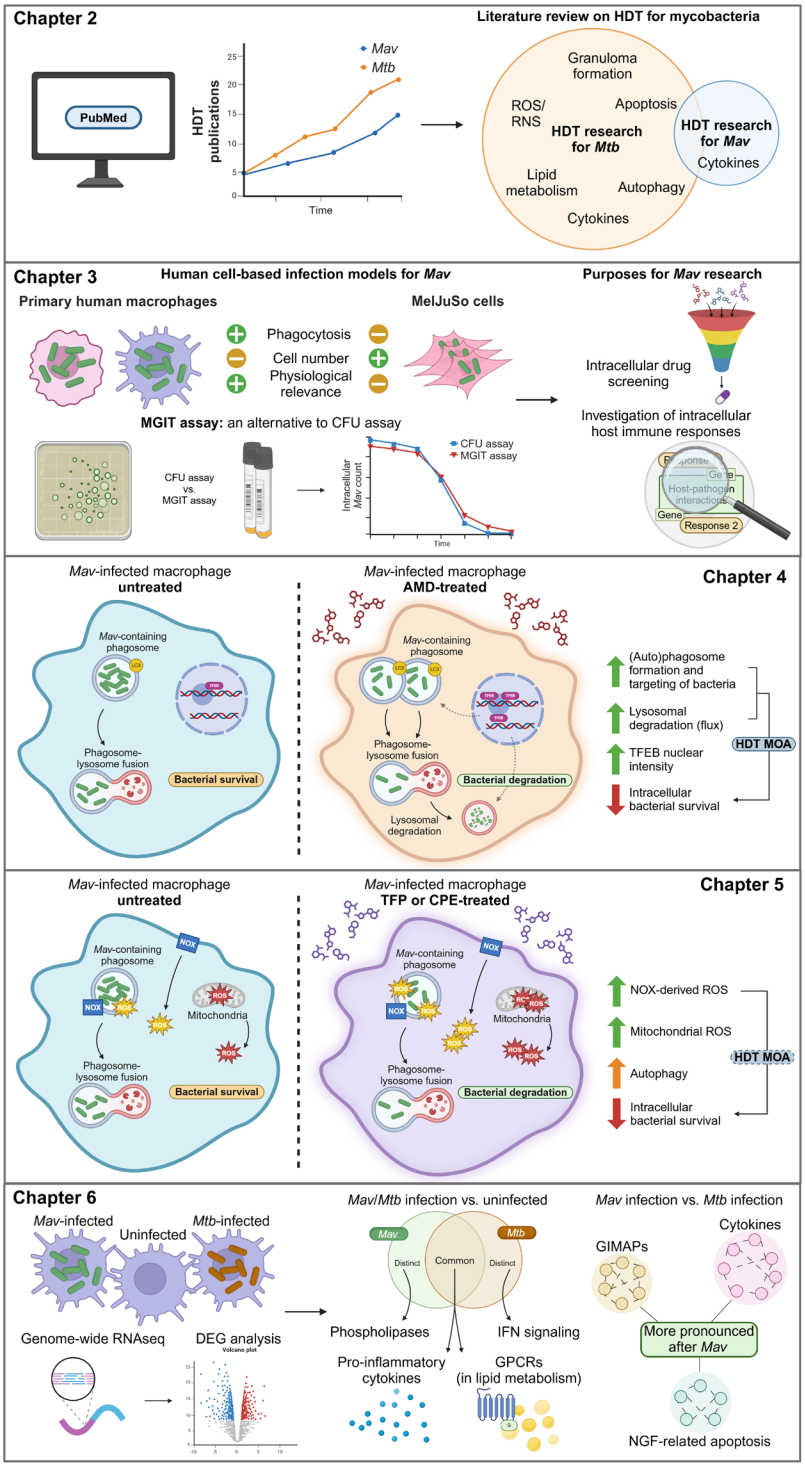


**Figure 1. Schematic overview of the main findings of this thesis.** *Mav*: *Mycobacterium*

*avium*, *Mtb*: *Mycobacterium tuberculosis*, HDT: host-directed therapy, ROS/RNS: reactive oxygen species/reactive nitrogen species, MGIT: Mycobacteria growth indicator tube, AMD: amiodarone, TFEB: transcription factor EB, MOA: mechanism of action, NOX: NADPH oxidase, TFP: trifluoperazine, CPE: chlorproethazine, RNAseq: RNA-sequencing, DEG: differentially expressed gene, IFN: interferon, GPCRs: G-protein coupled receptors, GIMAPs: GTPases of immunity-associated proteins, NGF: nerve growth factor. Created with BioRender.

**Identification of HDT for *Mav*: current status**

One of the aims of this thesis was to identify HDT for *Mav* since there is a compelling need for new therapies that augment the efficacy of current antibiotics and/or provide an alternative approach for decreasing host mycobacterial burden. In **chapter 2** of this thesis, we comprehensively reviewed HDT for mycobacterial infection. This review highlights the HDTs under investigation and describes host immune factors critical for controlling mycobacterial infection, which may be used as therapeutic targets. While the study of HDT in the context of *Mtb* has been extensively explored over the years, *Mav* remains understudied. Building upon the review, **Table 1** summarizes HDTs specifically investigated for *Mav* infections.

The table highlights the diversity of approaches targeting host immunity to enhance bacterial control. Most elaborate research has been performed on cytokines like GM-CSF and IFN-γ, which show potential against intracellular *Mav*, although inconsistent clinical outcomes undermine their therapeutic value. Furthermore, inducers of autophagy like lactoferrin and metformin have shown some evidence to combat *Mav* infection. While these efforts show that HDT in principle offers potential to provide the much-needed boost to the *Mav* complex (MAC) therapeutic pipeline, nearly all avenues of HDT research for *Mav* have been limited in scope and have not reached the level of efficacy to be considered an adjunctive to antibiotic treatment. In efforts to find drugs that may offer a contribution to the development of HDT for *Mav*, the next sectioof this discussion describes repurposing drugs as HDT candidates.

**Table 1. HDT investigated for MAC infection.**

| HDT | Model | Outcome | Ref. |
|---|---|---|---|
| **GM-CSF** | Case report MAC* | Improvement of infection control | (1) |
| | *In vitro* and *in vivo* MAC* | Reduced bacteria burden | (2) |
| | *In vitro* MAC | Enhanced inhibition of intracellular bacteria | (3) |
| | *In vitro Mav* [x] | Enhanced inhibition of intracellular bacteria | (4) |
| | (R)CT MAC*,[x] Ex vivo | Enhanced inhibition of intracellular bacteria, but no clinical improvement | (5) |
| | Case report leukemia and MAC[x] | Improvement of skin lesions | (6) |
| | Case report MAC* | Clinical and histological improvement | (7) |
| **IFN-γ** | Case report MAC*,[x] *In vitro Min* *In vivo* MAC | Limited control of bacterial burden in patients Limited inhibition of intracellular bacteria No effects *in vivo* | (8) |
| | (R)CT *Mav*[x] | Clinical and radiographic improvement | (9) |
| | (R)CT MAC[x] | (Earlier) improvement in clinical, radiographic, and bacteriological assessment | (10) |
| | (R)CT MAC[x] | No difference in treatment outcome | (11) |
| | Case report MAC* | Temporary limited clinical improvement | (12) |
| | *In vitro Mav* | Enhanced inhibition of intracellular bacteria | (13) |
| **IL-2** | *In vitro* MAC | Decreased bacterial burden | (14) |
| | Case report MAC* | Limited effects on clinical improvement | (15) |
| | Case report MAC | Sputum culture conversion and improvement of CD4+ T cell count | (16) |
| **ATP** | *In vitro Min*[x] | Enhanced inhibition of intracellular bacteria | (17) |
| **Lactoferrin** | *In vitro Mav*[x] | Enhanced inhibition of intracellular bacteria | (18) |
| **Metformin** | *In vitro* and *in vivo Mav*[x] | Reduced bacterial burden in mice and cells without synergism with antibiotics | (19) |
| **Thioridazine** | *In vitro*/HFS MAC[x] | Temporary reduction of bacterial burden | (20) |
| | *In vitro* MAC[x] | Enhanced inhibition of intracellular bacteria, with synergism with antibiotics | (21) |
| | *In vitro*/HFS MAC | Enhanced inhibition of intracellular bacteria | (22) |
| **CRL-1072** | *In vitro* MAC[x] *In vivo* MAC[x] | Improved activity of antibiotics on intracellular bacteria, limited effect when administered alone | (23) |
| | *In vitro* MAC | Improved bacterial clearance by macrophages | (24) |
| **Picolinic acid** | *In vitro Mav* | Enhanced inhibition of intracellular bacteria | (25, 26) |
| | *In vitro* MAC[x] | Enhanced inhibition of intracellular bacteria, with synergism with antibiotics | (27) |
| **Amiodarone (chapter 4)** | *In vitro Mav* *In vivo* Mmar | Enhanced inhibition of bacteria in cells and zebrafish | (28) |
| **TFP & CPE (chapter 5)** | *In vitro Mav* | Enhanced inhibition of intracellular bacteria | (29) |

MAC: *Mycobacterium avium* Complex, *Mav*: *Mycobacterium avium*, *Min*: *Mycobacterium intracellulare*, (R)CT: (randomized) clinical trial, HFS: hollow-fiber system, *Mmar*: *Mycobacterium marinum*. * Co-infection MAC and HIV, [x] Adjunctive to chemotherapy.

## Repurposing drugs as HDT for *Mav* infection

The rise in MAC infections and the limitations of current antibiotic treatments highlight the need for alternative strategies such as HDT. Given the limited research on HDT in

this context, we aimed to identify potential HDT candidates for *Mav*.

Many studies discovering HDT for mycobacterial infections use repurposed drugs and *in vitro* cell culture models, enabling rapid screening and identification of effective agents. Hit compounds are then forwarded to more advanced infection models to validate their efficacy *in vivo*. By conducting low-throughput screenings of repurposed drugs on our primary human macrophage *Mav* infection model described in **chapter 3**, we identified HDT candidates amiodarone (**chapter 4**) and two phenothiazines, trifluoperazine (TFP) and chlorproethazine (CPE) (**chapter 5**), that enhanced macrophage-mediated control of *Mav*.

**Repurposing amiodarone as HDT for *Mav*: targeting autophagy**
Amiodarone is an antiarrhythmic drug that blocks calcium, sodium, and potassium channels and inhibits alpha- and beta-adrenergic receptors. Furthermore, amiodarone has been shown to induce autophagy (30-34), and by accumulating in acidic organelles amiodarone may also interact with other intracellular degradation processes, like the endocytic pathway (35). We showed that amiodarone reduces the bacterial burden of *Mav* and *Mtb* in primary human macrophages and that of *Mycobacterium marinum (Mmar)* (another NTM species, mildly pathogenic in humans) in zebrafish, proving its efficacy can be translated from *in vitro* to *in vivo* (**chapter 4**). Moreover, amiodarone promoted the activity of a major autophagy-regulating transcription factor, TFEB, and induced the formation of LC3-positive (auto)phagosomes and targeting of bacteria to these vesicles in *Mav*-infected macrophages. Amiodarone enhanced autophagic flux both in primary human macrophages and in zebrafish. Importantly, lysosomal degradation was essential for the host-protective effect of amiodarone.

Lysosomal degradation is initiated by phagocytosis capturing the bacteria within phagosomes or, when mycobacteria like *Mtb* disrupt the phagosomal membrane escaping into the cytosol (36-38), by host cargo receptors targeting the cytosolic bacteria to autophagosomes in the process of specific autophagy, i.e. xenophagy, to overcome the bacterial immune evasion strategy. *Mav* has evolved mechanisms to resist lysosomal degradation by blocking phagosome maturation, preventing phagosome-lysosome fusion, and using the modulated phagosome as a niche for replication (39-41). Nevertheless, in contrast to *Mtb*, *Mav* has shown to remain phagosomal without cytosolic translocation although the opposite has not been disproven (36). It is therefore uncertain whether autophagy occurs during *Mav* and whether it could be an HDT target. In our study (**chapter 4**), amiodarone induced the formation of LC3-associated vesicles, indicative of both LC3-associated phagocytosis (LAP) and autophagy, however, due to the limited evidence for the role of autophagy cargo receptors, we could not with certainty determine the role of autophagy in the activity of amiodarone. Nonetheless, we observed that amiodarone was able to eliminate multiple mycobacterial species, indicating it stimulates a host defense degradation regardless of the specific immune evasion strategy (e.g. phagosomal escape) conducted that *Mav* and *Mtb* may or may not share.

While amiodarone has shown promise in inducing autophagy and enhancing bacterial clearance, understanding the precise mechanisms by which it activates autophagic pathways is crucial for the development of more effective autophagy-inducing

compounds for clinical translation. We showed that amiodarone enhanced TFEB activation in *Mav*-infected macrophages (**chapter 4**). Once activated, TFEB enters the cell nucleus, stimulating the expression of autophagy-related genes and the coordinated lysosomal expression and regulation (CLEAR) gene network genes (42, 43), and TFEB overexpression strengthens autophagy (31). While it remains to be elucidated whether the autophagy-inducing property of amiodarone is mediated through the activation of TFEB, TFEB activation by itself may be an interesting target for HDT. Acacetin has been shown to activate TFEB and promote autophagic clearance of bacteria such as *Salmonella Typhimurium* (44). Similarly, trehalose is known to induce autophagy via TFEB activation, although its effects during infection have yet to be investigated (45, 46). Other compounds that activate TFEB, such as bedaquiline and molecule 2062, may also hold potential against *Mav* (47, 48). Moreover, TFEB activation is mediated by TRPML1/MCOLN1, a lysosomal calcium channel (49). Chemical agonists of TRPML1 ML-SA5 have been shown to induce TFEB activation and (auto)phagosome formation and autophagy could be blocked using TRPML1 inhibitors. In addition, the activation of TFEB can be negatively regulated, for example, by mTOR (50). Amiodarone is known to inhibit mTOR and may in that way induce TFEB-mediated activation of autophagy (51). This mechanism could parallel the activity of other autophagy-inducing compounds like rapamycin or metformin. Rapamycin, a well-known mTOR inhibitor, was shown to induce autophagy and suppress intracellular survival of *Mtb* (52). Similarly, metformin, used to treat diabetes and an mTOR inhibitor, induces autophagy and has demonstrated efficacy in improving macrophage and murine control of *Mav* infections (10). The activity of these drugs suggests that the mTOR-TFEB axis may be modulated by mycobacterial infection and further exploration could reveal novel targets for HDT. Furthermore, amiodarone can induce autophagy via mTOR-independent pathways involving cAMP. Hence, amiodarone likely interacts with multiple players from the autophagy machinery. A deeper understanding of the molecular mechanisms by which amiodarone eradicates intracellular mycobacteria will enable the identification and development of agents that modulate components of autophagy that are safer and more effective in eradicating a spectrum of mycobacteria.

**Repurposing phenothiazines derivatives as HDT for *Mav*: multifaceted HDTs**

The other HDT candidates we identified were phenothiazines which are currently used as antipsychotic drugs. Though multiple studies have reported the direct antibacterial effects of phenothiazine against both planktonic and intracellular bacteria, we found no direct antimycobacterial effect of phenothiazines derivatives TFP and CPE on *Mav* in the concentrations that inhibited bacterial survival in primary human macrophages. These compounds may exert direct effects at higher concentrations achieved by intracellular accumulation, however, no correlation was found between tendency to accumulate and impairment of intracellular bacterial survival, indicating host-directed mechanisms are more likely at play (**chapter 5**). Another characteristic of phenothiazines is their ability to antagonize dopamine receptors, prompting us to investigate the role of dopamine receptor activity in the HDT activity of phenothiazines. The finding that dopamine agonists enhanced control of intracellular *Mav* suggests that the ability of TFP and CPE to improve control of *Mav* infection is likely independent of their dopamine receptor antagonism (**chapter 5**). Moreover, phenothiazines have been described to both induce and impair autophagy depending on the tissue investigated.

Although our studies showed an, albeit not significant, increase in (auto)phagosome formation and bacterial targeting in *Mav*-infected primary macrophages treated with TFP and CPE, autophagy was not required for the HDT activity of these compounds (**chapter 5**).

TFP and CPE were shown to induce reactive oxygen species (ROS) production which partially explained the improved macrophage activity against *Mav* upon treatment (**chapter 5**). ROS, including superoxides, hydrogen peroxide, hydroxyl radical, and singlet oxygen, play a fundamental role in host immunity by causing oxidative damage to intracellular bacteria and enhancing clearance (53). Recognition of bacteria by macrophages leads to ROS production mainly by NADPH oxidase (NOX) into the phagosome and by mitochondria releasing ROS into the cytosol or phagosomes (54, 55). Both sources primarily produce superoxides to impair *Mav* survival (56, 57). *Mav*, however, protects itself from the superoxide attack from the host with antioxidant enzymes such as superoxide dismutase (SOD) (MAV_0182 or MAV_2043), which catalyzes the conversion of superoxide radical to hydrogen peroxide and oxygen (58, 59). The activity of SOD MAV_0182 was found to increase upon phagocytosis by macrophages, and the absence of SOD on the surface of *Mav* has been associated with a significant decrease in bacterial viability (60, 61). Once hydrogen peroxides are formed, *Mav* responds by upregulating MAV_2838 (OxyR), which regulates detoxifying enzymes such as catalase-peroxidase (KatG) that convert hydrogen peroxide to water and oxygen, thereby neutralizing oxidative stress and enabling bacterial survival (57, 58, 62). Phenothiazines were found to induce both total cellular (e.g. NOX-derived) and mitochondrial ROS, as measured by the CellROX and MitoSOX assays, respectively (**chapter 5**). Since the CellROX assay detects both superoxides and hydrogen peroxide (54), treatment with MnTBAP (a SOD mimic that converts superoxide to hydrogen peroxide) might have altered the ratio of superoxides and hydrogen peroxides but did not affect the total ROS levels induced by phenothiazines. In contrast, the MitoSOX assay, which specifically detects superoxides, showed reduced superoxide levels in cells co-treated with MnTBAP. Notably, MnTBAP neither improved nor worsened the enhanced macrophage control of *Mav* mediated by phenothiazines, suggesting that their efficacy does not rely on one specific ROS species. A pan NOX-inhibitor did partially impair the improved host control by phenothiazines (**chapter 5**), indicating NOX-derived ROS in the phagosome, regardless of the species, is partially required, highlighting HDT with phenothiazines possibly overcomes the different antioxidant bacterial defenses.

The limited reliance of phenothiazines on ROS production suggests that these drugs must also act on ROS-independent pathways (**chapter 5**). HDTs may modulate multiple interconnected host pathways, complicating the identification of their mechanism of action(s). Using repurposed drugs for HDT discovery has, in theory, the advantage that their target processes are already known. In this thesis, we used chemical modulation by interfering with specific cellular molecular processes aiming to assess their role in HDT mechanism of action. While this approach informed us about the mechanism of action of amiodarone (**chapter 4**), the exact target remains elusive. Moreover, such work is a time-consuming trial-and-error process to fully evaluate the role of host pathways, as evidenced by the phenothiazines (**chapter 5**). Transcriptomics or proteomics of cells in the presence or absence of compound treatment may provide a global view of which proteins and/or host pathways are affected during treatment.

To fully elucidate the mechanisms of action of phenothiazines, complementary approaches could be used (63), which may include host genetic manipulation with for example a (whole-genome) siRNA library to pinpoint host pathways involved in the activity of HDT (64). With repurposed drugs, some ideas on the mechanisms exist and a more targeted siRNA library or highly specific CRISPR-Cas gene knockouts may be applied to find host pathways, as shown previously (64, 65). However, knockdown or knockout may also have pleiotropic effects, making it difficult to specify compound effects. Finally, affinity-based methods detect the binding of the compound of interest to proteins. However, sometimes the compound acts through indirect mechanisms and this method will fail to identify the true target. Above all, all approaches require the validation of the causality between the observed changes and the phenotype.

While drugs that act through multiple mechanisms complicate the identification of the mechanism of action, such multimodal compounds may remain effective even when certain immune responses are compromised as is often the case in subjects suffering from *Mav* infections. Moreover, pathogens like *Mav* employ diverse survival strategies, and drugs targeting several host mechanisms can counteract these multifaced bacterial defenses, making it more difficult for the pathogen to adapt or evade host immunity. Hence, HDTs that modulate multiple host pathways, which likely apply to phenothiazines, remain valuable.

**Clinical applicability of amiodarone and phenothiazine drugs**

Despite their promising efficacy, the concentrations of both amiodarone and phenothiazines required for activity make their clinical applications as HDT for *Mav* uncertain due to safety concerns. Amiodarone concentrations used in **chapter 4** for macrophage control of *Mav* can be achieved in patients treated for arrhythmia (1-11 uM, depending on the route of administration) (66, 67), however, plasma levels exceeding 3.9 uM are associated with serious side effects like pulmonary toxicity, thyroid dysfunction, and liver damage, making systemic use as an HDT improbable (68-70). Similarly, the concentration of TFP and CPE used in **chapter 5** exceeds the peak plasma levels achieved with standard oral doses for psychotic disorders (71). In addition, the binding of phenothiazines to dopamine receptors raises concerns about potential off-target effects and the risk of neuropsychiatric side effects. To address these issues, alternative drug delivery strategies such as encapsulation in liposomes or nanoparticles may limit systemic exposure and reduce toxicity risks, while enabling localized drug delivery to infected macrophages. Encapsulation of amikacin in liposomes has previously enhanced its uptake by macrophages and improved its *in vitro* and *in vivo* efficacy (72, 73). Also GM-CSF showed a 100-fold increase in efficacy in enhancing macrophage control of *Mav* when encapsulated in liposomes compared to free GM-CSF (4). Nanoencapsulation of phenothiazine derivative thioridazine reduces drug toxicity while retaining its synergistic efficacy (74). Furthermore, structural modifications to phenothiazines could minimize dopamine receptor binding while enhancing their antimycobacterial activity, as shown with phenothiazine derivatives effective in inhibiting intracellular *Mtb* growth (75). Hence, the HDT activity of amiodarone and phenothiazines demonstrated in this thesis highlights the value of repurposing clinically approved compounds with host-modulating potential for the rapid identification of HDT candidates for *Mav*. While challenges such as toxicity concerns and unclear mechanisms of action necessitate further refinement, repurposing drugs

could be an efficient initial strategy, providing a solid foundation for their optimization to safe and effective HDT to treat *Mav* infections.

## Unveiling the host response to *Mav*

Although our HDT studies highlighted the feasibility of targeting autophagy as an intracellular host pathway, our knowledge of the host immunity and pathogenesis of *Mav* infection remains limited, which significantly impairs the development of HDTs. Given the significant gaps in our understanding of host-pathogen interactions in *Mav* infection, the second key aim of this thesis was to investigate the host response to *Mav* infection and identify pathways that could serve as targets for novel HDT.

Transcriptomics of the host macrophage response to a range of mycobacteria like *Mtb* has greatly enriched the understanding of host-pathogen interactions involved in the pathogenesis of these infections (76, 77). Several studies investigated the host response on the transcriptional level during *Mav* infection (78-81), however, most, if not all, of this work relied on older RNA microarray technology, which is targeted and has limited sensitivity. In the last decades, RNA-sequencing (RNA-seq) has emerged as a more powerful tool for transcriptomic analysis of host cells in response to stimuli like pathogens (82). Although studies demonstrated the utility of RNA-seq in elucidating the host response to NTM infections (83-87), they often rely on animal models or cell lines, which may not fully represent the human host-pathogen response and/or do not include human-pathogenic *Mav* strains. To address this, **chapter 6** of this thesis used RNA-seq to examine the primary human macrophage transcriptomic response to *Mav* infection *in vitro*. By analyzing samples at 2 and 6 hours post-infection, we provided insights into early transcriptional changes associated with cellular pathways during *Mav* infection. Given that functional insights into transcriptional changes during *Mtb* infection are more established, we performed a comparative analysis between *Mav* and *Mtb* for interpreting and weighing the results of *Mav* infection responses.

### The role of proinflammatory cytokines and cell-mediated immunity in host defense to *Mav*

Proinflammatory cytokines are important for the host response to both mycobacterial infections, by affecting the macrophage antimycobacterial activity (IFN-γ/TNF), granuloma formation and maintenance (TNF/IL-1β), inducing differentiation of T cells (IL-12), increased (IL-6) and decreased (IL-10) responses in T cells and macrophages. Indeed, infection of macrophages with *Mav* or *Mtb* elicited strong upregulation of proinflammatory cytokine expression including *TNF*, *IL1B*, *IL12B*, *IL6*, and also *IL10* (**chapter 6**). Interestingly, the induction of many of these cytokines in the initial hours upon infection was stronger by *Mav* than *Mtb*. IL-12 is important for the induction of a Th1 response which is characterized by IFN-γ-producing CD4+ T cells. The protective immune response most likely resides in the production of IFN-γ as defects in the IL-12 and IFN-γ axis are associated with higher susceptibility to *Mav*, in particular, disseminated, disease (88-90). In our study (**chapter 6**), we observed the upregulation of genes involved in IFN-γ signaling upon infection with *Mav* and *Mtb*. It remains unknown, how IFN-γ exactly protects against *Mav*. While IFN-γ is produced by both CD4+ and CD8+ T cells upon *Mav* infection in mice, depletion studies have shown that CD4+ but not CD8+ T cells were required for protection from *Mav* disease in contrast

to *Mtb* (91-95). The role of CD4+ T cells in host defense against *Mav* is supported by the observation that particularly acquired immunodeficiency syndrome (AIDS) patients with a low count of CD4+ T cells develop disseminated *Mav* disease. Moreover, a study showed that the frequencies of IFN-γ-producing CD4+ T cells do not differ between patients with MAC-lung disease and healthy controls (96). Clinical trials using IFN-γ or GM-CSF as immunotherapy in *Mav* infection showed inconclusive efficacy, with only limited potential in those with IL-12/IFN-γ deficiencies (**Table 1**), suggesting that IFN-γ alone is necessary but not sufficient for host defense against *Mav*. This may be due to the fact that the optimal host response to *Mav* also requires TNF, as anti-TNF therapy also impairs host responses to *Mav in vitro* (97), which has a much more complex role *in vivo*. Hence, despite these cytokines being known to be essential, we do not fully understand how they are involved in the host defense against *Mav*. The limited efficacy of IFN-γ and GM-CSF-based HDT suggests that simply supplementing cytokines may not be sufficient for effective therapy of *Mav* infection. A better understanding of the immunity mediated by different immune cells during *Mav* infection to determine the most critical immune pathways for protection may therefore guide the development of more effective immunomodulatory HDT strategies.

**Lipid metabolism in *Mav* infection: balancing host defense and pathogen modulation**

Unlike *Mtb*, the intracellular interactions between *Mav* and the host, particularly macrophage immunometabolism, remain poorly understood. Macrophages undergo significant metabolic shifts in response to mycobacterial infection, including in energy metabolism (e.g. shift from oxidative phosphorylation to (aerobic) glycolysis) and lipid metabolism, which shape immune responses (56, 98-100). In *Mtb* infections, macrophage lipid metabolism is rewired toward increased lipid uptake, mobilization, and storage, while lipolytic pathways are suppressed (101, 102). This promotes the formation of foamy macrophages with lipid droplets that are enriched in cholesteryl esters and triacylglycerols (TAGs), a storage form of fatty acids, and serve as a nutrient reservoir for *Mtb* survival. There are various indications that *Mav* affects lipid metabolism (83, 103, 104), although the role of this host pathway in *Mav* pathogenesis remains less well understood. It is therefore intriguing that our RNA-seq analysis revealed that *Mav*, like *Mtb*, regulates genes involved in lipid sensing, accumulation, storage, and catabolism (**chapter 6**).

*Mtb* exploits host lipids through various virulence factors. The *Mtb* lipase LipY secreted through the ESX-5 efflux pump catabolizes TAGs into fatty acids (102). The *Mtb* protein Rv3723/LucA facilitates the uptake of these lipids in *Mtb* and is required for bacterial virulence *in vivo* (105). Notably, *Mav* possesses the homologs Rv3723 membrane protein (106), suggesting a conserved mechanism of lipid transport. Moreover, *Mtb* ESAT-6 promotes lipid accumulation by activating the antilipolytic receptor GPR109A (*HCAR2*), suppressing TAG catabolism, and preserving lipid droplets (107). While *Mav* lacks ESAT-6, our data indicate that both *Mav* and *Mtb* upregulate *HCAR2* (**chapter 6**), suggesting that *Mav* may induce similar lipid metabolic effects independently of this virulence factor. During *Mtb* infection, impairing lipid accumulation has been associated with reduced intracellular bacterial survival in foamy macrophages (108, 109). Similarly, during *Mav* infection, lipid-loaded macrophages showed impaired intracellular antimicrobial capacity (110), indicating a role of dysregulated lipid

metabolism in increased susceptibility to both *Mav* and *Mtb*. Interestingly, both *Mav* and *Mtb* downregulate *GPR34*, *GPR82*, and *FFAR2,* which all inhibit lipolytic activity (**chapter 6**), and may reflect a host attempt to enhance lipid breakdown to restrict bacterial survival or may also be an approach to yield nutrients or to synthesize (immunomodulatory) lipids.

Despite the pathogen's exploitation of host lipid droplets, they serve as major sites for eicosanoid synthesis, including prostaglandin E2 (PGE2). PGE2 is synthesized from arachidonic acids via *PTGS2* (e.g. COX2) and has been described to have antimycobacterial activity (111-115). Notably, *PTGS2* expression was found to be strongly upregulated by *Mav*, even more pronounced than by *Mtb,* early after infection (**chapter 6**), which suggests PGE2 synthesis during infection is increased. However, the use of COX-2 inhibitors in tuberculosis (TB) show conflicting results regarding the role of PGE2 during infection. Some report that high PGE2 levels impair host control of infection, with COX-2 inhibition reducing mycobacterial burden and improving clinical outcomes (116, 117). Others present that COX-2 inhibitors decrease the host's ability to control mycobacterial infection (114). Although the potential role of drugs targeting the COX2-PGE2 axis has not been identified for *Mav* infection, evidence suggests that macrophages from TB patients treated with COX inhibitors have impaired antimycobacterial activity against *Mav* (118). PGE2 exerts its functions through various receptors and its host-protective effects against *Mtb* in mice are linked to signaling via the receptor PTGER2/EP2 (119). We observed that the expression of *PTGER2* was significantly upregulated in macrophages infected with *Mav* or *Mtb* (**chapter 6**). Thus, these gene expression patterns from *Mav*- and *Mtb*-infected macrophages indicate that both infections similarly engage with this pathway, warranting further investigation into the balance between host defense and pathogen benefit. Beyond COX2/PGE2 signaling, our transcriptomic analysis showed that *Mav*, but not *Mtb*, significantly regulated the expression of phospholipase D (PLD) isoforms, upregulating *PLD1* and downregulating *PLD6* (**chapter 6**). PLD's role in phospholipid hydrolysis associated with *Mtb* killing (120, 121), suggesting another lipid remodeling strategy during *Mav* infection. Taken together, our data reinforce the idea that lipid metabolism plays an important but complex role in *Mav* infection, with many parallels to *Mtb*. Given this complexity of lipid metabolism and concomitant signaling, a deeper understanding is pivotal and could ultimately inform new therapeutic strategies targeting this host pathway to enhance host defense against *Mav*.

**Novel GIMAP genes identified in mycobacterial infection**

In addition to identifying pathways with established roles in macrophage antimicrobial responses, our RNA-seq analysis served as a tool for uncovering genes whose role in mycobacterial infection remains unknown but may be highly relevant. Notably, we identified several GTPases of immunity-associated proteins (GIMAP) genes that were significantly affected by infection, particularly by *Mav* (**chapter 6**). Specifically, *GIMAP1*, *GIMAP4*, *GIMAP6*, and *GIMAP7* were significantly downregulated in macrophages within 6 hours of infection with either *Mav* or *Mtb*. These genes were, in addition to *GIMAP2* and *GIMAP5*, overall stronger suppressed by *Mav* than *Mtb* (**chapter 6**). GIMAPs are broadly expressed in immune cells, with specific members involved in lymphocyte development and survival (122), and are associated with inflammatory disorders (123, 124). Moreover, GIMAPs are also thought to be important in intracellular

trafficking, autophagy, and the formation of lipid droplets (125-129), processes that are critical for immune defense against mycobacteria, as also discussed in this thesis. The involvement of GIMAP proteins in key immune processes raises questions about their role in host-pathogen interactions during mycobacterial infection. The downregulation of multiple GIMAP genes in *Mav*-infected macrophages may be either beneficial for the host or an immune evasion strategy employed by the bacteria (**chapter 6**). It remains therefore important to determine whether modulating the activity or expression of specific GIMAPs directly affects the host's ability to eradicate intracellular mycobacterial infections. Future research should focus on revealing the functions of GIMAPs in macrophage antimycobacterial responses, which may ultimately reveal them as promising targets for HDT.

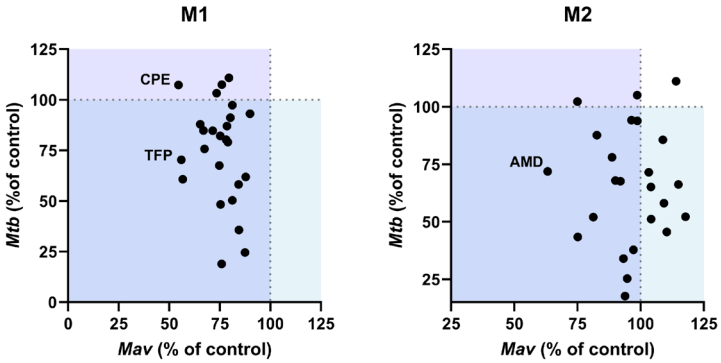


**Figure 2. Efficacy of HDT against intracellular *Mav* vs. *Mtb in* primary human M1 and M2 macrophages.**

**Comparing host responses to *Mav* and *Mtb* infections: insights**

Our RNA-seq results revealed, besides a few differences, a significant overlap in the early macrophage gene response to *Mav* and *Mtb*. While both mycobacteria can cause disease in healthy individuals, *Mav* primarily impacts individuals with immune deficiencies. This distinction in disease pathogenesis reflects differences in host-pathogen interactions. In addition to the clinical presentation, we also observed differences in the efficacy of various HDT candidates: while these treatments modulate host pathways, their efficacy in improving host control varied between *Mav* and *Mtb* infected macrophages (**Figure 2**), suggesting differential roles or manipulation of host pathways. This may suggest that the similarity observed in the host transcriptional response to *Mav* and *Mtb* (**chapter 6**) may be the result of the limited timeframe of 6 hours post-infection, with the divergence in host-pathogen interactions between *Mav* and *Mtb* occurring beyond this timepoint. However, a comparative study by McGarvey et al. also found that, eventhough only a small number of genes was evaluated by the microarray technique upon *Mav* and *Mtb* infection, there was a similarity up to 24 hours post-infection of U937 cells (81). Interestingly, a proteomics study of U937 cells showed a rather limited overlap of 35.7% (205/574) and 23.1% (682/887) of the proteins differentially expressed 24 hours after infection by *Mav* and *Mtb*, respectively (104). Furthermore, while our transcriptomic analysis provided insights into the host responses to *Mav* and *Mtb*, the findings from this study require biological validation.

Hence, further functional confirmation and multi-omics studies are therefore essential for a deeper understanding of the host-pathogen interactions and their consequences for the host control of *Mav*. Moreover, including avirulent *Mav* or *Mtb* strains may help us to understand modulation induced by bacteria which may also give us insights into new HDT strategies.

## Future directions in *Mav* (HDT) research

### Advancing preclinical infection models

*Mav* is an intracellular pathogen that evades host defenses to survive and persist within macrophages. Hence, *in vitro* human macrophage-based infection models are valuable for early drug discovery, in particular HDT, and for studying host-pathogen interactions. Commonly used cell systems in *Mav* research include human monocytic cancer cell lines THP-1 and U937 (130-132). U937 cells, however, have reduced phagocytosis activity compared to human monocyte-derived macrophages, and both THP-1 and U937 require stimulation for differentiation into mature macrophages, which may affect their cell surface markers and host response (133, 134). In addition, the human A549 alveolar epithelial cell line has been used to a limited extent (135-137), while murine macrophage cell lines RAW264.7 and J774 have been used next to *in vivo* murine studies (138-141). With regard to primary cells, human peripheral blood mononuclear cells (PBMCs) or (130, 142, 143), in studies involving mice, murine bone marrow-derived macrophages are used (144). Despite the availability of these existing cell systems, the lack of standardized and representative *in vitro* models of *Mav* infection hampers drug development. To address this, we developed primary human macrophage M1 and M2 models (**chapter 3**). Although these models are more complex to culture and limited in cell number, they offer more physiologically relevant *in vitro* systems, including macrophage spectral polarity, compared to human cell lines for *Mav* studies. These primary human macrophage models have proven valuable in studying HDT efficacy (**chapter 4 and 5**) and specific human pathways (**chapter 4**, **5 and 6**). Additionally, we developed a *Mav* infection model using the human MelJuSo cell line, which, while allowing larger drug screenings without cell number limitations and not requiring differentiation, has a lower phagocytosis capacity compared to primary human macrophages (**chapter 3**). While our primary human macrophage models are biologically relevant, the use of cells from healthy donors limits the application of results to immunocompromised conditions which is an important susceptibility factor for *Mav* disease. Developing models for *Mav* mimicking immunocompromised conditions, such as human immunodeficiency virus (HIV) co-infection (145, 146), IL-12/IFN-γ-deficient cell systems (e.g. CRISPR/Cas9-mediated knockout) (147), but also other immune defects, is therefore essential. Additionally, our model does not mimic interactions between different cell types and tissues during *Mav* infection. More advanced models like lung organoids (148-150), or gastrointestinal organoids (151) could better resemble the infection environment, but 3D cultures have yet to be developed for *Mav*. While this thesis focused on the identification of HDT candidates, our primary macrophage model has also been useful in evaluating intracellular antibiotic efficacy (**chapter 3**). Traditional drug susceptibility testing is performed in liquid broth, which lacks the role of the host immune system in affecting the bacteria, potentially explaining the poor translation of *in vitro* results to *in vivo* outcomes for many drugs used to treat *Mav* (152). This may be reflected in **chapter 3**, where the first-line *Mav* drug rifampicin effectively

impaired bacterial growth in broth, while showing limited efficacy against intracellular *Mav*. In summary, our primary human macrophage model represents a significant step forward in the development of *in vitro* infection models for *Mav* research.
The lack of standardized and reliable *in vivo* models is another hurdle in *Mav* research. Various mouse models have been used, including immunocompromised strains (beige and nude) (153-155), and immunocompetent (C57Bl/6 or Balb/c) (156). These models can develop granulomas and chronic infection as seen in human *Mav* disease (157) A head-to-head comparison of the different mouse models using one *Mav* strain showed that nude mice are highly susceptible to infection, while Balb/c mice were the most suitable to evaluate drug efficacy (158). However, a study found no correlation between the treatment outcomes in mice infected with patient-derived *Mav* strains and the treatment outcomes in those patients, which partly may be due to differences in drug dosing and determination of bacterial burdens across different tissue compartments (159), but is likely also due to the significant differences in immune responses between mice and humans (160). This discrepancy in host immune responses especially complicates studies on HDT targeting human-specific pathways. An alternative model may be zebrafish larvae, which have an innate immune system highly similar to humans (161). In **chapter 4**, we demonstrated that *in vitro* HDT activity of amiodarone could be translated to *in vivo* in *Mmar*-infected zebrafish. Furthermore, zebrafish' transparency with the use of fluorescently-labeled bacterial strains facilitates the investigation of host-pathogen interactions at a cellular level. More recently, the zebrafish model has also been developed for *Mav* infection (83), but the lack of adaptive immunity during the zebrafish larval stage may be a limitation in investigating innate and adaptive immune interactions (162). Taken together, current *in vivo* models fail to entirely recapitulate host immunity during *Mav* infection, highlighting the need for optimization of preclinical models.

**Evaluation of combinatorial HDT regimens**
While HDT has the potential to serve as a stand-alone treatment, particularly for patients unresponsive to standard-of-care, HDT is mainly envisioned as an adjunctive therapy to conventional antibiotics. Considering that antibiotics target bacteria and HDT target the host, they may complement each other and adjunction of HDT may shorten antibiotic treatment length or reduce the dosage of antibiotic regimens, minimizing side effects and probability of antibiotic resistance.

Evidence evaluating the efficacy of HDT, similar to treatment alone, combined with antibiotics during *Mav* infection is limited. Most available data involve combinations of HDT with cytokines and antibiotics. For instance, GM-CSF has been shown to enhance the efficacy of clarithromycin at clinically achievable concentrations, potentially due to increased intracellular uptake of clarithromycin following GM-CSF pre-treatment (4). Furthermore, it is also suggested that the impaired bacterial growth induced by cytokines, including GM-CSF, may be the result of phagosome acidification (163). Since macrolides, such as clarithromycin, accumulate in acidic vesicles, GM-CSF may enhance antibiotic activity by accumulating the drug at the site of bacteria by increasing phagosome acidification. Similarly, HDT that counteracts *Mav*-induced phagosome maturation arrest and promotes phagosome-lysosome fusion may not only enhance lysosomal degradation but also increase bacterial exposure to antibiotics localized in acidic lysosomes. However, at higher clarithromycin doses achieving serum peak

levels seen in patients, no additive effect with GM-CSF was observed (4). One possible reason is that both drugs are transported into cells via a similar uptake mechanism, and high clarithromycin concentration may saturate this process (164, 165), resulting in no enhanced antibiotic activity by GM-CSF. Alternatively, activating macrophages with cytokines like GM-CSF might render intracellular bacteria more susceptible to antibiotics, but in high clarithromycin concentrations, the bacteria are already killed, and GM-CSF has no additional effect.

Another approach in HDT as adjunctive therapy is the use of host efflux pump modulators. These pumps efflux ions and possibly antibiotics from vesicles like phagosomes and lysosomes, reducing antibiotic potency. Inhibiting these host cell pumps with HDT may therefore potentiate antibiotic efficacy. Verapamil, for example, has been shown to enhance the activity of antibiotics like rifampicin and bedaquiline against mycobacteria (166, 167), likely by inhibiting mycobacterial efflux pumps reducing drug tolerance (168, 169). This effect is linked to verapamil's ability to inhibit human p-glycoprotein (170), which may also reduce the efflux of antibiotics from vesicles where bacteria reside (171). However, verapamil may not potentiate antibiotics that have the same mechanism of action. Hence, considering the mechanism of action of both the HDT and antibiotic may inform the potential of combinations. In addition, drug metabolism should be considered in combinatorial regimens. For example, combining verapamil with clarithromycin has been observed to be fatal since clarithromycin impairs the metabolism of verapamil, leading to toxic levels (172). In summary, studying potential interactions between HDT and conventional antibiotics is critical in designing more effective and safe combinatory regimens for *Mav*.

Finally, there has been limited exploration of combining multiple HDTs. As discussed, mycobacteria like *Mav* are notorious for modulating host immune pathways via different mechanisms and a multi-targeted HDT approach could more effectively counteract these bacterial-induced modulations, resulting in improved host control of infection. For example, combining cytokines (173), or other immunomodulatory compounds have shown to have additive effects on the antimycobacterial activity of macrophages (174), including against *Mav* (175). However, combinations like vitamin D and PBA failed to show additive effects, potentially because both compounds target the same pathways, underscoring the importance of understanding the mechanism of action of HDT. Hence, further research is warranted to explore synergistic HDT combinations.

## Concluding remarks

This thesis highlights the potential of HDT as a promising strategy for combating intracellular *Mav* infections, using primary human macrophage-based infection models. Repurposed amiodarone and phenothiazines were shown to improve host control of *Mav* infection through immunomodulatory effects, and optimizing their safety and efficacy could improve their clinical applicability. Further investigation of their mechanisms of action may also reveal novel strategies to eliminate intracellular *Mav* infection. In our search for new host targets for HDT, we identified the macrophage response to *Mav* infection included cytokine immune responses, although the limited cytokine-based HDT emphasizes the need for a deeper understanding of protective immune pathways during *Mav* infection. Additionally, the regulation of lipid metabolism genes upon *Mav* infection, similar to *Mtb*, reinforces its potential as a therapeutic

target, while the identification of GIMAP gene modulation suggests additional host factors that may influence infection outcomes. The next challenge lies in deciphering the precise role of these responses in *Mav* infection and their potential as host targets for the development of HDT for *Mav*. Advancing preclinical models, particularly those mimicking immunocompromised conditions or incorporating multi-cell interactions, will be crucial for improving translational relevance. Moreover, combining HDT with antibiotics or other immunomodulators may enhance treatment efficacy, but understanding synergistic mechanisms and drug interactions is essential. Ultimately, these insights and refinements will pave the way for developing more effective HDT strategies against *Mav* infections to improve patient outcomes.

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