MycoJaca 26

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Foreword

We are delighted to welcome you to Jaca, a privileged spot in the Aragonese Pyrenees that combines a remarkable historical heritage, a long-standing University tradition, and an exceptional natural setting.

Known as the "Pearl of the Pyrenees," Jaca played a central role in the origins of medieval Spain, becoming the first capital of the Kingdom of Aragon under the reign of Ramiro I in the 11th century. Its history is still very much present in the city, from the Cathedral of Saint Peter, one of the most important examples of Romanesque architecture in the Iberian Peninsula and a landmark on the Aragonese Way of Saint James, to the Citadel, the remarkable 16th-century pentagonal fortress that has been preserved almost intact.

Jaca also has a long connection with academic life. It is the historical home of the University of Zaragoza's Summer Courses, which began in the 1920s and were among the first of their kind in Spain. Since then, the Residencia Universitaria has welcomed generations of professors, researchers, and students from Spain and abroad. Over the years, this has given Jaca a particular place in the academic life of the University of Zaragoza.

MycoJaca26 continues this tradition. The meeting will bring together researchers working on different aspects of mycobacterial biology and disease, with a common interest in understanding infection and finding better ways to prevent and treat it. The programme covers a broad range of topics, including new therapeutic approaches and antibiotic resistance, host–pathogen interactions, infection dynamics, genomic epidemiology and evolution, tuberculosis vaccines, and infections caused by non-tuberculous mycobacteria.

We hope that MycoJaca26 will be a good opportunity to share results, discuss ongoing work, meet colleagues from different fields, and start new collaborations. And we hope that, alongside the science, you will have the chance to enjoy Jaca and its surroundings.

The MycoJaca26 Organizing Committee

Zaragoza, 2026.

Program

Wednesday October 21st

Session 1: Wednesday, 17:00-19:30.
Palacio de Congresos de Jaca (Avenida Juan XXIII, 17)
Chairperson: Esther Julián

17:00–17:40	**REGISTRATION**
17:40–18:00	**OFFICIAL OPENING** • Carlos Serrano, Major of Jaca • Rosaura Pérez, Director of Secretariat for Research Resources, University of Zaragoza • Esther Julián, Coordinator of Myco_NET
18:00–18:30	**SPONSOR CONFERENCE: CERTEST** **Esther Pérez Herrán** Certest Biotech Abordaje integral de Certest frente a la tuberculosis: diagnóstico, vacunas y tratamiento. *CERTEST's comprehensive approach to tuberculosis: diagnosis, vaccines and treatment*
18:30–19:30	**PUBLIC OUTREACH LECTURE** **Prof. Carlos Martín Montañés** Universidad de Zaragoza MTBVAC: un viaje de la genética a una vacuna global contra la tuberculosis *MTBVAC: A Journey from Mycobacterial Genetics to a Global Tuberculosis Vaccine*

Thursday October 22nd

Session 2: Thursday 09:00-10:30.
Residencia Universitaria de Jaca (Calle de la Universidad, 3)
Chairpersons: Cristina Vilaplana & Alicia Aranaz.

09:00–09:30	**KEYNOTE PRESENTATION** **Mireia Coscollá** I2SysBio / FISABIO Host-Specific Infection Dynamics of *Mycobacterium tuberculosis* Complex Lineages in Human and Bovine Granuloma Models
09:30–10:00	**SHORT TALKS** **A. Arbués** Targeted nanocarrier-based drug delivery improves intracellular and granuloma-associated antitubercular activity **P. Gil-Berbel** Establishing an *in vitro* translation system to understand molecular translation and ribosome plasticity in mycobacteria **M. Silva** Conditional essentiality of *Mycobacterium tuberculosis* NDH-2 across environments
10:00-10:30	**KEYNOTE PRESENTATION** **Álvaro Chiner** I2SysBio / FISABIO / CIBERESP Novel phylogenetic association analyses reveal the complex architecture of drug resistance in *Mycobacterium tuberculosis*.

10:30-11:30	**COFFEE BREAK &** **POSTER SESSION #1 (Posters 1-12)**

Session 3: Thursday 11:30-13:30.
Residencia Universitaria de Jaca (Calle de la Universidad, 3)
Chairpersons: José Domínguez & Nacho Aguiló

11:30-12:00	**SHORT TALKS** **G. Pascual** Extracellular vesicles as morphotype-dependent effectors in *Mycobacterium abscessus* **A. Ruiz-Enamorado** The Non-Canonical Mismatch Repair Protein, NucS, Safeguards Mycobacterial Genomes Against Oxidative Damage **T. Silva** HOST-MAB: Understanding host-*Mycobacterium abscessus* interactions using macrophages with diverse genetic ancestry
12:00-12:30	**KEYNOTE PRESENTATION** **M. Vidal** IGTiP /UAB / CMCiB / HUGTiP *Drosophila melanogaster* as a cost-effective bridge model for antimycobacterial drug evaluation
12:30-13:00	**SHORT TALKS** **A. Jiménez-Alesanco** Optimization and preclinical development of oral BCG and MTBVAC vaccination for mucosal protection against tuberculosis **M. Casal** Prophylactic efficacy of MTBVAC in lung metastasis mouse models **P. Cuenca-Lara** Mucosal and parenteral BCG-based vaccination strategies differentially modulate Th1 immune responses intensity and confer protection against *Mycobacterium caprae* in goats
13:00-13:30	**Steering committee meeting MycoNET & CIBER**

13:30-15:00	**LUNCH BREAK AT THE RESIDENCE**

Session 4: Thursday 15:30-17:00.
Residencia Universitaria de Jaca (Calle de la Universidad, 3)
Chairpersons: María Jesús García & Sonia Gullón.

15:00-15:30	**KEYNOTE PRESENTATION** **Rafael Prados-Rosales** UAM. The Mtb RNAome in and out of extracellular vesicles: implications for diagnosis and pathophysiology
15:30-16:00	**SHORT TALKS** **A. Lucía** Impact assessment of inoculum size and recent modifications on the EUCAST reference method for MIC determination of anti-tuberculosis drugs **A. Roig-Guill** Role of the inhA S94A Mutation on drug resistance and the spread of an Isoniazid-Resistant Tuberculosis strain **A. Lacoma** Activity of fusidic acid against *Mycobacterium tuberculosis* H37Ra in a human macrophage infection model
16:00-16:30	**KEYNOTE PRESENTATION** **Emma Sáez** UNIZAR & CIBERES Patient-centered and health systems research integrated within the BLMs4BU clinical trial to shorten Buruli ulcer treatment in West Africa

16:30-17:30	**COFFEE BREAK &** **POSTER SESSION #2 (Posters 13-24)**

Session 5: Thursday 17:30-19:30.
Residencia Universitaria de Jaca (Calle de la Universidad, 3)
Chairpersons: José Domínguez & Nacho Aguiló

17:30-18:00	**SHORT TALKS** **S. Lin** Epidemiological, Clinical and Microbiological Characteristics of Tuberculosis in Aragon (Spain): A Population-Based Case-Control Study based on Real -Data (2017–2022). **J. Gonzalo-Asensio** Antimicrobial conjugates with vitamin B12 as a Trojan Horse strategy against *Mycobacterium tuberculosis* **L. Varón-Pozuelo** Early Translational Shutdown and Metabolic Reprogramming in *Mycobacterium tuberculosis* under Capreomycin Stress
18:00-18:30	**SHORT TALKS** **N. Ferrer-Luzón** Carbon Availability Orchestrates Small RNA Expression in *Mycobacterium tuberculosis* Lysates and Extracellular Vesicles **J. Arruego** Functional analysis of natural variants in the PhoPR virulence system of *Mycobacterium tuberculosis* **L. Vázquez** Preliminary Clinical Evaluation of a Ribosomal Operon Promoter as a Biomarker of *Mycobacterium tuberculosis* Viability and Bacillary Burden
18:30-19:30	**PLENARY LECTURE** **Prof. Margarida Saraiva** i3S - Instituto de Investigação e Inovação em Saúde. Universidade do Porto Understanding tuberculosis through the lens of *Mycobacterium tuberculosis*

21:00	**SOCIAL DINNER @ RESTAURANTE EL PARQUE** **(Gran Hotel de Jaca, Paseo de la Constitución, 1)**

Friday October 23rd

Session 6: Friday 09:00-11:00.
Residencia Universitaria de Jaca (Calle de la Universidad, 3)
Chairpersons: Cristina Vilaplana & Alicia Aranaz.

09:00-09:30	**KEYNOTE PRESENTATION** **Alisa Aliaga** IREC-CSIC / UCLM / MIVEGEC Shaping *Mycobacterium ulcerans* and Buruli Ulcer Distribution: Natural Drivers and Human-Induced Pressures
09:30-10:00	**KEYNOTE PRESENTATION** **Bernat Pérez del Val** CReSA / IRTA / UAB Connecting animal and human mycobacterial research: activities and perspectives from IRTA-CReSA
10:00-10:30	**SHORT TALKS** **M. Cortacans** Comparative macrophage responses to *Mycobacterium manresensis* and *Mycobacterium abscessus* **N. Traba** Mincle and TLRs coordinate macrophage-driven antitumour responses to mycobacterial lipid **M. Pinilla** Pericytes enhance macrophage bactericidal activity during *Mycobacterium tuberculosis* infection
10:30-11:00	**KEYNOTE PRESENTATION** **Daria Smalchuk** IGTiP / UAB Beyond phage diversity: Assessing mycobacteriophage-antibiotic combinations against *M. tuberculosis*

11:00-12:00	**COFFEE BREAK &** **POSTER SESSION #3 (Posters 25-36)**

Session 7: Friday 12:00-13:30.
Residencia Universitaria de Jaca (Calle de la Universidad, 3)
Chairpersons: María Jesús García & Sonia Gullón.

12:00–12:30	**SHORT TALKS** **F. Cordero** Genetic determinants of transmission success in *Mycobacterium tuberculosis* **M. Álvarez** MIPERA: automating Mtb Intra-Patient Evolution Reporting and Analysis **M. Caballer-Gual** Experimental evolution reveals lineage-specific trajectories of bedaquiline adaptation in the *Mycobacterium tuberculosis* complex
12:30–13:00	**KEYNOTE PRESENTATION** **Claire Brooks** UC3M Radiomic trajectory phenotyping of tuberculosis lesions reveals treatment response patterns in the marmoset model
13:00-13:30	**SHORT TALKS** **P. Soldevilla** Tuberculosis and stress: when two unstopable forces meet **F.J. Roca** The host-resistance factor Sam68 protects Mtb-infected macrophages from premature cell death **G. Goig** Therapeutic opportunities of high-dose rifampicin regimens against borderline rifampicin-resistant

13:30-15:30	**LUNCH BREAK AT THE RESIDENCE**

15:30	END OF THE MEETING

POSTER SESSION #1
Thursday 22nd October, 10:30-11:30

P_01 **Sofía Samper:** *Description of Two Novel Rapidly Growing Non-Tuberculous Mycobacterial Species Isolated from Human Clinical Samples*

P_02 **Isabel Otal:** *Evolution of a Mycobacterium tuberculosis strain during an outbreak transmission driven by IS6110-mediated recombination*

P_03 **Alexandre Pinto:** *Targeting Mycobacterium tuberculosis One-Carbon Metabolism to Disrupt Fatty Acid Resistance*

P_04 **Ilean Zitlali Aguilar-Elguea:** *Differential expression of exosomal miRNAs from macrophages with NOD2 receptor blockade challenged with M. bovis BCG*

P_05 **José Antonio Aínsa:** *A complementary photothermal treatment enhances the effect of BMNP-associated bacteriocin AS-48 and reduces the viability of Mycobacterium tuberculosis*

P_06 **Miriam Blay-Benach:** *Mucosal BCG and prime-boost vaccination strategies enhance functional reprogramming of alveolar macrophages in goats*

P_07 **Cristina Alierta:** *The IFN-γ/JAK1 axis modulates tumor aggressiveness and immune escape mechanisms in intravesical MTBVAC-based immunotherapy for bladder cancer*

P_08 **Paula Ruiz-Rodríguez:** *A closed pangenome reveals structural routes to host adaptation in the Mycobacterium tuberculosis complex*

P_09 **Carme Cucarella:** *Challenges in Precise Genome Engineering in Mycobacterium smegmatis: Insights from RecT-Mediated Editing of the rrnA Operon*

P_10 **Laura Villamayor-Belinchon:** *Structural basis of canonical translation initiation in Mycobacteria*

P_11 **Teresa Cortés:** *In vitro selection and characterisation of capreomycin resistant mutants in Mycobacterium smegmatis*

P_12 **Diego Aznar:** *Unravelling the transcriptome of the human tuberculosis lesion and its clinical implications*

POSTER SESSION #2
Thursday 22nd October, 17:00-17:30

P_13 **Irene Latorre:** *Assessment of lateral-flow and ELISA assays based on IP-10 detection for the diagnosis of Mycobacterium tuberculosis infection*

P_14 **Carlos Peña:** *Full-spectrum flow cytometry reveals distinct phenotypic immunological profiles in peripheral blood and bronchoalveolar lavage from patients with pulmonary tuberculosis*

P_15 **Núria Sabando-García:** *Immunomodulatory Effects of Mycobacterium brumae as an Adjuvant in Prophylactic Bladder Cancer Treatment*

P_16 **Iris Moret-Gómez:** *Non-tuberculous Mycobacteria-based Immunomodulation as a Prophylactic Strategy Against Pulmonary Infections*

P_17 **Arnau Seguí-Moll:** *Comparative genomics reveals the evolutionary diversification of asparaginases across the genus Mycobacterium*

P_18 **Arnau Seguí Moll:** *Exploring Mycobacteria as a Source of Therapeutic L-Asparaginases with Antileukemic Potential*

P_19 **Marta López-Llaría:** *Genomic epidemiology of mycobacteria involving paediatric population*

P_20 **Andrea Marcos-Abellán:** *Integrating INDELs to Refine Mycobacterium tuberculosis Transmission Networks*

P_21 **Sergio Sánchez Lorente:** *Paratuberculosis: the dual challenge*

P_22 **Vivian C. Salgueiro-Toledo:** *Cell envelope stress and Dynamin-like proteins IniA and IniC coordinate extracellular vesicle biogenesis in Mycobacterium tuberculosis*

P_23 **Carmen Menéndez:** *Lipid availability facilitates persistence of Mycobacterium tuberculosis under Iron deprivation*

P_24 **Mariana Moreira:** *Uncovering drivers of post-tuberculosis sequelae informed by host-pathogen interactions*

POSTER SESSION #3
Friday 23rd October, 11:00-12:00

P_25 **Juan Calvet-Seral:** *Exploring the rRNA synthesis ratio biomarker in Mycobacterium ulcerans for drug activity evaluation*

P_26 **Lidia de Tapia-Hernández:** *Multiplexed rRNA biomarkers for medium-to-high-throughput evaluation of Mycobacterium tuberculosis drug response*

P_27 **María Fernanda Raigosa Muñoz:** *New Cholic Acid Derivatives as Novel Anti-Mycobacterium tuberculosis Agents*

P_28 **Pedro Ruiz-Gutiérrez:** *Sam68 as a key regulator of pathological cell migration and host defense against tuberculosis*

P_29 **Víctor Castro-Ruiz:** *The host-resistance factor Sam68 protects Mtb-infected macrophages from premature cell death*

P_30 **Iris Moret-Gómez:** *Within-host evolution generates virulence diversity in Mycobacterium abscessus*

P_31 **Elsa Moreno-García:** *Context-dependent roles of progranulin in host defense: myeloid dysfunction with pathogen-specific effects beyond mycobacterial infection*

P_32 **Patricio López Expósito:** *Whole-Blood RNA-seq Captures Structured rRNA Patterns Linked to Tuberculosis State and Progression*

P_33 **Joaquín Sanz:** *Exploring the Effects of MTBVAC Vaccination on Innate Immune Responses to Sepsis Through Single-Cell Transcriptomics*

P_34 **Miriam Blay-Benach:** *In vitro Mycobacterium fortuitum priming enhances pro-inflammatory and bactericidal responses of caprine alveolar macrophages upon homologous re-stimulation*.

Talks abstracts

Abordaje integral de CERTEST frente a la tuberculosis: diagnóstico, vacunas y tratamiento.

CERTEST's comprehensive approach to tuberculosis: diagnosis, vaccines and treatment

Esther Pérez-Herrán, Esther Broset, Elena Mata, Javier Giménez, Diego Casabona, Henar Alonso, Rafa Clavelia, Jesús Aznar y Juan Martínez

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Tuberculosis (TB) remains a major global health challenge, further complicated by the emergence and spread of drug-resistant *Mycobacterium tuberculosis* strains. Tackling TB requires integrated strategies encompassing rapid diagnosis, antimicrobial resistance detection, effective vaccination and innovative therapeutic approaches.

CERTEST combines its established expertise in TB diagnostics with the development of next-generation vaccines and novel therapeutic strategies. Its diagnostic solutions enable the detection of *M. tuberculosis* and the identification of genetic determinants associated with resistance to anti-TB drugs, supporting rapid and informed therapeutic decision-making.

CERTEST has developed a proprietary RNA delivery platform based on a diverse library of high-performing ionizable lipids for tailored LNP formulations. Combined with proprietary UTRs, sequence optimization and in-house RNA production, the platform enables the development of nucleic-acid therapeutics for different applications. Building on this technology, CERTEST is developing a tuberculosis vaccine candidate currently undergoing preclinical evaluation for immunogenicity and protective efficacy.

In parallel, CERTEST is developing innovative therapeutic approaches targeting both the pathogen and the host. These include nucleic-acid-based strategies targeting essential bacterial functions and host-directed therapies (HDTs) aimed at modulating host pathways involved in the response to infection.

Together, these complementary technologies provide a comprehensive approach to TB diagnosis, prevention and treatment.

MTBVAC: un viaje de la genética a una vacuna global contra la tuberculosis

Carlos Martín[1,2,3]
en nombre del equipo y de la comunidad internacional de MTBVAC

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La tuberculosis continúa siendo uno de los grandes problemas de salud global, a pesar de disponer desde hace más de un siglo de la vacuna BCG. Esta charla propone un recorrido por la microbiología y la genética de *Mycobacterium tuberculosis* que condujeron al desarrollo de MTBVAC.

La historia comienza en los años ochenta, con los trabajos pioneros de Brigitte Gicquel en el Instituto Pasteur y el nacimiento de la genética molecular de las micobacterias. Herramientas como IS*6110* permitieron estudiar la transmisión de la tuberculosis durante las epidemias asociadas al VIH y a la multirresistencia de los años noventa. La secuenciación del genoma de *M. tuberculosis* permitió posteriormente identificar determinantes de virulencia y construir racionalmente MTBVAC, una vacuna viva atenuada derivada del patógeno humano.

Nacido en la Universidad de Zaragoza como un proyecto íntegramente europeo, MTBVAC avanzó desde los estudios moleculares y preclínicos hasta su transferencia a Biofabri y su desarrollo clínico con TBVI, IAVI y otros socios internacionales. Hoy es un proyecto global que busca una vacuna universal, eficaz y accesible, capaz de contribuir a transformar la lucha contra la tuberculosis.

Agradecimientos: *El desarrollo de MTBVAC ha sido posible gracias al trabajo y compromiso de una extensa comunidad científica y clínica y al apoyo continuado de numerosas instituciones y organizaciones, nacida en la Universidad de Zaragoza y como socio industrial y clínico la Biotecnológica española Biofabri, del Gobierno de Aragón, Gobierno de España, Unión Europea —desde los primeros Programas Marco hasta Horizon Europe y Global Health EDCTP3—, TBVI, IAVI, Gates Foundation, Norad, Coefficient Giving (anteriormente Open Philanthropy), Bharat Biotech y Fiocruz Bio Manguinhos FAP Brasil y además de otros socios y financiadores nacionales e internacionales.*

Host-Specific Infection Dynamics of *Mycobacterium tuberculosis* Complex Lineages in Human and Bovine Granuloma Models

Hellen Hiza[1], Julie Milena Galvis[1], Marta Caballer-Gual[1], Paula Ruiz-Rodriguez[1], Clara Picazo[1], Xavier Valldecabres [2], **Mireia Coscolla**[1]

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The *Mycobacterium tuberculosis complex* (MTBC) comprises human-adapted lineages (L1–L10) and several animal-associated lineages. While host spillover occurs, most lineages display strong host specificity. We hypothesize that host specificity arises from interactions between lineage-specific virulence mechanisms and host immune responses.

To investigate host-specific virulence, we used an in vitro granuloma model generated from human and bovine whole blood. PBMCs and bacilli were embedded in a collagen–fibronectin extracellular matrix and infected in parallel with human-associated lineages L4.3 and L6, and the animal-associated lineage *Mycobacterium bovis* (A4). Granuloma-like structures were monitored up to 8 days post-infection (dpi). Bacterial replication and dormancy were assessed at 1 and 8 dpi, while host immune responses were characterized by flow cytometry, cytokine and chemokine measurements, cell death assays, and transcriptomic profiling.

Bacterial replication was generally higher in the associated host and reduced in the non-associated host, whereas L6 showed reduced replication in both models. Higher levels of dormancy were observed in associated hosts. Distinct immune activation patterns were also detected, including increased macrophage activation during L4.3 infection. Ongoing host and bacterial transcriptomic analyses will help identify the molecular mechanisms underlying host adaptation. These findings provide insights into host-specific infection processes and may inform the development of future host-directed therapies.

Targeted nanocarrier-based drug delivery improves intracellular and granuloma-associated antitubercular activity

Zsuzsa Baranyai[1,2], Maria Alleva[1], **Ainhoa Arbués**[3,4], Ana C. Millán-Placer[3,4], Ana Picó[3,4], Héctor Soria-Carrera[1,5], Rafael Martín-Rapún[1,2,5], José A. Aínsa[3,4,6,7], Jesús Martínez de la Fuente[1,2]

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The efficacy of conventional anti-tuberculosis drugs is challenged by the persistence of *Mycobacterium tuberculosis* inside macrophages and granulomas, the hallmark of the disease. In this study, we developed bedaquiline (BQ)-loaded chitosan nanocapsules (NCs) as a platform for enhanced intracellular delivery and distribution within granulomas. To promote macrophage targeting, NCs were functionalized with mannose or formyl-methionyl-leucyl-phenylalanine (fMLF) peptide derivatives, enabling receptor-mediated interactions with macrophages. PEGylated and alginate-coated NCs were generated to investigate how stealth properties and surface charge affects the interaction. This NC library was evaluated *in vitro*, including drug release, cytocompatibility in macrophage and lung epithelial cell models, internalization and efficacy against extracellular and intracellular mycobacteria. Therapeutic performance was further assessed in in vitro granuloma-like structures. Mannose- and fMLF-functionalized chitosan NCs provided high drug loading and efficient BQ delivery, while enhancing macrophage uptake and intracellular antibacterial activity compared to free BQ. Importantly, encapsulated BQ also outperformed free BQ in the granuloma model, highlighting improved efficacy in infection-relevant microenvironments. These results demonstrate the potential of receptor-targeted chitosan NCs as a promising strategy for enhanced intracellular delivery of antitubercular agents and improved drug distribution within granuloma-like structures.

Establishing an *in vitro* translation system to understand molecular translation and ribosome plasticity in mycobacteria

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[*] Both authors contributed equally to this work.

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Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), remains a major global health threat. Many antibiotics used in TB treatment target the ribosome. Recent studies have revealed species-specific features and structural heterogeneity in mycobacterial ribosomes, suggesting that translational regulation and ribosome plasticity may contribute to antibiotic resistance and adaptation during infection. However, the molecular mechanisms governing translation regulation in *M. tuberculosis* remain poorly understood.

To address this, we have successfully established *in vitro* translation systems to investigate translation and ribosome function in mycobacteria, both with all *M. tuberculosis* translation factors purified through recombinant expression in Escherichia coli (PURE system) and with protein S150 extracts (extract-based system) from the non-pathogenic model organism *Mycobacterium smegmatis*. By systematically optimizing reaction conditions and components, we achieved efficient translation of an mRNA encoding superfolder GFP.

This platform is currently being used to characterize translation initiation mechanisms using mRNAs with altered ribosome-binding sites and by varying the composition of translation factors and ribosomal components. In parallel, we are investigating ribosome-mediated antibiotic resistance by adding mutant ribosomes and ribosome-targeting antibiotics to these *in vitro* translation systems. This work will provide new insights into translation regulation and ribosome plasticity, potentially identifying novel therapeutic targets against tuberculosis.

Conditional essentiality of *Mycobacterium tuberculosis* NDH-2 across environments

Marco Silva[1,2], Marta L. Silva[1], Rodrigo Monteiro[1], Tiago Beites[1]

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Mycobacterium tuberculosis adapts its respiratory metabolism to diverse host environments, hindering the identification of universally vulnerable drug targets. Type II NADH dehydrogenase (NDH-2) has long been considered an attractive target due to its central role in the mycobacterial electron transport chain. However, respiratory rewiring through NDH-1 can compensate for NDH-2 loss under standard laboratory conditions. We hypothesized that infection-relevant environments impose metabolic constraints that render NDH-2 essential despite this apparent redundancy. To test this, we investigated NDH-2 requirement using both a Δ*ndh-2* mutant and a conditional NDH-2 depletion (NDH-2-DUC) strain. While NDH-2 deficiency was tolerated under conventional growth conditions, loss of NDH-2 resulted in a reduction in bacterial viability following adaptation to an in vitro caseum model. Importantly, conditional depletion of NDH-2 after adaptation to caseum also reduced bacterial viability, although to a lesser extent than observed in the Δ*ndh-2* mutant. Consistent with these findings, NDH-2 depletion in the chronic phase of infection in both C57BL/6 and C3HeB/FeJ mice led to a significant reduction in bacterial burden and lung histopathology. Together, our results identify NDH-2 as an environment-dependent essential function and highlight lesion-specific metabolic states as sources of previously unrecognized therapeutic vulnerabilities.

Novel phylogenetic association analyses reveal the complex architecture of drug resistance in *Mycobacterium tuberculosis*.

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Past and present selective pressures acting on bacterial organisms drive the accumulation of genetic mutations that leave lasting imprints on their genomes. In obligate pathogenic bacteria, such as members of the *Mycobacterium tuberculosis* complex, antibiotic treatment represents a major selective force shaping evolutionary trajectories. In this study, we applied phylogeny-based approaches to identify mutations that either precede or emerge concurrently with drug-resistance phenotypes.

From the resulting candidates, we selected two mutations for functional characterization by constructing isogenic mutants and assessing their potential contribution to drug resistance. First, we analyzed the C61G substitution in the *hadA* gene. Variants at this codon have previously been associated with resistance to the now-discontinued antitubercular drugs isoxyl (ISO) and thiacetazone (TAC). However, our results show that the C61G mutation does not affect the minimum inhibitory concentration (MIC) or tolerance to rifampicin or isoniazid, nor does it exhibit a compensatory effect. We therefore propose that the phylogenetic signal detected for this mutation reflects past selective pressure driven by ISO and TAC usage, rather than ongoing selection, representing a historical imprint that persists in present-day genomes.

We also examined the synonymous V23V variant in the *prpD* gene. This mutation led to a marked increase in gene expression, with approximately a two-fold upregulation of *prpR* and a ten-fold increase in *prpD*. Besides these transcriptional changes, we observed differences in the OD decay after reaching the stationary phase between control strains and *prpD* mutants when growing under standard culture conditions. However, no differences were observed in MIC when the bacteria were grown in the presence of isoniazid or rifampicin.

Together, these findings highlight that evolutionary signatures identified through phylogenetic approaches may capture both current and historical selective forces. Importantly, not all such signals reflect direct contributions to contemporary drug resistance, but may instead reveal legacy effects of past treatments and broader adaptive processes shaping bacterial fitness.

Extracellular vesicles as morphotype-dependent effectors in *Mycobacterium abscessus*

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Mycobacterium abscessus is an emerging opportunistic pathogen causing severe pulmonary and soft tissue infections, particularly in cystic fibrosis and chronic lung disease patients. Two phenotypic morphotypes, smooth (S) and rough (R), differ markedly in virulence, immune evasion, and clinical outcomes. Extracellular vesicle (EV) production is an important mechanism in bacterial pathogenesis, yet vesiculation in *M. abscessus* remains poorly characterized, especially regarding morphotype-specific functions *in vivo*.

We isolated mycobacterial extracellular vesicles (MEVs) from S and R strains and compared their physicochemical properties, including concentration, size distribution, lipid and protein composition, proteomic profiles, and ultrastructure. To evaluate biological activity, we used an *ex vivo* human macrophage model for MEV stimulation, uptake, and infection assays. We also developed an *in vivo* zebrafish model in which MEVs from S and R strains were injected and immune cell recruitment quantified as a measure of host response.

S and R morphotypes produced MEVs with distinct physicochemical characteristics, including differences in vesicle yield, size distribution, and protein cargo. In macrophages, R-derived MEVs induced stronger and qualitatively different immune responses than S-derived MEVs. In the zebrafish model, R MEVs triggered a distinct pattern of immune cell recruitment, reflecting the differential pathogenicity of the parental strains.

These findings demonstrate that morphotype-dependent vesiculation in *M. abscessus* contributes to differential host–pathogen interactions ex vivo and in vivo, positioning MEVs as potential mediators of virulence and immune modulation.

The Non-Canonical Mismatch Repair Protein, NucS, Safeguards Mycobacterial Genomes Against Oxidative Damage

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Oxidative DNA damage is a major driver of mutagenesis in *Mycobacterium tuberculosis*, a pathogen that acquires drug resistance exclusively through chromosomal mutations. Although mycobacteria encode a robust GO system to counteract 8-oxo-dG lesions, the contribution of mismatch repair (MMR) to this process remains poorly understood. Here we demonstrate that NucS, the central component of the mycobacterial non-canonical mismatch repair pathway, plays a previously unrecognized role in repairing OG:A mismatches and cooperates with the GO system to limit ROS-induced lethality and mutagenesis. Together, our findings reveal an additional layer of genome protection in mycobacteria and provide new mechanistic insight into mutation rate regulation in *M. tuberculosis*, with implications for understanding the emergence of antibiotic resistance.

HOST-MAB: Understanding host-*Mycobacterium abscessus* interactions using macrophages with diverse genetic ancestry

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Mycobacterium abscessus (Mab) is an emerging opportunistic pathogen that poses a severe health problem worldwide. Unlike tuberculosis, infections caused by non-tuberculous mycobacteria (NTM), including Mab, are not systematically reported; thus, the epidemiology of Mab infection remains poorly understood. The incidence of disease is higher in people with cystic fibrosis, bronchiectasis, and other chronic lung diseases, but it varies widely within and between countries (higher in Asia and Oceania than in Europe and the Americas) and among individuals of different genetic ancestries. The host determinants that influence susceptibility to Mab are poorly understood.

We hypothesize that human genetic ancestry shapes macrophage responses to Mab infection, leading to differential intracellular bacterial survival and inflammatory responses. To our knowledge, the role of ancestry-related genetic variation in Mab infection remains unexplored. In this work, we will use human induced pluripotent stem cells (iPSCs) to generate macrophages from donors with different genetic ancestries (European, Asian, and African). These macrophages will be infected with a panel of Mab isolates from clinical and environmental sources, aiming to identify host and bacterial determinants driving infection outcome. The Mab isolates will be characterized in terms of growth rate, biofilm formation, and antibiotic susceptibility, and their relative virulence will be evaluated by infecting *Galleria mellonella* larvae. The response of the macrophages to Mab infection will be evaluated in terms of inflammatory response and permissiveness to intracellular bacterial growth. The host-Mab pairs with the most extreme behaviors in terms of bacterial proliferation and inflammatory response will be selected for further analysis, including dual RNA-seq and detailed macrophage responses, such as phagosome maturation, autophagy activation, and inflammatory signalling.

This work presents an innovative approach that integrates host genetic ancestry with the phenotypic diversity of Mab isolates to dissect host-pathogen interactions, an area largely unexplored in Mab research. Ultimately, this strategy has the potential to inform the development of host-directed therapeutic strategies against Mab infections.

Drosophila melanogaster as a cost-effective bridge model for antimycobacterial drug evaluation

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Antimicrobial resistance is rising globally, underscoring the urgent need for new therapeutic strategies. Traditional drug screening often relies on in vitro models, which may promote compounds with limited translational relevance to mammalian systems. D*rosophila melanogaster* (Dm) offers a promising alternative by reducing time and costs in preclinical antimicrobial screening. However, standardized protocols for its use in drug testing are still being refined. In this study, we validated a cost-effective oral treatment protocol using Dm infected with *Mycobacterium marinum* (Mm), highlighting its potential as an intermediary screening platform in tuberculosis research. Adult flies were infected with 50 CFUs of Mm and treated orally with compound-infused yeast paste from three days post-infection. Treatment continued for seven days, during which survival was monitored daily, and bacterial burden was assessed on day 10 post-infection. Our results showed that antibiotics, host-directed therapies, and anti-virulence compounds can be successfully screened for efficacy in this model, as most treatments improved survival and significantly reduced bacterial load compared with untreated controls. In addition, a new Nanostring gene expression panel provided insight into previously unknown mechanisms of action of some host-directed therapies against tuberculosis. Overall, our findings confirm Dm as a cost-effective intermediary screening tool for antimicrobial development.

Optimization and preclinical development of oral BCG and MTBVAC vaccination for mucosal protection against tuberculosis

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Oral delivery of live tuberculosis (TB) vaccines represents a potentially scalable and clinically feasible strategy to induce protective mucosal immunity. MTBVAC, a live attenuated *Mycobacterium tuberculosis* vaccine, has previously demonstrated superior protection to Bacillus Calmette-Guérin (BCG) in preclinical models and may be particularly suitable for oral administration.

We evaluated the preclinical development of oral MTBVAC vaccination in mouse models, including proof-of-concept efficacy, optimization of formulation and vaccination regimen, and assessment of protection, immunogenicity and safety. Initial studies showed that oral BCG and MTBVAC reduced pulmonary bacterial burden following M. tuberculosis challenge, although heterogeneous responses highlighted the need for optimization. Vaccination conditions were subsequently refined by adjusting dose, administration volume and dosing schedule, while a sucrose-assisted semi-voluntary uptake strategy improved the consistency of vaccine administration.

Under optimized conditions, oral BCG and MTBVAC achieved protection levels comparable to subcutaneous BCG, with homogeneous responses across animals. Efficacy was confirmed across different mouse strains and was associated with antigen-specific immune responses in lungs and spleen. Biodistribution studies demonstrated progressive clearance of vaccine bacteria and a favorable safety profile, with no long-term adverse effects detected up to six months post-vaccination. These findings support oral delivery of live TB vaccines as a promising needle-free vaccination strategy.

Prophylactic efficacy of MTBVAC in lung metastasis mouse models

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Tuberculosis vaccines are known to induce broad immunomodulatory effects beyond protection against *Mycobacterium tuberculosis* infection. MTBVAC is a novel live tuberculosis vaccine, currently under clinical evaluation as prophylactic vaccine against tuberculosis. In this study, we investigated whether MTBVAC could exert a prophylactic antitumor effect in murine models of metastatic cancer, using established models of pancreatic, bladder, and melanoma metastasis. Mice were immunized with MTBVAC 1 month prior to tumor challenge; survival and immune responses were subsequently assessed. Vaccinated animals showed improved survival across all tumor models compared with untreated controls. Immunological analyses revealed modulation of immune responses in peripheral blood, spleen, and lung, with changes in effector function. Specifically, we assessed IFN-γ and granzyme B expression in NK and T cell populations, observing an increase in effector function in vaccinated animals compared with controls. These findings suggest that MTBVAC induces systemic immune modulation characterized by enhanced cytotoxic effector function, consistent with an antitumor immune profile across distinct metastatic models and potentially involving trained innate immunity mechanisms, further supporting tuberculosis vaccine repurposing strategies in clinically relevant metastatic models. Although evaluated in a prophylactic setting, these results provide a proof-of-concept that may be translated into future neoadjuvant approaches aimed at enhancing antitumor immunity prior to surgical intervention.

Mucosal and parenteral BCG-based vaccination strategies differentially modulate Th1 immune responses intensity and confer protection against *Mycobacterium caprae* in goats

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Tuberculosis (TB) remains a major global challenge, with limited understanding of the immune mechanisms underlying effective protection. Mucosal vaccination strategies and boosting of Bacillus Calmette–Guérin (BCG)-induced immunity have been proposed to improve protection against respiratory mycobacterial infections. Here, we evaluated the immunogenicity and protective efficacy of different mucosal-based vaccination strategies, including single-dose intranasal (i.n.) BCG, and a heterologous prime-boost approach (BCG s.c. followed by mucosal HIMB boost), compared with traditional subcutaneous BCG, in goats challenged with *Mycobacterium caprae*.

All vaccination strategies induced antigen-specific Th1 responses, characterized by increased IFN-γ production and higher frequencies of $CD4^+$IFN-γ^+ and $CD8^+$IFN-γ^+ T cells. Subcutaneous BCG elicited the highest systemic immunogenicity, followed by the prime-boost and intranasal strategies. Nevertheless, all vaccinated groups showed partial protection compared to non-vaccinated controls. Notably, mucosal strategies were associated with a more consistent reduction in both pulmonary lesion dissemination and clinical outcomes.

Importantly, differences in systemic immune responses did not directly correlate with protection. BCG i.n. conferred protection through mechanisms independent of systemic IFN-γ, whereas BCG s.c. relied on a peripheral Th1 response in blood, highlighting the complexity of identifying reliable correlates of protection in TB and and supporting mucosal vaccination as a promising strategy to enhance TB control.

The *Mtb* RNAome in and out of extracellular vesicles: implications for diagnosis and pathophysiology

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Extracellular vesicles (EVs) are central to the physiology and virulence of *Mycobacterium tuberculosis* (*Mtb*). We previously showed that mycobacterial EVs (mEVs) carry diverse RNA classes with important functional roles. Because EVs protect RNAs from extracellular nucleases, they were initially considered the primary source of extracellular RNA (exRNA). Nevertheless, accumulating evidence indicates a substantial EV-independent exRNA population in conditioned media and clinical samples, underscoring the need to characterize both EV-associated and non-EV-associated RNA fractions.

Beyond this vesicle-centric view, conventional small RNA sequencing (RNA-seq) faces major limitations in detecting RNAs with modifications that interfere with adapter ligation and reverse transcription, particularly tRNAs and rRNAs. To overcome this, approaches as T4 polynucleotide kinase (T4PNK) treatment enable “end repair” of modified RNAs, allowing comprehensive sequencing of species that elude traditional RNA-seq and yielding a more complete picture of the RNAome.

Here, we isolated intravesicular and extravesicular RNA from axenic *Mtb* cultures, *Mtb*-infected THP-1 macrophages, and uninfected THP-1 macrophages. We applied T4PNK-based end repair prior to RNA-seq and a bioinformatic analysis to examine RNA species distributions across conditions. We propose that a molecular definition of the extracellular RNAome is highly relevant to understanding *Mtb* physiology and may hold future potential for diagnostic applications in tuberculosis.

Impact assessment of inoculum size and recent modifications on the EUCAST reference method for MIC determination of anti-tuberculosis drugs

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Background. The EUCAST reference method for MIC determination of anti-tuberculosis drugs underwent key recent updates in version 7.0 in 2024, consolidated in version 8.2 in early 2025. These updates addressed inoculum range, readout schedules, growth interpretation, media and plate preparation, and solvent handling, although some points remain debated among users.

Methods. Within the ERA4TB consortium, seven trained laboratories assessed these methodological changes using harmonized workflows. MIC testing under SOP version 7.0 assessed isoniazid across three inoculum levels spanning the EUCAST-recommended range for the ATCC 27294 strain, while evaluation under version 8.2 generated MIC data for isoniazid, bedaquiline and TBAJ-587.

Results. High inocula increased MICs by roughly one doubling dilution, although median values remained stable. When faint borderline growth or pinpoint effects were considered, MICs increased by more than one doubling dilution or could not be determined, underscoring borderline growth as a critical readout challenge.

Conclusions. Clarity is needed on faint borderline growth interpretation and optimal readout timing, particularly for new drugs and large clinical isolate panels, which may affect MIC determination and detection of low-frequency resistant subpopulations.

Role of the inhA_S94A Mutation on drug resistance and the spread of an Isoniazid-Resistant Tuberculosis strain

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Isoniazid is a key drug for treating drug-susceptible tuberculosis (TB), however, ~5% of isoniazid-resistant (INH-R) cases have unknown resistance mechanisms. While *katG* is the main gene involved in INH resistance, some *inhA* mutations have also been associated with resistance, whereas others, such as *inhA*_S94A, still have limited evidence. This mutation confers low-level resistance, confirmed in allele exchange experiments, but often produces discordances in phenotypic drug susceptibility testing (pDST). Since 2014, a genomic epidemiology study in the Valencia Region has sequenced ~80% of culture-positive TB cases. Within this framework, we identified a large cluster of 31 cases carrying *inhA*_S94A that circulated between 2016 and 2025. To further investigate the clinical, epidemiological, and evolutionary significance of this mutation, we expanded the analysis also to global genomic datasets. Our results confirm that *inhA*_S94A confers borderline resistance, showing inconsistent microdilution test results (S/R), pointing to the challenges of using phenotypic susceptibility testing as gold standard and the difficulties to link genotype-phenotype in borderline mutations. In addition, S94A is associated with the acquisition of secondary INH-R mutations, particularly epidemiologically rare *katG* variants such as S315N. Our data from Valencia suggest that the mutation tends to be associated with worse clinical outcomes while it is a clear risk factor for developing MDR-TB.

Activity of fusidic acid against *Mycobacterium tuberculosis* H37Ra in a human macrophage infection model

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Background: Drug-resistant tuberculosis remains a major global health challenge, and repurposed antibiotics represent a promising strategy to expand the limited antitubercular drug pipeline.
Aims: This study aimed to evaluate whether fusidic acid (FA) reduces intracellular and extracellular *Mycobacterium tuberculosis* (Mtb) burden in an infected human macrophage model, compared with rifampicin (RIF).
Methods: PMA-differentiated THP-1 macrophages were infected with Mtb H37Ra (MOI 10). After elimination of extracellular bacteria, infected cells were treated from Day 3 post infection with FA (4-120 mg/L), RIF (4 µg/mL), or no drug. Intracellular and extracellular
bacterial loads were quantified by CFU enumeration at Days 3, 5, and 7 post-infection.
Results: FA reduced intracellular CFU in a concentration-dependent manner at Days 5 and 7 relative to untreated cells ($p<0.0001$). The highest concentration (120 mg/L) achieved the greatest intracellular inhibition and reduced intracellular CFU by 1.6 $\log_{10}$ at Day 7 compared with the untreated control, but remained less potent than RIF. Lower concentrations (4-60 mg/L) produced partial but significant reductions. Extracellular CFU were also reduced by FA at Day 7 ($p<0.05$), although without a clear concentration-response relationship.
Conclusions: FA demonstrates measurable intracellular and extracellular antitubercular activity in a macrophage infection model, supporting its further investigation as a repurposed candidate for DR-TB.

Patient-centered and health systems research integrated within the BLMs4BU clinical trial to shorten Buruli ulcer treatment in West Africa

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Buruli ulcer (BU) is a neglected tropical disease (NTD) caused by *Mycobacterium ulcerans* that can lead to permanent disability, with major psychosocial and socio-economic consequences. WHO-recommended treatment consists of 8 weeks of daily rifampicin and clarithromycin (RC8), together with wound care, physiotherapy, and, in some cases, surgery.

The BLMs4BU clinical trial (*https://blms4bu.org/*) conducted in Benin, Côte d'Ivoire and Togo (NCT05169554, PACTR202209521256638), evaluates whether a 4-week regimen of amoxicillin/clavulanate combined with rifampicin and clarithromycin (RCA4) is non-inferior to RC8 in achieving cure at month 12 after treatment initiation. If proven effective, RCA4 could shorten treatment duration, reduce hospitalization and associated costs, and improve adherence, thereby supporting patient-centered and sustainable BU care in endemic settings.

Complementary nested studies will further assess pharmacokinetics, bacterial clearance, patient-reported outcomes, treatment adherence, healthcare access, and economic burden. Together, these data will provide implementation-oriented evidence to inform feasibility, scalability and integration of the shortened regimen into national BU control programs.

By combining clinical efficacy data with patient-centered and health systems evidence, the project seeks to promote equitable access to simplified, effective, and sustainable BU treatment, in line with the WHO NTD Road Map 2030 targets.

Epidemiological, Clinical and Microbiological Characteristics of Tuberculosis in Aragon (Spain): A Population-Based Case-Control Study based on Real -Data (2017–2022). Preliminary Results

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Purpose: To identify factors associated with susceptibility and clinical progression of active TB in Aragon, aiming to improve disease control and public health strategies.
Methods: Retrospective case-control study using the BIGAN health big-data registry of patients with active TB diagnosed between January 2017 and December 2022. Control group matched by age, sex and basic health zone (1,860 cases; 1,734 controls).
Results: Foreign-born individuals were significantly overrepresented (68% vs. 32%, $p<0.001$), with lower socioeconomic status and higher mortality ($p<0.01$). Key comorbidities included COPD, bronchiectasis, HIV, chronic kidney disease, malnutrition, autoimmune disorders, depression and malignancies. Immunosuppressive therapies, corticosteroids, antiretrovirals and vitamin D/B12 deficiency showed significant associations. Hospitalization was required in 55%, with a mean stay of 14 days.
Microbiologically, 91.7% had a single positive sample, while 8.3% showed multiple or previous positive cultures. Only 4% had documented contact tracing, with a mean contact-to-diagnosis interval of 31 days. Mean symptom-to-diagnosis delay was 50 days (symptomatic data available in only 58 cases). BCG coverage was negligible (0.81%).
Conclusions: TB in Aragon predominantly affects foreign-born, socioeconomically vulnerable patients with immunosuppressive comorbidities, vitamin deficiencies and lifestyle risk factors. Prolonged diagnostic delays, low contact-tracing registration and minimal BCG coverage reveal critical surveillance gaps requiring targeted intervention.

Antimicrobial conjugates with vitamin B12 as a Trojan Horse strategy against *Mycobacterium tuberculosis*

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Tuberculosis treatment regimens are prolonged and often associated with significant adverse effects. Besides, every year there is a significant number of cases of drug-resistant *Mycobacterium tuberculosis* (*Mtb*), all of which highlights the need for novel therapeutic strategies. *Mtb* has undergone a genomic decay for B12 biosynthesis, relying on exogenous uptake from the host. Hence, we propose to use vitamin B12 as a Trojan horse to enhance the delivery of antimicrobial compounds into *Mtb*.

First, we selected an inhibitor of the InhA enzyme ($InhA_i$), which was subsequently conjugated to B12 separated by a 1-carbon linker ($InhA_i$-1C-B12). The conjugate retained antimicrobial activity against *Mtb*. The transport mechanism of the conjugate via the B12 transporter (encoded by *bacA*) was evaluated in a Δ*bacA* strain. The $InhA_i$ maintained its activity, whereas $InhA_i$-1C-B12 showed a complete loss of antimicrobial effect in the mutant strain. Further confirming the mechanism of action, we demosntrated that a chiral, inactive enantiomer of the $InhA_i$ showed no activity even at the highest concentration. We next optimized linker length by synthesizing conjugates with 4- and 9-carbon linkers (InhAi-4C-B12 and InhAi-9C-B12), resulting in a decrease in IC_{50} as the linker length increased. Besides, all compounds showed comparable activity across *Mtb* complex lineages.

Overall, this strategy represents a promising approach to improve the bioavailability of existing compounds for TB treatment and it is currently being assayed with other antimicrobials.

Early Translational Shutdown and Metabolic Reprogramming in *Mycobacterium tuberculosis* under Capreomycin Stress

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Human tuberculosis, caused by *Mycobacterium tuberculosis*, remains one of the world's deadliest infectious diseases, with over 1.3 million deaths annually and rising multidrug- and extensively drug-resistant strains. Capreomycin, previously used in MDR-TB therapy, inhibits protein synthesis by ribosome binding and serves as a model to study transcription-translation coupling in Mtb. Because these processes are tightly linked, ribosomal inhibition triggers broad physiological adaptations beyond protein synthesis. We integrated matched RNA-seq and Ribo-seq datasets from *M. tuberculosis* exposed to a sublethal capreomycin dose for 45 minutes to investigate early transcriptional and translational responses to antibiotic stress. Capreomycin exposure induces a rapid physiological shift in *M. tuberculosis* characterized by global repression of the translational machinery and extensive metabolic reprogramming. Ribosome-related functions, including translation, ribosomal structure and protein folding, are strongly downregulated, indicating a transition to a low-protein-synthesis, non-growing state. In parallel, stress and survival pathways are activated. The alkB-rubAB operon suggests lipid utilization and redox adaptation, while induction of the efflux pump EfpA supports drug tolerance and membrane remodeling. Core biosynthetic functions are partially maintained, including leucine biosynthesis, while shikimate pathway flux is redirected toward tryptophan and folate synthesis. SOS response and siroheme pathways indicate DNA damage and respiratory adaptation. Overall, capreomycin triggers coordinated shutdown of growth and metabolic reorganization to promote survival under antibiotic stress.

Carbon Availability Orchestrates Small RNA Expression in *Mycobacterium tuberculosis* Lysates and Extracellular Vesicles

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During infection, *Mycobacterium tuberculosis* encounters distinct carbon environments, ranging from glycerol-rich conditions to lipid-rich niches dominated by long-chain fatty acids (LCFAs). Adaptation to these metabolic shifts requires extensive gene expression reprogramming, in which small RNAs (sRNAs) play a key regulatory role. Extracellular vesicles (EVs) may further contribute to adaptation by transporting regulatory molecules, including potentially sRNAs.

To investigate how nutrient availability shapes sRNA-mediated regulation, we characterized sRNA profiles in cellular lysates and EVs under distinct carbon and iron conditions. We show that the EV-associated sRNA signature is strongly influenced by carbon source while remaining largely robust to iron availability. Carbon availability also drives variation in lysate sRNA profiles through distinct responses to glycerol depletion and LCFA acquisition. Rather than reflecting a single progressive adaptation, these transitions involve separable gene programs associated with the loss of glycerol and the gain of lipid substrates. In contrast, iron-dependent effects on sRNA expression are comparatively modest in EVs and are conditioned by the carbon environment in lysates.

Overall, our findings identify carbon availability as a major determinant of sRNA expression in *M. tuberculosis*, shaping both intracellular and EV-associated regulatory landscapes.

Functional analysis of natural variants in the PhoPR virulence system of *Mycobacterium tuberculosis*

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Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), remains the deadliest infectious disease caused by a single pathogen. MTB virulence depends largely on the PhoPR two-component system, which regulates key host–pathogen interaction phenotypes. Despite >99.99% genomic conservation among clinical isolates, *phoPR* genes accumulate mutations, with 580 unique nonsynonymous clinical variants reported, suggesting a natural selection with unknown phenotypic consequences.

This study evaluates the functional impact of these variants using a dual genetic platform: a PhoPR-dependent GFP reporter and a second module expressing MTB *phoPR* under its native promoter. The system was integrated into a vector and introduced into a *phoPR*-defective *Mycobacterium smegmatis* surrogate. In parallel, a bioinformatic analysis characterized mutation distribution, frequency, and potential structural implications.

For functional assessment, metabolically inactive bacteria were excluded prior to flow cytometry. Active populations were then stratified according to PhoPR activity using GFP fluorescence. This approach enables discrimination and enrichment of functional versus non-functional *phoPR* variants, followed by genotypic identification by amplicon sequencing and correlation with PhoPR-regulated virulence phenotypes.

Preliminary Clinical Evaluation of a Ribosomal Operon Promoter as a Biomarker of *Mycobacterium tuberculosis* Viability and Bacillary Burden

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Rapid and accurate diagnosis of tuberculosis (TB) remains essential for timely treatment initiation and effective disease control. Although molecular assays such as Xpert MTB/RIF Ultra have significantly improved the detection of *Mycobacterium tuberculosis* (Mtb), the identification of additional biomarkers may provide complementary information regarding bacillary burden and bacterial viability. Previous studies conducted by the home laboratory in Mtb, identifying a promoter within the ribosomal operon as a potential biomarker of bacterial viability. As this target corresponds to a precursor ribosomal transcript that is rapidly processed following transcription, its detection may reflect ongoing transcriptional activity and, therefore, the presence of viable bacilli. To evaluate its clinical applicability, respiratory samples from patients with suspected TB were classified according to Xpert MTB/RIF Ultra results. Positive samples with different bacillary loads were included to assess assay performance, while Xpert-negative samples were analyzed to determine the potential of the selected targets for detecting low-burden infections. To compare analytical performance and evaluate biosafety considerations, both raw and decontaminated specimens were processed. Human β-globin was used as a sample quality control marker. In addition, Mtb-specific 23S rRNA and 16S rRNA were analyzed to provide complementary information for result interpretation.

Preliminary findings demonstrate successful detection of the selected ribosomal marker in clinical specimens and support the feasibility of RNA-based quantitative analyses to assess bacillary burden and bacterial viability. Clinical data will allow to determine more accurately its diagnostic value and clinical utility.

Understanding tuberculosis through the lens of *Mycobacterium tuberculosis*

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Tuberculosis (TB) is a very heterogeneous disease: upon infection with *Mycobacterium tuberculosis* most individuals remain healthy, in some a latent infection is established, and in a few active disease develops. In addition to host and environmental factors, the outcome of *M. tuberculosis* infection also depends on genetic factors in the bacteria. We previously showed that *M. tuberculosis* isolates recovered from patients with severe TB presentations evaded host macrophage cytosolic surveillance mechanisms, ultimately inducing low levels of IL-1β. In contrast, isolates recovered from patients with mild TB presentations were readily recognized by macrophages, leading to the activation of the NLRP3 inflammasome and the secretion of high levels of IL-1β. More recently, using selected high IL-1β/mild TB (*M. tuberculosis* 4I2) and low IL-1β/severe TB (*M. tuberculosis* 6C4) isolates, we showed differences in bacterial burden, lung transcriptomics, histopathology between these isolates in the mouse model of infection. To gain insights on the mechanisms driving early protection, we are exploring at the functional level the transcriptomic signatures detected in these models, by multiparametric flow cytometry, antibody blockade and by analysing the dynamics of *M. tuberculosis* transition from the alveolar space to the lung tissue. Moreover, we are expanding the distinct *in vivo* outcomes of *M. tuberculosis* infections to include slow progressing isolates, such as those recovered from patients in Guinea Bissau, where the *M. tuberculosis* complex lineage 6 is highly prevalent. We have shown before that *M. tuberculosis* isolates from lineage 6 are slow progressors in mice and are currently addressing the cellular and molecular immune events dictating such outcomes, using human and mouse based samples and models. Our studies illustrate the potential of combining clinical data with experimental systems and are unravelling important links between the pathogen genetic diversity, the host immune responses and ultimately TB outcomes.

Shaping *Mycobacterium ulcerans* and Buruli Ulcer Distribution: Natural Drivers and Human-Induced Pressures

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Buruli ulcer, a neglected tropical disease caused by the environmental mycobacterium *Mycobacterium ulcerans*, poses a major health challenge in regions with limited access to diagnosis and treatment. Its transmission and geographic distribution are closely linked to environmental and ecological conditions.

We investigated how soil type, mineral composition, climate, and native and introduced species may influence the distribution of *M. ulcerans*. Using pathogeographic tools and predictive models, we analyzed environmental, ecological, demographic, and socioeconomic variables to identify spatial patterns and risk factors. Our results suggest that climate and soil conditions constrain the bacterium's range, while invasive grasses and gold deposits may favor its presence. Across Africa, the distribution of Buruli ulcer cases was associated with interactions between UV-B radiation and climate, soil moisture and altitude, as well as the presence of animal and plant species.

These findings support an interdisciplinary approach to understanding the pathogen and the disease it causes. Closer collaboration among ecologists, epidemiologists, and public health experts could improve control strategies as environmental conditions change.

Connecting animal and human mycobacterial research: activities and perspectives from IRTA-CReSA

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Our research group at IRTA-CReSA focuses on animal tuberculosis (TB) within a One Health framework, aiming to understand host-pathogen interactions and translate this knowledge into improved strategies for disease prevention, diagnosis and control. Our research spans immunology, vaccinology and genomic epidemiology, combining *in vivo* and *in vitro* approaches that provide complementary perspectives to those traditionally developed in human TB research.

A central component of our work is the development and evaluation of vaccination strategies against TB, particularly BCG-based approaches and mucosal vaccination. We use the goat-*Mycobacterium caprae* system as a natural host-pathogen model to investigate vaccine-induced immunity and protection, including cellular and humoral responses, disease progression and bacterial dissemination.

We also investigate innate immune mechanisms involved in mycobacterial control, focusing on alveolar macrophages, macrophage activation, phagosome maturation and trained immunity through integrated *in vitro* and *ex vivo* approaches.

A further research topic focuses on the genomic epidemiology of *M. bovis and M. caprae*. Phylogenomic and phylodynamic analyses are being used to investigate transmission and improve our understanding and resolution of outbreaks.

Together, these activities integrate experimental and epidemiological research, from host-pathogen interactions and vaccine development to field-oriented applications at the livestock-wildlife-human interface.

Comparative macrophage responses to *Mycobacterium manresensis* and *Mycobacterium abscessus*

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Mycobacterium manresensis is an environmental mycobacterium with no known human infections, yet previous data suggest host-context-dependent pathogenicity and immunomodulatory capacity. Here, we established a murine bone marrow-derived macrophage infection model to compare the behaviour of *M. manresensis* with the clinically relevant *M. abscessus*, and to assess whether heat-killed *M. manresensis* (hkMm) modulates macrophage responses to infection.

Macrophages were infected with live *M. manresensis* or *M. abscessus*, with or without prior hkMm treatment. Intra- and extracellular CFUs were quantified over time, and infected cells were analysed by fluorescence microscopy, microscopy-based phenotypic profiling, TNF-α ELISA, and RT-qPCR for M1/M2-associated genes. *M. manresensis* and *M. abscessus* showed broadly similar infection kinetics in macrophages, although *M. manresensis* displayed higher early internalization. Imaging suggested intracellular localization and replication of *M. manresensis*, and infection with *M. manresensis* was associated with larger, rounder macrophages and induced a marked MOI- and time-associated TNF-α response. Overall, these findings suggest that *M. manresensis* actively shapes macrophage responses during infection and support its potential as an immunomodulatory organism capable of inducing trained immunity-like phenotypes. These results provide a foundation for further studies exploring the therapeutic and prophylactic applications of heat-killed *M. manresensis* in infectious diseases.

Mincle and TLRs coordinate macrophague-driven antitumour responses to mycobacterial lipid

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Mycobacterial lipids contribute to the immunotherapeutic activity of *Mycobacterium bovis* BCG in non-muscle-invasive bladder cancer, but the receptor networks controlling these effects remain undefined. We investigated whether purified mycolate-derived lipids from BCG and *Mycobacterium brumae* trigger Mincle-dependent responses and whether their effects act directly or indirectly on tumour cells. Human and murine Mincle-expressing HEK cells responded to glycerol monomycolate (GroMM), trehalose monomycolate (TMM) and, most prominently, trehalose dimycolate (TDM) from both mycobacterial species, supporting conserved lipid sensing across species. In contrast, direct exposure of MB49 bladder cancer cells to these lipids had no detectable effect, consistent with the absence of Mincle expression. To assess indirect effects, murine J774A.1 macrophages were exposed to the same lipids under basal conditions or in the presence of TLR2/TLR4 stimulation, with or without Mincle blockade. Macrophages showed modest responses to lipids alone, whereas TLR co-stimulation markedly enhanced TNF-α and IL-6 production. The strongest indirect antitumour effect on MB49 cells was observed with supernatants from macrophages exposed to TDM under combined TLR2/TLR4 stimulation. Mincle blockade abolished the effect of bacterial lipids in these conditions. Overall, these findings identify mycobacterial lipids as key immunomodulatory players in the antitumour activity of BCG and *M. brumae*.

Pericytes enhance macrophage bactericidal activity during *Mycobacterium tuberculosis* infection

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Vascularization of the tuberculous granuloma is increasingly recognized as a key determinant of tuberculosis pathogenesis. In particular, infected macrophages produce vascular endothelial growth factor (VEGF), driving aberrant angiogenesis and contributing to granuloma expansion, bacterial replication and tissue pathology. Although VEGF inhibition reduces bacterial burden in animal models and restores vascular integrity, the mechanisms underlying this protective effect remain poorly understood. Pericytes are mural cells essential for vascular stability, commonly identified by markers such as alpha-smooth muscle actin (α-SMA). Granuloma-associated vessels are often poorly covered by pericytes, whereas VEGF inhibition restores the presence of α-SMA-positive perivascular cells. Beyond their structural role, pericytes may participate in inflammatory and antimicrobial responses, but their contribution to tuberculosis immunity remains largely unexplored.
Here, we hypothesize that pericytes enhance the bactericidal activity of Mtb-infected macrophages. Using a co-culture system of THP-1-derived macrophages infected with Mtb and primary mouse lung pericytes, we show that pericytes reduce intracellular bacterial burden. Our findings identify pericytes as previously underappreciated regulators of macrophage antimicrobial function during tuberculosis infection.

Beyond phage diversity: Assessing mycobacteriophage-antibiotic combinations against *M. tuberculosis*

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Background: The emergence of drug-resistant tuberculosis necessitates the development of new therapeutic strategies. Phage therapy represents a promising alternative, although its efficacy may be enhanced by the additional use of antibiotics at subinhibitory concentrations.

Objectives: To evaluate the antimycobacterial activity of mycobacteriophages D29 and TM4 against *Mycobacterium tuberculosis* individually, in combination, and in combination with a suboptimal concentration of rifampicin.

Methods: Time-kill kinetic assays were conducted with *M. tuberculosis* H37Ra. Conditions were tested at a multiplicity of infection of 10 and included individual phages, a dual-phage combination, and each supplemented with 0.5 µg/mL RIF. Bacterial burden was quantified by colony-forming units on days 3 and 7.

Results: TM4 showed a higher killing activity than D29. The combination of both phages did not provide a relevant advantage over TM4 used alone. The subinhibitory rifampicin activity was higher than the use of the phages used alone or in combination. However, combining subinhibitory rifampicin with either D29 or TM4 resulted in a slight improvement in activity compared with rifampicin alone.

Conclusion: D29 and TM4 showed lytic activity against *M. tuberculosis*, but no added benefit when combined together. Subinhibitory rifampicin was more effective than phages alone. Rifampicin combination with phages, particularly both phages together, resulted in the highest antimycobacterial activity.

Genetic determinants of transmission success in *Mycobacterium tuberculosis*

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Understanding the genetic basis of differential transmission success in tuberculosis remains a major epidemiological challenge. We combined large-scale phylogenetic analyses and machine learning to identify genomic variants associated with increased transmissibility in *Mycobacterium tuberculosis* (Mtb). Using ~60,000 globally distributed Mtb genomes, we generated a whole-genome SNP alignment containing positions with at least one fixed SNP among all samples, reconstructed a phylogeny, and mapped recurrent mutations (homoplasies) through ancestral state reconstruction. To focus on recent transmission dynamics, analyses were restricted the analysis to nodes arising after 1950. We then applied the Ratio of Homoplasic Offspring (RoHO) index, which compares descendant numbers in sister clades with and without a given mutation across independent mutation events. Statistical significance was assessed using 1,000 permutation-based redistributions of homoplasic events, and robustness was evaluated through 1,000 jackknife replicate phylogenies. This approach identified several variants significantly associated with increased epidemiological success, including mutations in genes linked to Mtb adaptation and virulence, such as *whiB6* and *phoR*. We also developed an XGBoost-based framework to identify variants associated with larger transmission clusters in densely sampled countries. Despite class imbalance limiting predictive performance, the model achieved moderate accuracy and highlighted biologically plausible candidate variants. Together, these results demonstrate the value of integrating phylogenetic and machine learning approaches to investigate long and short term genomic determinants of Mtb transmission.

MIPERA: automating Mtb Intra-Patient Evolution Reporting and Analysis

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Studying the genetic variation within individual hosts over time is essential for understanding how microorganisms evolve and adapt. Research has often focused on fixed variants, while low-frequency within-host variants remain relatively underexplored. We have developed MIPERA (*Mycobacterium* Intra-Patient Evolution Reporting and Analysis): a modular, reproducible Snakemake workflow that automates the analysis of long-term *M. tuberculosis* (Mtb) infections from high-throughput sequencing data to study short-term evolution. MIPERA extends VIPERA, which we developed to study chronic SARS-CoV-2 infections (https://github.com/PathoGenOmics-Lab/VIPERA), and it updates its framework and biological assumptions to the challenges of the Mtb genome. Our new tool detects anti-microbial resistance (AMR)-associated mutations, identifies high-variability genomic regions via a Bayesian hierarchical model of polymorphism counts, and assesses co-variation of allele frequencies over time. A user-friendly HTML report facilitates the interpretation of analyses to a range of user backgrounds, while structured raw outputs enable in-depth downstream analyses; for instance, identifying of clusters of co-variating mutations involving AMR. By applying MIPERA to published long-term intra-host datasets, we demonstrate that incorporating low-frequency variants alongside majority alleles provides more robust estimates of within-host evolutionary metrics and recapitulates the reported AMR emergence events with minimal setup.

Experimental evolution reveals lineage-specific trajectories of bedaquiline adaptation in the *Mycobacterium tuberculosis* complex

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Bedaquiline is a key component of new anti-tuberculosis regimens, but emerging resistance threatens its long-term effectiveness. This study investigated the evolutionary mechanisms of bedaquiline adaptation in the *Mycobacterium tuberculosis* complex using longitudinal experimental evolution in two distinct genetic backgrounds: an animal-associated A4 strain and a human-associated L7 strain. Populations were exposed to increasing bedaquiline concentrations over 19 weeks and assessed resistant phenotype, growth curve, metabolomics, genomics and methilomics. Both strains developed increased bedaquiline resistance through different trajectories. A4 showed an earlier increase in MIC, accompanied by a visible growth cost and broad intracellular metabolic remodeling. In contrast, L7 displayed a later MIC increase, without fitness cost and with a more selective, biphasic metabolic response. Genomic analyses revealed heterogeneous population dynamics rather than a single universal resistance pathway. A4 adaptation involved low- to intermediate-frequency variants, including late emergence of canonical candidates such as *atpE* and *Rv0678*. L7 resistance was more strongly associated with late, replicate-dependent *atpE* expansion. Overall, bedaquiline adaptation combines shared resistance targets with lineage-specific phenotypic, metabolic, and genetic trajectories.

Radiomic Trajectory Phenotyping of Tuberculosis Lesions Reveals Treatment Response Patterns in the Marmoset Model

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Previous work established a reproducible radiomics framework for longitudinal PET/CT imaging of pulmonary tuberculosis lesions in marmosets, and defined a subset of robust imaging features associated with HRZE treatment response. Here, we investigated whether lesion-level radiomic changes over time could reveal distinct response patterns associated with additional treatment regimens.

Longitudinal PET and CT radiomic features were extracted from individual lesions in animals treated with BDQ, PaL, and BPaL regimens, and analyzed to characterize temporal response patterns during early (first month) and later (two months) treatment phases. Analyses were restricted to previously validated reproducible features to ensure robustness of the extracted metrics and reduce multiple-testing burden.

Radiomic features exhibited heterogeneous temporal response patterns across early and late treatment phases. PET features showed clearer early separation of lesion response trajectories than CT features, with BPaL enriched for favorable response patterns. These findings suggest that longitudinal radiomics can capture biologically meaningful heterogeneity in lesion response, reflecting both early and evolving treatment effects.

Tuberculosis and stress: when two unstopable forces meet

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Tuberculosis (TB) pathology is characterized by exacerbated inflammation, which ultimately contributes to tissue damage and disease progression. In this context, endogenous stress-response glucocorticoids (GCs) are of particular interest due to their intrinsic anti-inflammatory properties. To investigate the bidirectional relationship between TB and stress-response GCs, two experimental approaches were performed. First, movement restriction (RST) was applied as an inducer of stress-response GC production in *Mycobacterium tuberculosis* (Mtb)-infected C3HeB/FeJ mice of both sexes and two age groups, young and aged, in order to assess the impact of age and sex as biological variables. Aged animals, particularly females, showed enhanced stress responses, increased bacterial burden in all analyzed organs, and pulmonary histological alterations associated with stress status. Second, a comparative infection study was performed using two Mtb strains, HN878 and H37Rv, to evaluate the effect of bacterial virulence on the stress response in C3HeB/FeJ mice, with sex as the main biological variable. HN878-infected animals displayed stronger inflammatory responses together with a tendency toward increased stress-response GC production. Finally, a re-exploratory analysis of public transcriptomic datasets from HN878-infected mice revealed a stronger glucocorticoid-associated transcriptional signature in this infection context.

The host-resistance factor Sam68 protects Mtb-infected macrophages from premature cell death

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The way that an infected macrophage dies impacts tuberculosis outcome. Here, we have identified that Sam68, a multifunctional RNA-binding protein involved in RNA metabolism, stress responses, cell cycle and signal transduction, is required for survival of infected macrophages both in the zebrafish and in human.
We have found that deletion of *sam68* promotes caspase 8-mediated macrophage necrosis after infection, leading to increased bacterial burdens and susceptibility to infection in vivo in the zebrafish model. In contrast, uninfected *sam68*-deficient animals showed normal macrophage numbers, indicating that Sam68 is specifically required for macrophage survival during infection and is not essential for macrophage production.

Therapeutic opportunities of high-dose rifampicin regimens against borderline rifampicin-resistant tuberculosis

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Rifampicin-resistant/multidrug-resistant tuberculosis (RR/MDR-TB) remains a major global health challenge. Although newer regimens based on bedaquiline have improved outcomes, their limited availability and emerging resistance highlight the need to optimize the use of existing drugs. A substantial proportion of rifampicin resistance is associated with specific *Mycobacterium tuberculosis* mutations causing low-level resistance, also known as borderline resistance. Accumulating evidence from clinical trials shows that doses as high as triple the standard dose are safe and well tolerated. Therefore, the inevitable question is whether high-dose rifampicin could overcome low-level resistance and become a therapeutic alternative against RR/MDR-TB. Repurposing HiRIF for borderline RR/MDR-TB could provide an affordable and scalable therapeutic alternative, particularly in resource-limited settings. In this study, we integrated pharmacokinetic data from dose-finding studies of high-dose rifampicin together with the analysis of *M. tuberculosis* genomes isolated worldwide to estimate the proportion of borderline RR/MDR-TB cases that may benefit from HiRIF on a global scale. Our results suggest that up to 64% of borderline RR/MDR-TB could be effectively treated using HiRIF, accounting for 6% of all RR/MDR-TB worldwide.

Poster abstracts

P_01

Description of Two Novel Rapidly Growing Non-Tuberculous Mycobacterial Species Isolated from Human Clinical Samples

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Introduction. Non-tuberculous mycobacteria (NTM) comprise a heterogeneous group of over 200 species, particularly affecting immunocompromised individuals and patients with underlying pulmonary diseases.
This study aimed to describe two novel rapidly growing NTM species isolated from human clinical samples.
Methods. Sputum samples were processed and cultured in BBL™ MGIT™ liquid medium and incubated at 37 °C using the Bactec™ MGIT™ system, and subcultured on Coletsos, Löwenstein–Jensen, and Middlebrook 7H11 media. Preliminary identification used MALDI-TOF MS. Whole-genome sequencing was conducted with Illumina and Oxford Nanopore platforms. Bioinformatic analyses included average nucleotide identity (ANI), digital DNA–DNA hybridization (dDDH), and phylogenomic reconstruction.
Results. 12766410_HUMS strain was isolated from a patient with ciliary dysfunction, and strain HUMS_1102779-3 from a patient with breast adenocarcinoma and respiratory symptoms. Both strains showed growth within 7 days. MALDI-TOF MS failed to achieve species-level identification. ANI (<95%) and dDDH (<70%) values were below species delineation thresholds. The closest related species was *Mycobacterium helveticum*, within the *M. simiae* complex. Both isolates formed independent monophyletic clusters in phylogenetic reconstructions.
Conclusion. Genomic and phylogenetic data support that both isolates represent novel rapidly growing NTM species, named *Mycobacterium servetii* and *Mycobacterium cajalii*. Despite their close relationship, distinct genotypic and phenotypic features justified their classification as separate species.

P_02

Evolution of a *Mycobacterium tuberculosis* strain during an outbreak transmission driven by IS*6110*-mediated recombination

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Insertion sequence *6110* is a major source of genomic variation in *Mycobacterium tuberculosis* and has historically been used as an epidemiological marker. However, its role in the short-term evolution of circulating strains remains incompletely understood. Within the framework of routine population-based tuberculosis surveillance in Aragón (Spain), we investigated a large outbreak, including three members of a family, in which a derived IS*6110*-RFLP pattern emerged during outbreak evolution. Whole-genome sequencing showed that isolates, belonging to L4.1.2, derived RFLP patterns closely related, differing by only 2-4 SNPs, consistent with recent transmission.

Mapping of IS*6110* insertion sites revealed two copies located in close proximity within the genome. Inspection of sequencing reads using Integrative Genomics Viewer (IGV) supported a homologous recombination event between these elements, resulting in deletion of the intervening genomic region and loss of one IS*6110* copy, explaining the observed RFLP change.

Remarkably, population-based surveillance identified an isolate—apparently unrelated to the outbreak—that exhibited an identical RFLP pattern, including the same loss of IS6110, suggesting that similar IS*6110*-mediated rearrangements may arise independently in distinct transmission chains.

These results provide evidence of microevolution occurring during recent transmission and highlight the genomic plasticity conferred by IS*6110*, emphasizing its contribution to the ongoing evolution of *M. tuberculosis* populations.

P_03

Targeting *Mycobacterium tuberculosis* One-Carbon Metabolism to Disrupt Fatty Acid Resistance

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Targeting *Mycobacterium tuberculosis* (Mtb) metabolism is a promising strategy to combat infections. Mtb infection foci are lipid-rich environments, containing long-chain free fatty acids (LCFA), that present antimicrobial activity. Interestingly, Mtb survives this toxicity and uses LCFAs as a preferred carbon source. As such, inhibition of LCFA resistance mechanisms may turn lipid-rich environments toxic to Mtb.

To define the Mtb LCFA resistome, we performed transposon sequencing (Tn-seq) and identified 38 LCFA resistant factors. Among the top hits, hits were two predicted essential genes involved in one-carbon metabolism: *glyA1*, encoding a serine hydroxymethyltransferase (folate cycle), and *rv3038c*, encoding a S-adenosylmethionine (SAM)-dependent 6-O-methyltransferase that transfers one-carbon units to methylglucose lipopolysaccharides biosynthesis.

We have generated knockout strains for *glyA1* and *rv3038c* in Mtb H37Rv and phenotypically validated both enzymes as LCFA resistant factors. A chemical-genetics screen revealed a LCFA-dependent pattern of increased antibiotic susceptibility in the knockout strains, suggesting that these enzymes are intrinsic resistance factors to some antibiotics in lipid-rich environments. Currently, we are evaluating possible compensatory mechanisms and determining the relevance of these enzymes for infection using in vitro and in vivo models.
This project expands Mtb's one-carbon metabolism drug target space, offering new avenues for tuberculosis drug development.

P_04

Differential expression of exosomal miRNAs from macrophages with NOD2 receptor blockade challenged with *M. bovis* BCG

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Recognition of mycobacteria by innate immune cells is mediated by several pattern recognition receptors, including members of the NOD-like receptor (NLR) family. Among them, NOD2 recognizes muramyl dipeptide derived from the mycobacterial cell wall and activates NF-κB and MAPK signaling pathways, thereby shaping macrophage inflammatory responses and contributing to antibacterial immunity.

Mycobacteria also modulate the expression of host microRNAs (miRNAs), which act as key post-transcriptional regulators of immune pathways involved in inflammation, autophagy, apoptosis, and macrophage polarization. Numerous studies have identified miRNAs that influence host responses during *Mycobacterium tuberculosis* and other mycobacterial infections, highlighting their role in balancing protective immunity and pathogen persistence.

Although NOD2 signaling and miRNA-mediated regulation have independently been implicated in the control of inflammatory and antibacterial responses to mycobacteria, the relationship between NOD2 activity and exosomal miRNA expression remains poorly understood. The present study aimed to characterize the exosomal miRNA signature induced by BCG in macrophages with functional or disrupted NOD2 and to investigate its potential association with NF-κB/MAPK signaling and the resulting pro-inflammatory response.

To address this question, we compared the exosomal miRNA transcriptomes of BCG-infected wild-type and NOD2-knockout human macrophages using small RNA sequencing. Alternative mapping and quantification strategies optimized for miRNA profiling were evaluated, followed by transcriptome-wide exploratory and differential expression analyses. Our study aims to provide a comprehensive characterization of the exosomal miRNA response to intracellular BCG infection and to establish a framework for investigating the contribution of NOD2 signaling to the regulation of host miRNA programs during mycobacterial infection.

P_05

A complementary photothermal treatment enhances the effect of BMNP-associated bacteriocin AS-48 and reduces the viability of *Mycobacterium tuberculosis*

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Conventional treatments against TB are long-lasting and cause systemic toxicity. To overcome these drawbacks, anti-TB drugs could be immobilized on nanocarriers and combined with thermal therapy, an innovative approach that has proven effective in enhancing the efficacy of cancer chemotherapy. Our work has explored the use of biomimetic magnetic nanoparticles (BMNP) as nanocarriers for bacteriocin AS-48, an antimicrobial peptide active against *M. tuberculosis*, and as photothermal agents against *M. tuberculosis*.

We have found that the survival of *M. tuberculosis* decreases with increasing three factors: BMNP concentration, laser power density, and time. Also, we succeeded in immobilizing AS-48 on BMNP, control their distribution using magnetic fields and mediate localized photothermia. Finally, the combination of BMNPs_AS-48 and photothermia was able to eradicate *M. tuberculosis* in infected THP-1 macrophages.

Therefore, we can conclude that i) photothermia is a useful alternative for improving the efficacy of AS-48 and reducing the survival of *M. tuberculosis*, and ii) the combination of BMNPs_AS-48 and photothermia could reduce the duration of the treatment and potential side effects.

P_06

Mucosal BCG and prime-boost vaccination strategies enhance functional reprogramming of alveolar macrophages in goats.

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Tuberculosis is a major health concern for livestock and wildlife, and vaccination may provide a long-term alternative to test-and-cull approaches. Alveolar macrophages (AMs) constitute the first innate defence against mycobacteria and can develop trained immunity, contributing to non-specific innate immune protection. This study evaluated the effects of different vaccination regimens on the pro-inflammatory, activation, and phagocytic capacities of caprine AMs. Thirty-two goats were allocated to five vaccinated groups (n=6) and two unvaccinated controls. Vaccination strategies included intranasal BCG (BCGin), intranasal heat-inactivated *Mycobacterium bovis* (HIMBin) with or without mucosal adjuvant (HIMBmuc), and prime-boost strategies combining parenteral and mucosal delivery (HIMBpar-HIMBmuc; BCGsc-HIMBmuc). AMs were collected by bronchoalveolar lavage 16 weeks after prime vaccination and stimulated *ex vivo* with LPS for 24 h. Cytokine production (IL-1β, IL-6, TNFα), iNOS expression, and phagocytic activity against pH-sensitive labelled *Mycobacterium fortuitum* were assessed. BCGin and prime-boost strategies enhanced pro-inflammatory cytokine production following LPS stimulation compared with single HIMB vaccines. BCGin also increased iNOS expression in activated and pro-inflammatory AMs subsets. Enhanced phagosomal acidification following *M. fortuitum* challenge was observed in these groups. Overall, mucosal BCG and prime-boost strategies induce functional reprogramming of AMs, supporting enhanced innate pulmonary immunity in TB vaccination of goats.

P_07

The IFN-γ/JAK1 axis modulates tumor aggressiveness and immune escape mechanisms in intravesical MTBVAC-based immunotherapy for bladder cancer

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The live tuberculosis vaccine BCG represents nowadays a first line treatment for non-muscle invasive bladder cancer. MTBVAC is a novel live tuberculosis vaccine, currently under clinical evaluation as prophylactic vaccine against tuberculosis. Preclinical experiments have shown a good antitumoral potential for MTBVAC, in models of BCG-refractory bladder cancer. Mechanistically, MTBVAC induces antitumor responses dependent on CD8+ T lymphocytes and IFN-γ–mediated signaling. Its efficacy requires activation of inflammatory programs in tumor cells, including induction of major histocompatibility complex class I (MHC-I) molecules, which are essential for recognition by cytotoxic T cells. However, the mechanisms underlying response and resistance to this type of immunotherapy remain incompletely understood.

To address this, we obtained in vivo a MTBVAC-resistant bladder cancer cell line, called MB49Res42, derived from the parental cells MB-49. Res42 cells showed a higher induction of IFN-induced molecules as MHC-I and PDL1, following IFN-γ stimulation. Given the central role of the IFN-γ/JAK1 pathway in regulating MHC-I expression, we generated JAK1 deficient Res42 cells and evaluated their behavior in orthotopic bladder cancer mouse models.

JAK1 inactivation in Res42 cells, but not in MB49 parental cell line, was associated with a significant increase in survival compared to tumors derived from control cells. These results indicate that the IFN-γ/JAK1 pathway might modulate the acquisition of tumor resistance to immunotherapy, and suggest that the modulation of IFN signaling by specific inhibitors could be an effective approach to subvert these evasion mechanisms.

P_08

A closed pangenome reveals structural routes to host adaptation in the *Mycobacterium tuberculosis* complex

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Host adaptation in the MTBC is often described through reference-defined deletions, yet this view potentially misses much of the structural diversity that separates human- and animal-associated lineages. We built a robust MTBC pangenome of 423 closed genomes using cAMAP that includes 1) AMAP to harmonise CDS annotation against curated MTBC proteins, and 2) Recore to recover false absences before clustering. The resulting cAMAP pangenome was compact and closed, comprising 4,029 clusters with an 90% core and a Heaps' alpha of 0.0042. It revealed 84 CDS clusters and 682 kb absent from H37Rv, including virulence-associated loci and siderophore export functions. Genome graphs then revealed the central paradox of MTBC evolution: the chromosome remains 96% syntenic, yet residual variation concentrates in PE/PPE-rich hotspots, Regions of Difference and mobile-element-associated loci. This restricted structural layer captured a coherent host-adaptation continuum. Accessory gene content classified host tropism with near-perfect performance, driven by 41 loci including MmpL4/MmpS4, an animal-specific regulator and a human-enriched glycosyltransferase. Human lineages largely preserve the metabolic backbone and accelerate mainly in drug-resistance loci, whereas animal lineages accumulate stepwise deletions and broader diversification across metabolism, cell envelope and iron homeostasis. *M. africanum* occupies an intermediate position, sharing ancestral losses with animal lineages while retaining key human-associated structural features.

P_09

Challenges in Precise Genome Engineering in *Mycobacterium smegmatis*: Insights from RecT-Mediated Editing of the *rrnA* Operon

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Our group investigates the mechanisms of translation initiation and the role of ribosomal protein bS1 in mRNA recognition. To enable fluorescent probing of specific rRNA regions, we aimed to introduce a short hairpin into selected helices of *Mycobacterium smegmatis* rRNA, following strategies previously established in *E. coli*. Because *M. smegmatis* contains two ribosomal operons, we used strain SZ380, in which the *rrnB* operon had been deleted through classical homologous recombination. The 22-nt hairpin was inserted into the remaining *rrnA* operon using oligonucleotide-mediated homologous recombination in the presence of the Che9c phage RecT protein. For two of the three targeted positions, the engineered sequence coexisted with the wild-type allele. This heterogeneity persisted after multiple rounds of re-isolation, ruling out mixed populations and strongly suggesting duplication of the ribosomal operon.

RecT-mediated recombineering is widely used because it increases homologous recombination frequencies by 10^3–10^4-fold in mycobacteria, greatly facilitating genome engineering. Yet such stimulation can also enhance non-allelic homologous recombination between repeated sequences longer than ~1 kb. In *M. smegmatis*, insertion sequences (IS) represent major sources of long homology tracts. The *rrnA* operon is flanked by two IS1096 elements in direct orientation positioned in close proximity, providing an ideal substrate for RecT-stimulated duplication. Because obtaining a pure mutant in SZ380 proved unfeasible, we are now generating a *rrnA*-deletion strain to introduce the hairpins into *rrnB* which lacks close IS elements.

P_10

Structural basis of canonical translation initiation in *Mycobacteria*

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Mycobacterium tuberculosis, the causative agent of tuberculosis, is one of the deadliest human pathogens worldwide. During infection, this bacterium can persist for prolonged periods in a non-replicating state and must adapt to a variety of hostile environments within the host. Recent studies have revealed important differences in translational control between *M. tuberculosis* and other organisms, which may contribute to its remarkable adaptability during infection. In particular, the translational machinery of *M. tuberculosis* displays species-specific features. Furthermore, we have previously shown that more than 50% of its genes lack canonical translation initiation signals, yet are robustly translated during exponential growth and under conditions that mimic host persistence, suggesting the existence of alternative mechanisms of translation initiation. However, the molecular basis and physiological relevance of non-canonical translation remain poorly understood. Therefore, structural and functional characterization of translation initiation in *M. tuberculosis* is essential to better understand translational regulation in this pathogen.

Although several structures of mycobacterial ribosomes have been reported, no functional translation initiation complex has been structurally characterized to date. Here, we obtained high-resolution structures of canonical translation initiation complexes using cryo-electron microscopy (cryo-EM). We previously processed a dataset collected on a 200-kV microscope and obtained maps around 4.3Å. Following multiple rounds of 3D classification, we identified distinct initiation states that differ in both conformation and composition, including several functional states that have already been described in other organisms. We are currently processing a larger dataset collected on a 300-kV microscope, in which we aim to apply more exhaustive 3D classification approaches to isolate additional states. Preliminary analysis indicates that the majority of particles contain at high occupancy tRNA, mRNA harbouring a Shine–Dalgarno sequence, IF1, and IF3, whereas a subset of particles additionally includes IF2 at lower occupancy.

We now aim to perform structural comparisons with initiation complexes from other organisms in order to identify potential species-specific differences. Additionally, we seek to characterize non-canonical initiation complexes to uncover key elements associated with alternative translation initiation mechanisms. We anticipate that these structures, together with complementary biochemical approaches, will provide important insights into translational control in *M. tuberculosis*.

P_11

In vitro selection and characterisation of capreomycin resistant mutants *in Mycobacterium smegmatis*

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Mycobacterium tuberculosis, the causative agent of tuberculosis, remains one of the deadliest infectious diseases worldwide. In 2023, approximately 10.8 million new cases and 1.25 million deaths were reported, with multidrug-resistant strains contributing significantly to mortality. Over one tenth of deaths caused by antibiotic-resistant bacteria are linked to multidrug-resistant *M. tuberculosis*. Many clinically used antibiotics target the ribosome, making ribosomal mutability a key factor in the emergence of resistance. Capreomycin, a ribosome-targeting antibiotic, has been used to treat multidrug-resistant tuberculosis. To investigate resistance mechanisms, capreomycin-resistant *Mycobacterium smegmatis* clones were generated *in vitro* under varying drug concentrations. Whole genomes were sequenced to identify resistance-associated mutations. Most resistant mutants carried mutations in tlyA, encoding a ribosomal methyltransferase, while only 13% showed mutations in the 16S rRNA. Structurally, tlyA mutations clustered in the C-terminal domain, likely disrupting methyltransferase activity and conferring resistance. All 16S rRNA mutants shared a single nucleotide change associated with an in vitro fitness cost and reduced capreomycin binding. Furthermore, capreomycin-resistant ribosomes were devoid of two structurally important ribosomal proteins. These findings improve understanding of ribosomal mutability and resistance mechanisms in tuberculosis, supporting the development of new therapeutic strategies.

P_12

Unravelling the transcriptome of the human tuberculosis lesion and its clinical implications

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The tuberculosis (TB) lesion is a complex structure, contributing to the overall spectrum of TB. We characterised, using RNA sequencing, 44 fresh human pulmonary TB lesion samples from 13 TB individuals (drug-sensitive and multidrug-resistant TB) undergoing therapeutic surgery. We confirmed clear separation between the TB lesion and adjacent non-lesional tissue, with the lesion samples consistently displaying increased inflammatory profile despite heterogeneity. Using weighted correlation network analysis, we identified 17 transcriptional modules associated with TB lesion and demonstrate a gradient of immune-related transcript abundance according to spatial organization of the lesion. Furthermore, we associated the modular transcriptional signature of the TB lesion with clinical surrogates of treatment efficacy and TB severity. We show that patients with worse disease present an overabundance of immune/inflammation-related modules and downregulated tissue repair and metabolism modules. Our findings provide evidence of a relationship between clinical parameters, treatment response and immune signatures at the infection site.

P_13

Assessment of lateral-flow and ELISA assays based on IP-10 detection for the diagnosis of *Mycobacterium tuberculosis* infection

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Aim: To assess IP-10 detection for tuberculosis infection (TBI) diagnosis, comparing the results with IFN-γ detection.

Methods: Active TB patients (aTB; n=8) and individuals undergoing TBI screening (n=73) were enrolled. For IP-10 detection, an ELISA and a lateral-flow (LF) assay was performed (R-Biopharm). IFN-γ was detected by QFN-Plus (Qiagen). Blood was incubated at 37oC during 16-24h for plasma collection and analysis. Samples were processed according to manufacturer's instructions.

Results: Percentage of positivity by IP-10 ELISA, IP-10 LF, and QFN was 41.9%, 39,5%, and 46.9% respectively. A total of 6.2% and 7.4% of indeterminate results were obtained by IP-10 ELISA and LF assays. Combined used of IP-10 with IFN-γ increased positivity to 54.3%. In aTB patients, positivity was 87.5% (7/8) for both IP-10-based assays and 100% (8/8) for IFN-γ detection. Among individuals screened for TBI, positivity was 36.9%, 34.3%, and 41.1% for the IP-10 ELISA, IP-10 LF, and QFN, respectively. Concordance between IP-10 assays and QFN was 80.2%. IP-10 levels were significantly higher compared with IFN-γ ($p<0.0001$ for IP-10 ELISA or LF vs. TB1/TB2 QFN tubes).

Conclusions: IP-10 could be a marker for TBI diagnosis. Its detection enables miniaturization, as the levels of secretion are significantly higher than the IFN-γ ones.

P_14

Full-spectrum flow cytometry reveals distinct phenotypic immunological profiles in peripheral blood and bronchoalveolar lavage from patients with pulmonary tuberculosis

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The immune response to *Mycobacterium tuberculosis* (Mtb) may differ between systemic and local compartments, due to the presence of specialized populations involved in pathogen control. Here, we aim to evaluate phenotypic characteristics of T and B cells from peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage fluid (BALF) involved in immune responses against Mtb. For that purpose, cells were obtained from blood and BALF of patients with presumptive pulmonary TB. PBMCs were isolated by density sedimentation using CPT tubes. BALF cells were obtained by filtration and centrifugation. Cells were phenotype to assess T- and B-cell subsets using a 30-color Cytek Aurora spectral flow cytometer panel. We expect to observe distinct percentages of expression of T- and B-cell subpopulations according to the tissue of origin. T cells are classified into naive, central memory, effector memory, and terminally differentiated subsets using CD45RA and CCR7. Tissue-resident memory T cells are identified by CD69 and CD103 markers. B cells are characterized using CD19, CD20, CD27, IgD, IgM, IgG, IgA, CD38, HLA-DR, CD80, and CD86 to identify maturation, activation, and antibody-associated phenotypes. This data may reveal immunological differences in T and B cells that contribute to local immunity against Mtb in human lung.

P_15

Immunomodulatory Effects of *Mycobacterium brumae* as an Adjuvant in Prophylactic Bladder Cancer Treatment

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Bladder cancer is among the most common cancers worldwide and ranks among the 11 most frequently diagnosed cancers in men. Currently, non-muscle-invasive bladder cancer (NMIBC) is primarily treated with intravesical instillations of *Mycobacterium bovis* BCG. However, BCG therapy is often associated with adverse effects that may compromise patient compliance and treatment continuation. *Mycobacterium brumae*, a non-pathogenic environmental mycobacterium, has emerged as a promising alternative due to its favorable safety profile. To further investigate its immunomodulatory properties, *M. brumae* has been evaluated as an adjuvant in prophylactic bladder cancer treatment strategies. Animals receiving *M. brumae* as an adjuvant exhibited a reduced tumor incidence compared with control groups. Furthermore, these animals displayed a distinct pattern of immune cell infiltration, suggesting enhanced antitumor immune responses. In addition, immune cell populations isolated from *M. brumae*-treated animals showed differential activation profiles upon stimulation, further supporting the ability of *M. brumae* to modulate the immune response and promote tumor protection. Collectively, these findings highlight the immunomodulatory properties of *M. brumae* and suggest its potential application as an adjuvant in prophylactic bladder cancer treatment.

P_16

Non-tuberculous Mycobacteria-based Immunomodulation as a Prophylactic Strategy Against Pulmonary Infections

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The rise of multidrug-resistant (MDR) bacteria represents a critical global health challenge, limiting therapeutic options and increasing morbidity and mortality. *Pseudomonas aeruginosa*, a leading cause of respiratory infections, particularly in patients with cystic fibrosis or chronic obstructive pulmonary disease, exhibits intrinsic and acquired antibiotic resistance, highlighting the urgent need for alternative therapeutic approaches. In this context, we evaluated the prophylactic potential of *Mycobacterium brumae*, a safe, non-pathogenic non-tuberculous mycobacterium with known immunomodulatory properties. Using a C57BL/6 murine model, we investigated the immunological and protective effects of *M. brumae* administered via different routes. Immune responses were initially characterized in non-infected animals, after which oral and intranasal routes were selected for challenge experiments with *P. aeruginosa*. Both administration routes resulted in significantly reduced pulmonary bacterial burdens in *M. brumae*- treated animals compared to controls, demonstrating enhanced protection. This effect correlated with increased activation and modulation of local inflammatory mediators, as observed in bronchoalveolar lavage fluid. Our findings demonstrate that *M. brumae* elicits a protective immune response capable of reducing *P. aeruginosa* pulmonary infection. These results support the development of immunomodulatory non-tuberculous mycobacteria as a non- antibiotic prophylactic strategy targeting MDR pathogens through host-directed immune modulation.

P_17

Comparative genomics reveals the evolutionary diversification of asparaginases across the genus *Mycobacterium*

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L-asparaginases (ASNases) are enzymes best known for their antileukemic activity, but they have also been implicated in the intracellular survival and virulence of *Mycobacterium tuberculosis* during macrophage infection. Despite their biological relevance, the evolutionary distribution and diversification of ASNase-encoding genes across the genus *Mycobacterium* remain poorly characterized. Here, we performed a comparative genomic analysis of approximately 220 curated *Mycobacterium* type-strain genomes to investigate the conservation and evolutionary dynamics of ASNase-related genes. Genome quality assessment using BUSCO showed consistently high completeness values, with most genomes exceeding 98–99% completeness. Orthologous groups were inferred using OrthoFinder and functionally annotated with eggNOG, generating a comprehensive phylogenomic framework for comparative analyses integrated into the ARX genomic platform.Pan-genome analysis revealed a highly diverse accessory gene pool, representing the large majority of inferred orthogroups, consistent with extensive lineage-specific genomic diversification across the genus. Within this dynamic genomic landscape, ASNase-related orthogroups displayed variable conservation patterns, including differential retention across clades and evidence of gene copy expansion in selected taxa. Comparative analyses also identified multiple ASNase-associated orthogroups with distinct functional annotations, supporting the evolutionary diversification of these enzymes within mycobacteria. Overall, this work establishes a genome-scale comparative framework to investigate the evolution of ASNases in *Mycobacterium* and provides a foundation for future studies addressing their potential contribution to mycobacterial adaptation and host-pathogen interactions.

P_18

Exploring Mycobacteria as a Source of Therapeutic L-Asparaginases with Antileukemic Potential

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L-asparaginase (ASNase) is a key component in the treatment of acute lymphoblastic leukaemia. However, currently available ASNases derived from *Escherichia coli* are frequently associated with toxicity events, highlighting the need for alternative therapeutic enzymes with improved safety profiles. In this study, seven mycobacterial ASNases were recombinantly produced in *E. coli*, purified, biochemically characterised. Their enzymatic activity and toxicity were subsequently evaluated using both *in vitro* and *in vivo* models.

Mycobacterial ASNases showed diverse biochemical and kinetic properties, including distinct optimal pH and temperature profiles, as well as varying substrate affinities. Their antitumoral activity was assessed in THP-1, HL-60, Jurkat and Raji haematopoietic tumour cell lines, in which all enzymes induced dose-dependent growth inhibition compared to controls. Remarkly, treatment with mycobacterial ASNases triggered cytokine release, whereas the *E. coli*-derived ASNase inhibited cytokine production. *Ex vivo* experiments using murine splenocytes suggested a selective cytotoxic effect towards tumour cells, with lower toxicity compared to commercial *E. coli* ASNase. Consistently, *in vivo* administration of mycobacterial ASNases demonstrated a favourable safety profile in *Galleria mellonella*. Finally, three mycobacterial ASNases were intravenously administrated to mice. Among the candidates, three enzymes were further evaluated by intravenous administration in mice. Toxicity profiles varied between enzymes, but one mycobacterial ASNase showed no detectable toxicity compared to *E. coli* ASNase, while effectively depleting circulating asparagine levels.

Overall, these findings identify mycobacteria as a previously underexplored source of ASNases with potential therapeutic applications and support further investigation of mycobacterial enzymes as safer antitumoral biotherapeutics.

P_19

Genomic epidemiology of mycobacteria involving paediatric population

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Genomic epidemiology enhances tracking of mycobacteria transmission. We have applied WGS to *M. tuberculosis* and non-tuberculous mycobacteria involving pediatric cases to i) analyze more in-depth the transmission dynamics of pediatric tuberculosis and ii) to understand the emergence of *M. lentiflavum* in pediatric lymphadenitis in Madrid. In the first study, we sequenced 47 *M. tuberculosis* pediatric isolates (2022-2024), 17 isolates from their epidemiologically-related available adults and sequences from non- epidemiologically related cases from Madrid and Almería. Our integrated genomic analysis frequently identified pediatric cases clustered with pediatric/adult cases non-suspected to be epidemiologically related; even an interterritorial Madrid/Almeria cluster was identified. For the second study we analyzed 29 *M. lentiflavum* strains from paediatric cases and 47 control adult isolates from Madrid and other regions. The isolates grouped in two genomic clades (>20,000 SNPs appart), one containing most isolates (n=81) and all pediatric cases. Median pairwise distances were unexpectedely short, even for unrelated controls (28 and 29 SNPs), which impaired the assessment of epidemiological links. No clear clustering was observed by Spanish region, or age group. A single network branch included all but one pediatric isolates (7/8) diagnosed in central Spain, which might suggest a common exposure link among them.

P_20

Integrating INDELs to Refine *Mycobacterium tuberculosis* Transmission Networks

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After the COVID-19 pandemic, tuberculosis has recovered its position as the deadliest infectious disease. Effective epidemiological surveillance and transmission control require a detailed characterization of the genomic diversity among *Mycobacterium tuberculosis* (MTB) circulating strains. Current approaches to genomic surveillance rely predominantly on SNP-based analyses derived from short-read sequencing data. Despite being highly effective, these analyses focus mainly on single nucleotide variation and may overlook other sources of genomic diversity, such as insertions and deletions (INDELs), which could provide further discriminatory power for reconstructing transmission events. In this project, we integrate INDEL calling into genomic analyses to improve the characterization of diversity between MTB strains and therefore refine the relationships among cases and the chronology of the transmission. We are including in this enhanced analysis 31 transmission clusters involving 290 patients. Our results show that 21 clusters (68%) contain INDELs, with 1-7 INDELs detected per cluster, compared with 1-32 SNPs per cluster. The inclusion of INDELs increases the overall genetic diversity captured within transmission clusters and provides additional resolution beyond SNP-based analyses. Consequently, INDELs can reshape transmission networks, refining both cluster structure and the inferred chronology of patient-to-patient transmission.

P_21

Paratuberculosis: the dual challenge

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Paratuberculosis is an infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) that primarily affects the gastrointestinal tract, resulting in a chronic wasting disease. It has a high prevalence in ruminants world-wide, and there is no antibiotic treatment available; consequently, its control relies on appropriate animal management and vaccination. In addition, several studies link *MAP* to Crohn`s disease –a controversial issue–, indicating a role as a zoonotic agent which could be transmitted by dairy products or meat.

Identification of *MAP* by bacteriological culture is slow, due to the long-time incubation period, and it also has a limited sensitivity; therefore, the trend is towards the use of molecular methods, preferably based on clinical (faeces, lymph nodes, ...) or environmental samples.

Existing vaccines consist of a suspension of *MAP* in an adjuvant, intended for use on sheep, goats and cattle. Despite the advantages that vaccination offers in reducing the development of lesions and infectivity, the use of vaccines is largely hampered by its interference with the official diagnosis of tuberculosis (*Mycobacterium bovis*, *M. caprae*), and is currently banned in cattle.

A tuberculosis-free status across large areas of Spain would allow the use of paratuberculosis vaccines; to this end, it would be essential to unequivocally classify the status of the animals. This requires sensitive and specific diagnostic methods available to identify these infections, which presents a scientific and technical challenge.

P_22

Cell envelope stress and Dynamin-like proteins IniA and IniC coordinate extracellular vesicle biogenesis in *Mycobacterium tuberculosis*

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Extracellular vesicles produced by *Mycobacterium tuberculosis* (MEVs) are important mediators of host–pathogen interactions, contributing to virulence, immunomodulation, nutrient acquisition, and bacterial adaptation. However, the molecular mechanisms regulating MEV biogenesis remain poorly understood. Previous studies demonstrated that loss of the envelope-associated protein VirR leads to altered cell envelope integrity, increased permeability, and hypervesiculation concomitant with induction of the iniBAC operon. Likewise, exposure of *M. tuberculosis* to sublethal concentrations of isoniazid induces iniBAC expression, suggesting a mechanistic link between envelope stress and vesiculogenesis, highlighting the iniBAC operon as a potential key regulator of MEV production. IniA and IniC belong to the dynamin-like protein (DLP) superfamily, known to mediate membrane fusion and fission events during vesicular trafficking. Here, biochemical characterization revealed that both IniA and IniC associate with lipid membranes in flotation assays, supporting their direct involvement in membrane remodeling. Native PAGE analyses suggested that both proteins assemble into high-order oligomeric structures in solution, consistent with membrane-remodeling DLPs. In addition, a split-GFP approach in *Mycobacterium smegmatis* was engineered to investigate potential IniA–IniC interactions. Altogether, these findings identify IniA and IniC as central determinants of MEV biogenesis and support vesiculogenesis as a regulated adaptive response to cell envelope stress in *M. tuberculosis*.

P_23

Lipid availability facilitates persistence of *Mycobacterium tuberculosis* under iron deprivation

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Lipid availability and iron deprivation are key features of the granuloma environment during tuberculosis infection (TBI), yet the adaptation of *M. tuberculosis* (*Mtb*) to iron limitation under different carbon sources remains unknown. In this study, *Mtb* was exposed to iron-deprived conditions in media containing different carbon sources.

Phenotypic characterization revealed a dormancy-associated state marked by cell envelope remodeling, loss of acid-fastness, and carbon source-dependent changes in lipid composition.

Transcriptomic analysis refined the iron deprivation signature by identifying genes required for survival under iron deprivation regardless of carbon source. However, carbon availability strongly shaped the overall response. In lipid-free media, *Mtb* showed a pronounced stress response involving cell wall remodelling and activation of stress-related genes. In contrast, lipid-rich conditions induced a global metabolic shutdown, with fewer differentially expressed genes, particularly when long-chain fatty acids were the sole carbon source, suggesting that lipids buffer stress and promote a more balanced physiological state.

Moreover, lipid environments appeared to activate alternative regulatory networks, with genes such as Rv3840 and Rv2621c potentially complementing or replacing IdeR in maintaining iron homeostasis. These findings suggest the idea that host lipids function not only as nutrients but also as environmental signals that facilitate long-term bacterial persistence during TBI.

P_24

Uncovering drivers of post-tuberculosis sequelae informed by host-pathogen interactions

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Tuberculosis (TB) affects over 10 million individuals and kills over 1.25 million annually. Millions of TB survivors suffer from post-TB complications, living with permanent lung dysfunction and other health problems, and with enormous socio-economic burdens. Despite this, post-TB lung disease (PTLD) is vastly neglected and the mechanistic drivers of disease remain poorly understood. With this in mind, our goal is to identify molecular drivers of disease that can be targeted to mitigate PTLD.

Based on distinct host-*Mycobacterium tuberculosis* interactions, our group has recently established tractable mouse models capturing the spectrum of TB disease severity, recapitulating human lung pathology and immune response. Taking advantage of this unique in vivo platform, we plan to study the molecular trajectory of TB resolution or persisting damage, in the lung and systemically, throughout the course and after therapy. We expect to find cellular and/or molecular candidates of lesion resolution or persisting damage, both at the site of infection and systemically. The findings of the mouse model will be used to interrogate available human TB datasets to uncover and test common predictive and therapeutic candidates. We anticipate this project to depart from classical TB research and contribute with key knowledge on post-TB sequelae.

P_25

Exploring the rRNA synthesis ratio biomarker in *Mycobacterium ulcerans* for drug activity evaluation

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Buruli ulcer (BU), caused by *Mycobacterium ulcerans* (Mul), is treated with an 8-week regimen of rifampicin (RIF) plus clarithromycin (CLA). BLMs4BU clinical trials are evaluating the addition of amoxicillin/clavulanate (AMX/CLV) to shorten treatment duration. The ribosomal RNA synthesis ratio (RS-ratio), a marker of rRNA biogenesis and a bacterial metabolic activity proxy, has shown promise as a predictor of treatment-shortening potential in tuberculosis but has not been explored in Mul.

We evaluated the RS-ratio retrospectively from Mul time-kill assays exposed to RIF, CLA, and AMX/CLV, alone and in combination. Two precursor rRNA and two total rRNA targets were quantified to calculate four RS-ratios.

Combinations containing RIF+AMX/CLV produced the strongest and fastest reductions, decreasing from baseline values (≈2000) to 200 by day 3 and reaching maximal inhibition (50–60) between days 7 and 10. This decline preceded CFUs and luminescence reductions previously observed in the same experimental system. The RS-ratio also detected a metabolic recovery after day 14, suggesting persistent bacterial viability not captured by conventional readouts.

This is the first study applying the RS-ratio to Mul. Our findings support its use as a biomarker of sterilizing drug activity and further support the RIF+CLA+AMX/CLV regimen in evaluation in the BLMs4BU clinical trials.

P_26

Multiplexed rRNA biomarkers for medium-to-high-throughput evaluation of *Mycobacterium tuberculosis* drug response

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Tuberculosis drug development requires in vitro platforms able to prioritise new regimens before animal or clinical evaluation, while providing information beyond conventional viability endpoints. We developed a miniaturised, multiplexed workflow to quantify two rRNA-based biomarkers of *Mycobacterium tuberculosis* drug response directly from 96-well plate cultures: rRNA burden and the rRNA synthesis ratio (RS-ratio). Cultures of *M. tuberculosis* H37Ra were sampled using 50 microlitres, stabilised in DNA/RNA Shield containing an external RNA control, and heat-inactivated using validated conditions compatible with safe downstream processing. RNA extraction and reverse transcription were followed by a single-tube droplet digital PCR assay targeting precursor rRNA regions, mature rRNA targets and the external control. The workflow was integrated with resazurin-based growth inhibition, colony-forming unit and most probable number readouts in a proof-of-concept panel of antitubercular drugs with diverse mechanisms of action. rRNA burden generally reflected bacterial load but was less informative about the metabolic state of the bacteria. In contrast, the RS-ratio detected drug- and dose-dependent effects on ribosomal activity, particularly after exposure to treatments affecting core bacterial physiology, providing a more realistic indicator of bacterial state. Overall, this approach provides a practical platform for integrating molecular and culture-based biomarkers into early tuberculosis drug evaluation.

P_27

New Cholic Acid Derivatives as Novel Anti-*Mycobacterium tuberculosis* Agents

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Mycobacterium tuberculosis (Mtb) relies on host-derived cholesterol as its principal carbon source. Several strategies have exploited cholesterol metabolism as a compelling target for drug development, including compounds that regulate cholesterol metabolism, inhibit early catabolic enzymes, or trigger the accumulation of toxic intermediates. Ceragenins, bile acid-derived mimics of endogenous antimicrobial peptides, represent a complementary approach, acting through physical disruption of the bacterial membrane. Building on these precedents, cholic acid (CA) emerges as an attractive drug scaffold: its rigid triterpenoid core, amphipathic character, and chemically versatile hydroxyl and carboxyl groups allow regioselective functionalization. Prior work has shown that conjugation of steroidal backbones with cationic moieties enhances electrostatic interactions with the negatively charged mycobacterial surface, conferring selectivity. Herein we report the preliminary antitubercular effects in glycerol and cholesterol growth media of the AZM series, novel cholic acid derivatives bearing a tricationic functionalization, developed as potential anti-Mtb agents.

P_28

Sam68 as a key regulator of pathological cell migration and host defense against tuberculosis

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Egress of infected macrophages from the tuberculous granuloma is a pathogenic event leading to bacterial dissemination and establishment of new lesions in the context of tuberculosis pathogenesis. Using the zebrafish model of tuberculosis, we have found that absence of the host protein Sam68, a multifunctional member of the STAR protein family that acts as a critical link between cellular signaling and RNA metabolism, promotes uncontrolled dissemination of infected macrophages throughout the animal. We have discovered that Sam68 is a host-resistant factor which differentially regulates macrophage migration depending on the inflammatory stimulus and also on the stage of infection.

P_29

The host-resistance factor Sam68 protects Mtb-infected macrophages from premature cell death

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The way that an infected macrophage dies impacts tuberculosis outcome. Here, we have identified that Sam68, a multifunctional RNA-binding protein involved in RNA metabolism, stress responses, cell cycle and signal transduction, is required for survival of infected macrophages both in the zebrafish and in human.
We have found that deletion of *sam68* promotes caspase 8-mediated macrophage necrosis after infection, leading to increased bacterial burdens and susceptibility to infection in vivo in the zebrafish model. In contrast, uninfected *sam68*-deficient animals showed normal macrophage numbers, indicating that Sam68 is specifically required for macrophage survival during infection and is not essential for macrophage production.

P_30

Within-host evolution generates virulence diversity in *Mycobacterium abscessus*

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Mycobacterium abscessus is a rapidly growing non-tuberculous mycobacterium that causes chronic, difficult-to-treat infections, particularly in individuals with cystic fibrosis. Its intrinsic antibiotic resistance and capacity for long-term persistence make it a major clinical challenge. Recent genomic studies have shown that prolonged infections can promote extensive within-host diversification, generating heterogeneous bacterial populations whose functional significance remains poorly understood.

This study investigates whether within-host genomic diversification is associated with differences in virulence and host interaction. We analyzed five clinical variants selected from a previously described chronic *M. abscessus* infection spanning 192 months and involving 23 sequential isolates. During infection, an initial persistent genotype that rapidly acquired macrolide resistance was followed by the emergence of additional genotypes differing by dozens to hundreds of single-nucleotide variants.

Virulence-associated phenotypes were evaluated in macrophages and two invertebrate infection models: *Drosophila melanogaster* and *Galleria mellonella*. Host survival, bacterial burden, and innate immune activation were assessed across models, alongside comprehensive phenotypic characterization of the isolates. Marked differences in phenotypic traits and host survival were observed among variants, indicating that within-host diversification can generate alternative strategies for infective success.

By integrating a well-characterized longitudinal clinical case with complementary infection models, this work provides a functional framework to investigate how within-host diversification may influence infection outcomes in *M. abscessus.*

P_31

Context-dependent roles of progranulin in host defense: myeloid dysfunction with pathogen-specific effects beyond mycobacterial infection

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Progranulin (PGRN) is a cytokine of the Tumor Necrosis Factor alpha (TNF-α) family that is primarily secreted and displays pleiotropic biological functions. It acts as a growth factor involved in tissue repair, embryonic development, cellular differentiation, and immune regulation. PGRN is also the precursor of several 6 kDa peptides known as granulins, which are generated through proteolytic processing. Both PGRN and granulins are biologically active, and their production is tightly controlled by processing inhibitor proteins and by the regulation of proteases responsible for PGRN cleavage.

The functions of PGRN and granulins have been extensively investigated in cancer, where PGRN promotes cell proliferation, survival, angiogenesis, migration, immune evasion, and treatment resistance. PGRN also participates in inflammatory responses to viral, bacterial, and fungal infections, as well as in autoimmune diseases, where its effects may vary depending on the context.

Here, we investigated the role of PGRN and granulins in tuberculosis using the zebrafish model. Although PGRN deficiency impaired myeloid differentiation, it did not increase susceptibility to mycobacterial infection. Unexpectedly, the absence of PGRN increased susceptibility to other bacterial pathogens, including Salmonella enterica and Pseudomonas aeruginosa, due to functional defects in both macrophages and neutrophils that compromised infection control.

P_32

Whole-Blood RNA-seq Captures Structured rRNA Patterns Linked to Tuberculosis State and Progression

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Monitoring treatment response in tuberculosis (TB) remains a challenge, especially due to low bacterial burden in patients or limited access to sputum-based diagnostics. To overcome such limitation, whole-blood RNA sequencing is being investigated to profile host responses, but pathogen transcriptomic signatures remain underexplored for infection monitoring. We performed an exploratory analysis to assess whether non-human reads from whole-blood RNA-seq carry patterns associated with TB disease state. Using longitudinal data from healthy controls and pulmonary TB patients sampled at diagnosis through week 24 of standard therapy we quantified non-human fragment alignments to fixed bins of the 16S and 23S rRNA subunits of *Mycobacterium tuberculosis* and other species. Logistic regression models trained on controls and patients at diagnosis showed above-chance separation between healthy and diseased individuals across three mycobacterial species, with the strongest performance in *M. tuberculosis*. The models also captured a mild but significant longitudinal decline in estimated disease probability over the course of treatment. These findings suggest that whole-blood RNA-seq data may contain latent, structured rRNA patterns that correlate with TB disease state.

P_33

Exploring the Effects of MTBVAC Vaccination on Innate Immune Responses to Sepsis Through Single-Cell Transcriptomics.

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Accumulating epidemiological and experimental evidence suggests that mycobacterial vaccines can induce long-lasting innate immune memory, potentially enhancing protection against unrelated infectious challenges. Bacillus Calmette-Guérin (BCG) vaccination is the best-characterized example, with numerous studies documenting sustained functional reprogramming of innate immune cells and heterologous protective effects beyond tuberculosis. These findings underscore the importance of investigating whether next-generation tuberculosis vaccines retain or enhance the broader immunomodulatory properties of BCG while improving protection against *Mycobacterium tuberculosis* infection.

MTBVAC, a live-attenuated M. tuberculosis vaccine candidate developed at the University of Zaragoza, is the first tuberculosis vaccine derived from an attenuated human clinical isolate. Here, we investigate the capacity of MTBVAC to induce heterologous immune responses by characterizing transcriptional changes following systemic lipopolysaccharide (LPS) challenge in a murine model of endotoxemia. Single-cell RNA sequencing was employed to assess vaccination-associated changes in immune cell composition and transcriptional responses to a systemic LPS challenge in a murine model, with particular emphasis on innate immune populations.

Our aim is to identify cellular and molecular signatures of MTBVAC-induced innate immune reprogramming and explore their potential implications for heterologous protection beyond tuberculosis.

P_34

In vitro Mycobacterium fortuitum priming enhances pro-inflammatory and bactericidal responses of caprine alveolar macrophages upon homologous re-stimulation.

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Trained immunity involves epigenetic and metabolic reprogramming of innate immune cells, enhancing their responsiveness upon re-stimulation. *Mycobacterium fortuitum* (MF), a rapidly growing non-tuberculous mycobacterium, activates innate immune pathways while being safer than members of the *Mycobacterium tuberculosis* complex, making it a suitable model for studying macrophage functional reprogramming. This study evaluated whether *in vitro* MF priming enhanced the pro-inflammatory and bactericidal responses of caprine alveolar macrophages (AMs) following homologous re-stimulation. Cytokines (IL-1β, TNFα, IL-6) were quantified by multiplex assay, iNOS+ frequencies were assessed by flow cytometry, and phagocytosis of pH-sensitive labelled MF was evaluated by live-cell imaging and flow cytometry. Primary MF stimulation induced a pro-inflammatory response compared with non-stimulated (NS) controls. Upon homologous re-stimulation, MF-MF cells showed increased IL-1β and TNFα production and higher iNOS+ frequencies compared with NS-MF controls. MF-MF cells also exhibited greater phagosome acidification, reflected by higher pH-sensitive fluorescence intensity and area under the curve over 48 h post-infection. Flow cytometry confirmed increased acidification at 8 h post-infection in MF-MF cells. Overall, in *vitro M. fortuitum* exposure induces trained immunity features in caprine AMs, enhancing pro-inflammatory responsiveness and bactericidal capacity upon re-stimulation, supporting the caprine model as a relevant framework for studying mycobacterial trained immunity.