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MEETING REPORT



# Early diagnosis and effective treatment regimens are the keys to tackle antimicrobial resistance in tuberculosis (TB): A report from Euroscicon's international TB Summit 2016

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**ABSTRACT**

To say that tuberculosis (TB) has regained a strong foothold in the global human health and wellbeing scenario would be an understatement. Ranking alongside HIV/AIDS as the top reason for mortality due to a single infectious disease, the impact of TB extends far into socio-economic context worldwide. As global efforts led by experts and political bodies converge to mitigate the predicted outcome of growing antimicrobial resistance, the academic community of students, practitioners and researchers have mobilised to develop integrated, inter-disciplinary programmes to bring the plans of the former to fruition. Enabling this crucial requirement for unimpeded dissemination of scientific discovery was the TB Summit 2016, held in London, United Kingdom. This report critically discusses the recent breakthroughs made in diagnostics and treatment while bringing to light the major hurdles in the control of the disease as discussed in the course of the 3-day international event. Conferences and symposia such as these are the breeding grounds for successful local and global collaborations and therefore must be supported to expand the understanding and outreach of basic science research.

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## Introduction

For a disease that should largely be under control, tuberculosis (TB) still claims over 1.5 million lives every year with children accounting for 140,000 of them.[1] Among the multiple factors that contribute to the success of the disease are the recalcitrant nature of the causal pathogen *Mycobacterium tuberculosis* and the growing drug-resistance acquired by it through decades of treatment with a fixed regimen of drugs. Lengthy diagnostic procedures, incorrect diagnoses followed by inadequate prescription of treatment enable resistant strains to spread within and outside communities. This is further complicated as TB is largely associated with resource-poor countries that have high population densities where contact tracing programmes are compromised due to lack of infrastructure.[2] However, the bleak environment has fuelled the need for innovative, rapid and accurate diagnostic tests based molecular techniques such as the GeneXpert® MTB/RIF that are being widely used all over the world.[3] These technologies require further development to suit the need of the hour in poor areas with low infrastructural support. For effective control of the disease, advances in diagnosis must be mirrored by the advent of effective therapeutics to treat the infection.

Antimicrobial drug discovery is often viewed as a "high-risk/low-reward endeavor" And this sector was ignored by the pharmaceutical companies and concurrently overlooked by public health policies alike. This has resulted in a critical shortage of novel drug entities in clinical trials. As extensively drug-resistant (XDR)-TB has now been detected in 105 countries, the US FDA has granted accelerated options for anti-TB drug development.[1] After around half-decade of no novel drugs for treatment, multi-drug resistant (MDR) TB patients finally have the option, albeit under strict regulations, of receiving bedaquiline or delamanid for treatment.[4,5]

The public cost of TB treatment in most cases ranges between US$ 100-500, increasing to US$ 10,000 in the case of MDR TB patients.[1] The rise of antimicrobial resistance (AMR) has been predicted to cost the global

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economy between ≤2.1 trillion to over ≤100 trillion in the year 2050, based on the interventions taken and their effectiveness.[6] This burden would be a direct outcome of the predicted annual loss of 10 million lives in their most productive years if AMR was to spread without control. TB holds a major share of the burden caused due to AMR. Adding to this, the impact of TB in animal husbandry has severe implications as there are no vaccines or practical, cost-effective means of treating infected animals. Parts of Wales in the United Kingdom have reported a staggering increase in the number of cattle slaughtered owing to bovine TB.[7] These cullings have put an inadvertent strain on the animal farming industry and local governments.

In today's global world and economy, an amalgamation of efforts starting from the student at the bench needs to go through to the supply chain which eventually lands the finished diagnostic or therapeutic product used at the point-of care or by the patient's bed. Scientists need to understand the ground realities and the difficulties faced by healthcare workers to come up with effective solutions and strategies. In a similar manner, physicians need to be informed of the latest advancements in basic sciences research to generate quicker feedback mechanisms that can highlight areas of pressing need for development. It is therefore essential to initiate lines of communication between these diverse set of individuals united by their efforts to control TB.

The TB Summit 2016, the third such event organized by Euroscicon, was fittingly held at North Greenwich, London (UK) as it is one of the boroughs with the highest incidences of TB in London.[8-10] The 3 day event was well attended with representatives from universities and government agencies from all over the world. The informative oral presentation and poster sessions were categorised for a systematic discussion on the different aspects of TB as is discussed in more detail in the following sections of the report.

## Tuberculosis: The disease, the pathogen and the host

### *Studying the spread of tuberculosis*

TB, in its most common form is an air-borne disease that invades the lung tissues and resides in the macrophages and other phagocytic immune cells of the host. The host immune responses serve to sequester the organism in compartments known as granuloma.[11] These granulomas mature further to form necrotic lesions which are finally broken down by mechanical shear and released into air by the act of coughing or talking by the infected persons.[12] Though the airborne route for transmission is widely accepted, adequate investigation into the different stages of disease, namely: expulsion of bacteria, transmission via air, inhalation and infection of new host needs to be carried out.

The act of coughing is considered important for TB transmission as it is a common symptom of pulmonary TB. As *M. tuberculosis* is encased within the lung cavities in thick biofilms a lot of force is required to shed the bacilli into the airways and out. Coughing releases more droplets than other maneuvers such as talking or singing as higher force is involved in the former act and is thus considered to be the major factor involved in the transmission mechanism.[13,14] Several studies studying cough patterns and household transmission have shown that patients cough more during the day than at night and that cough frequency is an important predictor of transmission.[15,16] As noted by **Richard Turner** (NHS Foundation Trust, UK), the act of coughing in the context of infectiousness of TB has not been looked into. The cause of cough in a TB patient; its necessity for the transmission of TB, correlation with infective/non-infective strains of the pathogen and characteristics of the optimum cough droplet for disease transmission are yet to be completely understood. Factors such as airway anatomy and the quantity, composition and viscosity of sputum have been overlooked and methodologies to investigate them need to be developed.

Investigating transmission patterns of TB can help develop effective intervention strategies to control the disease. Exhaustive transmission tracing studies should be able to detect or rule out transmission between cases, track the successive acquisition of single nucleotide polymorphisms (SNPs) along the chain of transmission and differentiate between primary and acquired resistance phenotypes while accounting for host diversity. However, TB infections are notorious for their heterogeneity within the same host as the lengthy infection period allows for microevolution of the bacilli within the duration of one infection cycle.[17] On the other hand, the pathogen has a slow-replication rate and a proclivity toward low genetic diversity. Thus the bacilli may either show no genetic diversity or the presence of multiple SNPs between contacts making it extremely difficult to trace transmission using their gene sequence alone. **Caroline Colijn** (Imperial College London, UK) suggests combining dated phylogenetic trees with statistical modeling to reveal the likelihood of a transmission tree using Markov chain Monte Carlo simulation/Bayesian inference methods.[18] However, using statistical methods to infer the transmission of the tubercle bacilli requires computationally intensive inference methods which suffer from the limitations of the available data and assumptions made to build the models as indicated by **Jarno Lintusaari** (Aalto University, Finland).[19] The complexity

of the process makes it difficult to draw reliable inferences from statistical models of transmission dynamics.

**Albert Nienhaus** (University Medical Center, Germany), **James Seddon** (Imperial College London, UK) and **Robert Wilkinson** (University of Cape Town, SA) discussed the hurdles in the control of transmission among different sets of high-risk individuals- healthcare workers, children and HIV patients.

Talking about occupational acquirement of disease, Prof Nienhaus stated that TB is one of the most infectious diseases affecting healthcare workers (HCW) in Germany.[20] The situation is worse in countries such as Portugal and France where occurrence rates are decidedly higher at 33% and 19% compared to Germany's 10%.[21,22]

HCW, especially physicians and nurses are at a higher risk of acquiring active and latent TB infection (LTBI) because of prolonged and frequent contact with infected patients. In contrast, this group has lower transmission rates to contacts than other groups of infected individuals as a result of frequent health checks.[23] After close scrutiny of the screening data of 32 practitioners, Prof Nienhaus recommended an annual screen of HCW at high-risk due to contact with highly-infectious patients. However, screening guidelines issued by the public health systems of different countries suggest a varied combination of X-ray, tuberculin skin test (TST) and interferon-$\gamma$ release assays (IGRA) with no consensus on the best strategy.[24,25] He suggested that in circumstances when IGRA is positive and X-ray negative, LTBI can be confirmed and followed by prophylactic measures if the risk of avoiding treatment is deemed higher than the risk posed by administering it. Encouragingly the detection of LTBI in HCW has been increasing over the years as diagnostic technologies improve and it is expected that investigations into the risk-reward dynamics of treatment will allow for the development of a uniform global solution.

Another group at high-risk of acquiring the disease owing mainly due to their association with infected adults, are children. In spite of one million cases of TB being reported in children, very little is being done to understand the pathogenesis and treatment options available for them. Dr Seddon has been working toward improving our understanding of treatment and prevention option of MDR-TB in children.[26] TB in children and infants is difficult to diagnose as sputum collection is difficult. Complicating matters further, the disease manifests in multiple forms including infection of the intra- or extrathoracic lymph nodes.[27] Hence, treatment must be tailored according to child's needs and health status. Though studies point out that almost all children who are diagnosed complete their therapy and have very good outcomes, regimens for MDR-TB in children require an overhaul as the pharmacokinetics and toxicity of drugs in children are significantly different from that observed in adults.[28,29] The regimen for drug-susceptible TB in children is the standard 6 month combination therapy where rifampicin, isoniazid, and pyrazinamide are administered for the initial 2 months followed by 4 months of rifampicin and isoniazid.[30] In children suffering from HIV and TB, additional ethambutol is advised in the first 2 months. Future trials to assess TB prevention strategies include TB-CHAMP to evaluate levofloxacin in children under 5 and ACTG A5300 to evaluate levofloxacin and isoniazid in adults and children among several others.[26,31] As new and repurposed drugs enter the therapeutic regimens, evaluation of their pharmacokinetic properties in children remains essential.

Prof Wilkinson and his team are looking at the association between TB and HIV in Khayelitsha, South Africa. Khayelitsha is an impoverished township where the prevalence of TB is one of the highest seen around the world. Antenatal HIV prevalence is reported to be 25% of the total population wherein almost 40% have the risk of developing active TB.[32] Furthermore, 76% of TB cases are also HIV positive.[32] The team carried out a randomized controlled trial of antiretroviral therapy (ART) alone and isoniazid preventive therapy (IPT) combined with ART (cART) for prevention of HIV-associated TB. The results indicated that isoniazid added to the anti-viral therapy reduces the risk of TB by 35% as observed after a year of IPT therapy.[33] He also estimated that individuals infected with HIV have around 30 times the risk of acquiring active TB due to immunosuppression and in very high incidence environments reinfection is more common than endogenous reactivation of disease.[34]

## *Mycobacterium tuberculosis: A closer look*

The genus *Mycobacterium* has undergone several speciation and genetic decay events to give rise to a variety of non-pathogenic and pathogenic species with varying host-specificities. Unlike other bacteria, mycobacterial species are not involved in horizontal gene transfer hence evolution is gradual, driven by small genetic changes regulated by natural selection or associated with human evolution and their paleomigration. The course of these genetic drifts has caused *M. tuberculosis* to become an obligate pathogen with the host-specificity restricted primarily to humans.[35] **Frédéric Veyrier** (INRS-Institut Armand-Frappier, Canada) has been using gene sequencing technologies to reveal the factors that make *M. tuberculosis* human-specific. Two approaches were tried: (a) the 'aimless' method which included next-generation sequencing (NGS) and mycobacterial homolog

investigation tool (mycoHIT) and (b) the 'focused' approach which narrowed in on the loci of interest. Their main interest was to investigate the genetic mutations that are selected during the process of evolution toward becoming an obligate pathogen.[35]

Whole-genome sequencing can help differentiate between fast and slow growing mycobacteria. These studies revealed *M. kansasii* to be a potential candidate to model an ancestral image of *M. tuberculosis* being an environmental species that can cause TB-like disease that cannot be transmitted.[36] The genetic analyses revealed an explosion of toxin-antitoxin modules in *M. tuberculosis* after speciation, mostly by gene duplication. Another specific adaptation is anaerobic respiration.[37,38] Ancestral genetic events could explain the sequential evolution of the pathogen and the ongoing functional characterization of these events could be reconstructed in *M. kansasii* or *M. marinum*.

Moving on from the clues in the genetic code to the message–relay system, **Eric Galburt** (Washington University School of Medicine, USA) discussed the kinetics and energetics of transcription initiation in mycobacteria. Though a fundamental biological process that is common to, and conserved in bacteria; transcription rates vary largely between *E. coli* and mycobacterial species owing to the differences in stability of the intermediate complexes formed in this process. Hence it is important to study the central processes in the pathogen itself rather than inheriting information from other model organisms. The essential transcription initiation factors CarD (Rv3583c) and RbpA (Rv2050) play important roles in the biology of *M. tuberculosis*.[39-42] Initiation requires the formation of RNA polymerase (RNAP) holoenzyme with the bound sigma factors. A series of kinetic steps bring about the formation of a closed complex leading to the open complex with the transcription bubble. The initiation factors are positioned near the upstream edge of the transcription bubble.[43,44] Techniques such as fluorescent labeling were used to study the open complex using *M. bovis* RNAP which is known to form open complexes that are more unstable as compared to *E.coli* RNAP. It was found that the initiation factors act independently and cooperatively to regulate transcription and that only in the presence of both factors does the transcription kinetics in mycobacteria appear to be similar to that observed in *E. coli*.[45,46] As both factors are known to play important roles in pathogenesis, global rRNA/gene expression levels and resistance to stress and antibiotics, they would serve as good targets for drug design.

A major challenge to the development of effective drugs and treatment regimens against *M. tuberculosis* is the heterogeneity of the infecting population of bacilli which include actively replicating and dormant cells known as persisters. Persister cells are quiescent, highly tolerant toward antibiotics and other external stresses and under favorable conditions can revert to actively dividing states.[47] Detection of these cells is near impossible as conventional microbiological techniques of culture cannot identify them. These cells can be generated *in vitro* in prolonged stationary phase culture or gradual acidification of culture medium. They can then be reverted to their actively replicating physiology by exposure to resuscitation promoting factors (Rpfs) and are also known as Rpf-dependent cells.[48]

Rpfs are secreted or cell-wall bound enzymes produced by actively dividing mycobacteria. They are structurally and functionally similar to lysozymes and are essential for resuscitating dormant cells.[49] There are 5 Rpfs in *M. tuberculosis* and they have been found to be important for persistence and reactivation of infection in mice. RpfA, B and E are secreted and found in culture filtrates whereas RpfC is cell-bound.[50] RpfD is upregulated during infection. Once revived, Rpf-dependent cells lose this characteristic dependency during *in vitro* passages.

Rpf-dependent mycobacteria are very important in the clinical aspect.[48] These cells are highly abundant in sputum and extra pulmonary TB samples with their relative proportions varying from 0 to 99.99% of the total *M. tuberculosis* population. **Jaishree Garhyan** (Jawaharlal Nehru University, India) reported bone marrow to be a niche for persisters as the mesenchymal stem cells survive for an extended period of time and reside in immune suppressed environments.[51,52] Additionally, stem cells have active drug efflux cells providing a second wall of defense for the pathogen. Their work indicates that *M. tuberculosis* hijack mesenchymal stem cells, reprogramme them through hypoxia inducible factor (Hif-1) and targeting this pathway could potentiate standard treatment regimens.

Rpf-dependent cells are inherently more resistant to several drugs such as rifampicin, isoniazid and streptomycin.[53] As it is more difficult to get rid of Rpf-dependent mycobacteria they are found to be enriched in treated TB patients. As persisters are often a cause of treatment failure and post-treatment relapses it is essential that evaluation of TB treatment regimens accounts for elimination/induction of this group of cells.[54] **Yanmin Hu** (St George's University of London, UK) believes the invisible persisters represent a promising therapeutic target and shorter chemotherapy regimens such as the one trialled with fluoroquinolones can only be successful if they are effective in decimating this subset of the pathogenic population. Under *in vitro* conditions, her group observed that high doses of rifampicin are required to kill stationary cultures of *M.*

*tuberculosis*.[47] This is mainly owing to the Rpf-dependent bacilli which are highly tolerant to the drug and appear to decrease with increasing concentrations of rifampicin but cannot be completely eradicated.[47] When they incubated infected mouse lung tissues (2 week infection time) with culture filtrate containing Rpfs, the persister cells could be revived even after the mice were administered with high-dose rifampicin treatment. They used the Cornell model to study the efficacy and relapse rate of different drug treatment regimens, wherein the infection was allowed to establish for 3 weeks and then treated with drug combinations for 14 weeks. At the end of treatment period the mice were given hydrocortisone to suppress the immune system to study relapse rate. As noted with the earlier model, dose-dependent killing of bacteria was observed. It was also noted that higher doses of rifampicin can shorter treatment times however, a small subset of Rpf-dependent bacilli still persist in the host tissues.

**Galina Mukamolova** (University of Leicester, UK) introduced the concept of drug-induced Rpf-dependency in *M. tuberculosis*. Treatment with rifampicin, isoniazid and ethambutol was shown to induce Rpf-dependency, whereas no such effect was observed in the case of treatment with streptomycin and amikacin.[53] Thereby it is critical that drugs be tested for their propensity to induce Rpf-dependency. Toward this end, Mukamolova and co-workers strived to design a simple assay to determine the potential of drugs to induce Rpf-dependency. Rpf-dependent mycobacteria can be generated during infection in mice. However, these models are difficult to process in a high-throughput setting. To adapt this to *in vitro* settings, the Mukamolova group synthesized novel nitric oxide donors which could induce persister phenotype in *M. bovis* cells. These *in vitro* systems can thus be used for validation of novel drugs and the search for Rpf-dependency biomarkers alike.

Another model for persistence, using the toxin-antitoxin (TA) modules found littered in the *M. tuberculosis* genome, was discussed by **Shaleen Korch** (Midwestern University, USA). Out of the 88 putative TA modules identified, around 30 have been assigned functions.[55] These are typically 2-gene operons, wherein one encodes a toxic protein and the other encodes an antitoxin that binds to and inhibits the toxin.[56] Regulation of these modules is controlled mostly through auto-repression of transcription of the operon by the antitoxin. However, upon exposure to stresses encountered during infection a certain subset of these modules are activated. Given that in *E. coli* these systems are known to be involved in generating persisters, their role in controlling *M. tuberculosis* division rates during infection needs further investigation.[57] Korch chose to focus on the RelBE system which inhibits translation through mRNA cleavage.[58,59] By generating strains that overexpress the toxins followed by antitoxin rescue they demonstrated a persistence phenotype wherein only 20% of total translation was observed.[60] This model will enable further investigation into the stages of persistence namely, entrance, maintenance and exit.

## Host responses to M. tuberculosis

Macrophages are the sentinel of the innate immune system. Once *M. tuberculosis* gains entry into the airways of a suitable host it encounters phagocytic cells and invades alveolar macrophages and monocytes preferentially. Once infected, these macrophages release a host of cytokines that recruit several immunologically active cells such as the T-cells which release interferon-$\gamma$ (IFN$\gamma$) which in combination with tumor necrosis factor-$\alpha$ (TNF$\alpha$) activate macrophages to eliminate the pathogen.[61] **Marcin Wlodarczyk** (University of Lodz, Poland) is interested in the profile of mycobacterial antigen driven cytokine responses and the expression of signal transduction receptors in TB. Two hundred and eighteen BCG vaccinated, HIV-/TB+ patients were chosen for the study. The main objective of the research was to assess the production of IFN$\gamma$, TNF$\alpha$, interleukins and the cell-surface expression of receptors such as TLR2, LFA-1, CD14 as well as the soluble counterpart of CD14 between the patients and healthy volunteers. They report to observe quantitative rather than qualitative changes in the expression levels of the cytokines studied when serum from infected individuals were challenged with mycobacterial antigens. TB patients were also found to have a significantly higher frequency of monocytes with CD14, TLR2 and LFA-1 receptors.[62]

Another Toll-like receptor family member, RP105 (CD180) is under investigation by **Antje Blumenthal**'s group (The University of Queensland Diamantina Institute, Australia). RP-105 mainly expressed by macrophages and monocytes. It has a short cytoplasmic tail and it has been suggested that it cannot signal directly without a signaling partner. *CD180* mRNA expression levels are higher in whole-blood of cured TB patient versus those who relapse.[63] It was also shown that RP105 deficient mice are more susceptible to TB and RP105 deficient macrophages produced significantly reduced amounts of inflammatory cytokines than their wild-type counterparts.[64] However, neither mRNA nor protein expression of inflammatory cytokines such as TNF were affected in RP105 deficient mutants macrophages.[64] Through their work the Blumenthal group have demonstrated the RP105-mediated macrophage activation to be distinct from classical Toll-like receptor (TLR) signaling where RP105 directs cytokine trafficking.[65,66] This novel

innate immune signaling pathway involves RP105, BTK and the p110δ subunit of phosphoinositide 3-kinase.

Another family of small proteins, the interferon induced transmembrane (IFITM) proteins, and their role in restricting *M. tuberculosis* infection was introduced by **Shahin Ranjbar** (Harvard Medical School, USA).[67] TLR2 and 4 and MyD88-dependent signaling pathways engage in the induction of the IFITM proteins 1, 2 and 3. Their work revealed that expression levels of the IFITM proteins directly correlated to the growth rates of *M. tuberculosis* in THP-1 cells. IFITM3 was seen to co-localize with mycobacterial cells in alveolar epithelial cells and overexpression of the protein enhanced endosomal acidification thereby limiting the infection.[67]

Bringing the discussion from receptors to signaling molecules, **Maria Regina D'Imperio Lima** (Universidade de São Paulo, Brazil) spoke about the role of purines, especially extracellular ATP (eATP) in the immune response to severe TB. Hypervirulent mycobacterial strains show varying degrees of pathogenicity. The disease severity is often associated with suppression of effector cytokine production.

eATP and its breakdown product adenosine, are known to act as a chemotactic signals leading to enhanced migration of neutrophils and macrophages to the site of inflammation.[68,69] eATP is known to activate several receptors that in turn induces cell death by necrosis, formation of ion channels or pyroptosis.[70] These mechanisms enable release of bacteria from the phagocytic cells and it has been observed that TB caused by hypervirulent mycobacteria is attenuated in mice that lack the P2X7 receptor and there is a decrease in disease dissemination.[71] The wild-type cells with active P2X7 die of necrosis which contributes to release of the hypervirulent mycobacteria.[71] Histological analyses also revealed less inflammation-induced damage in the knockout mice thus demonstrating that P2X7 signaling contributes to the pathology in a severe infection model. The group also noted that specific CD4 cells in the pulmonary parenchyma of mice infected with virulent mycobacterial strains are suppressed.

**Annemarie H. Meijer** (Leiden University, Netherlands) uses 1-day old zebrafish embryos infected with *M. marinum*, a close relative of *M. tuberculosis* to study the mechanisms of host defense in the early stages of infection. The advantage of studying these organisms is that they are optically transparent and cell aggregates and their dispersal can be easily followed. The young embryos do not have adaptive immunity and the earliest immune cells are macrophages and neutrophils. Within minutes of infection the bacteria are phagocytosed. The infected cells recruit new macrophages and consequently granulomatous aggregates are formed. The granuloma wards the pathogen from further immunological intervention and thereby offers it a great advantage.[72] A steady secretion of early secretory antigen-6 (ESAT-6) by the pathogen ensures maintenance and growth of the granulomas. This agrees with the finding that ESAT-6 deficient mutants can only sustain small granulomas. Prof Meijer and her group focus on the chemokine network that facilitates the migration of the macrophages to form the granuloma.

In zebrafish with mutated CXCR3 receptor, random motility of macrophages is reduced.[73] This motility defect correlates with reduction in number and size of granuloma translating to the decrease in total burden of infection. Inhibition of the CXCR3 receptor showed reduction in granuloma formation and previous studies showed CXCR3 deficient mice to exhibit an increased resistance to infection.[73] Hence targeting granuloma formation through the use of immunomodulatory drugs could help develop host-directed strategies that may work synergistically with chemotherapy and lower the risk of new resistance arising.

Using zebrafish as a preclinical model they tried several methods to (a) inhibit granuloma expansion (b) reverse the inhibition of phago-lysosome fusion and (c) stimulate anti-mycobacterial autophagy and autophagic efflux.[74] Autophagy is an immunological defense system and numerous *in vitro* studies show that inhibition of autophagy promotes the growth of *M. tuberculosis* within host tissues.

DNA damage related autophagy modulator1 (DRAM1) is upregulated during *M. marinum* infection dependent on the TLF/MyD88 pathogen recognition pathway.[75] On overexpressing *dram1* the fish larvae develop lower infection levels and smaller granuloma.

## Early diagnostics: The key to tackling TB?

Out of the 9.6 million estimated cases worldwide in 2014 only 6 million were reported through national surveillance centers. Most of the 3 million undiagnosed cases are believed to be from regions where health services are weak. This is mainly owing to under-reporting and under-diagnosis. Under-reporting occurs when diagnosis and treatment are done in the private sector which is disassociated from nationalised schemes. Secondly, pediatric services are often poorly-linked to the national TB services and children often go undiagnosed as detection of TB in these cases is especially difficult. Poor access to health care in low-income countries is the primary cause for under-diagnosis and many patients are lost to follow-up. Most health centers carry out passive case finding whereas **Luis E. Cuevas** (Liverpool School of Tropical Medicine, UK) believes a more active approach

incorporating outreach services could improve the situation. He compared 2 case studies of active case finding; one carried out in Ethiopia which has an active health extension program and the other in Nigeria, which discontinued its health extension program several decades ago. The study was carried out in the slums around Abuja in Nigeria. With the help of retired health extension workers (HEWs) and using resources such as the polio eradication census data, house-to-house visits were made to collect 2 sputum samples 40 minutes apart. The results were notified at the individual's home or through a phone call. The case detection rate was doubled after the effort with increased diagnoses for women and the older population.[76]

Ethiopia has a well organized health extension program. In the study, the HEWs were provided basic health packages that were delivered on their house visits every 14 d. The main aim of the exercise was to improve TB case findings and treatment outcome. The HEWs were trained in case identification, collection of sputum and preparation of smears which were then dropped at the local laboratories. Once results confirmed TB infection the supervisor initiated treatment at home and domestic contacts would be screened. There was at least a 2-fold increase in detection from the previous year thereby clearly demonstrating improvements in TB case findings using the active case-finding approach.[77,78] **Andrew Curtis** (Kent State University, USA) also supported this approach, he discussed the use of spatial video-camera footage and geo-narratives to enable active-contact tracing of the homeless and drug-user population who are at high-risk of acquiring the disease in developed countries.

Sputum smear microscopy remains the mainstay of classical diagnostic tests used in low and middle-income countries with high-TB burden.[79] This method is easy to perform, cheap and rapid but its use is limited due to poor sensitivity and specificity especially in pediatric cases, HIV patients and patients with low sputum bacterial loads.[80] Liquid and solid culture techniques can take up to 3 weeks to obtain results, at which point patient tracing may become difficult resulting in TB-infected individuals receiving no treatment and disseminating the disease within their communities and households precipitating the situation further.

In an effort to develop a non-invasive, diagnostic strategy for TB using breath samples, **Thu-Hoa Tran-Thi** (Center National de la Recherche Scientifique, France) discussed the potential of volatile metabolites of *M. tuberculosis* as biomarkers for the disease.[81,82] Nicotinic acid (NA), methyl phenylacetate (MPA), methyl p-anisate (p-MA), methyl nicotinate (MN) and o-phenylanisole are 5 volatile compounds that were identified from *M. tuberculosis* cultures grown *in vitro*.[82] NA can be detected in breath of active TB patients although present in low amounts. It was also found to be a promising indicator of *M. tuberculosis* strains resistant to pyrazinamide. Dr Tran-Thi and group investigated a method to detect NA in complex solutions with different metabolites and also biological media. Luminescence techniques to detect NA using terbium III ($Tb^{3+}$) as a probe owing to its property to luminesce on energy transfer from NA to the $Tb^{3+}$ ion were developed.[83] Potential interference by other metabolites such as MPA, p-MA and MN that have similar excitation wavelengths as NA in samples was identified as a challenge. Breath condensate was identified as the best sample as there is less interference compared to saliva and urine samples. The cost of this diagnostic process is estimated to be less than the World Health Organization (WHO)-approved molecular diagnostic test, the GeneXpert® MTB/RIF, making its development a priority for subsequent use in developing countries.

Blood biomarkers can also be exploited for use in diagnostic tests in detecting TB in endemic areas. As a whole host of antibodies specific to the various antigenic epitopes found in *M. tuberculosis* are generated in TB patients, single antibody profile-based detection methods are likely to fail. **Imran Khan** (University of California, USA) has looked into the use of a multiplex sero-detection panel for TB diagnosis.[84] This test uses a panel of 28 *M. tuberculosis* antigen-coated microbeads which enables the universal identification of antibody profiles in patients by a single test. Using the same method, Dr Khan examined 10 plasma cytokine/chemokine/growth-factor and 8 antibodies against 8 *M. tuberculosis* antigens biomarkers that are elevated in TB patients. Interestingly, they found the antibody profile levels among TB patients are gender biased and linked to treatment success.[85] Immunobiomarkers in TB are valuable to understand host responses against the pathogen and this area could be further explored for development of TB diagnostics and treatment.

Genomics, proteomics and transcriptomics are approaches that have been extensively utilised to characterize *M. tuberculosis*. Recently, metabolomics, a newer approach, has been complemented into the "omics" revolution to provide a more complete insight into the pathogen's complex internal systems.[86,87] Metabolomics investigates all intra- and extra-cellular metabolites (small molecule intermediates and product) in a biological sample using highly specialized analytical techniques to create a metabolic profile and measures any dynamic responses to stimuli or genetic modifications.[88-90] **Du Toit Loots** (North-West University, SA) is interested in characterizing *M. tuberculosis* using metabolomics and identifying its characteristic metabolite signatures. This

approach can identify between the different species of mycobacteria using specific metabolite markers, although the species are known to have minor genetic variations, illustrating the sensitivity of this approach.[86] Prof Loots conveyed his optimism of the possibility of using metabolite markers to build diagnostic models based on systematic approaches in metabolomics research.[91-93] It could also assist in finding the association between TB and its symptoms; for example, increase epinephrine levels can predict weight loss and insomnia in patients. In the future, this approach could also be used to diagnose drug resistant TB and for prognosis.[94]

Nucleic acid amplification tests (NAATs) are molecular diagnostic approaches that were developed to improve the sensitivity and specificity in diagnosing TB, and most importantly, to detect MDR-TB infections. The GeneXpert® MTB/RIF Ultra was introduced to this year's TB Summit, highlighting the significant improvements on its earlier version. **Elisa Tagliani** (IRCCS San Raffaele Scientific Institute, Italy) promoted the new molecular diagnostics tool and hailed it to be transformational for TB detection/elimination efforts. The latest format uses bigger cartridges and extra probes detecting 4 new *rpoB* mutations, IS6110 and IS1081 from *M. tuberculosis* to enable detection of 30 different rifampicin-resistant *M. tuberculosis rpoB* mutations. With these advancements they hope to enhance the sensitivity of the assay close to liquid culture.[95]

**Eric R. Houpt** (University of Virginia, USA) spoke about the TaqMan array card (TAC). It is a genotypic method to detect pathogen mutation which provides 2 layers of accurate detection of mutations via sequence-specific probes and high-resolution melt analyses (HRM). It can detect mutations in genes that are indicated in resistance, such as *rpoB, inhA, embB, gyrA* to name a few.[96] The method is easy to perform, reproducible and has good sensitivity and specificity. It has the potential to improve MDR-TB and XDR-TB detection hence improving the appropriateness and efficacy of TB treatment.

Many of the commercially available NAAT formats are inaccessible in resource-poor centers due to the high costs related to instrumentation and consumables. PURE-TB-LAMP, a TB NAAT test, claims to be simple, fast, robust, sensitive (compared to smear microscopy) and affordable (compared to other NAAT platforms). **Yasuyoshi Mori** (Eiken Chemical Co., Ltd., Japan) spoke about this screening test kit that uses loop-mediated isothermal amplification (LAMP) that removes the needs for costly thermocycler or detection systems.[97] The kit consists of Loopamp™ PURE DNA Extraction Kit and Loompamp™ MTBC detection kit. Three main technical aspects of the test are (a) amplification of DNA with LAMP, (b) ultra-rapid extraction (PURE) of DNA that can remove inhibitors of the test without significant loss of genetic material and (c) straightforward visual detection of the result using fluorescence under ultra-violet light.[98,99] The Loopamp™ LF-160 machine does not need maintenance or calibration which makes the overall process simple and easy to pick up; dismissing the need for extensively trained personnel. The sensitivity of TB LAMP is 100 times higher than sputum smear microscopy.[100] Currently, the screening test has been approved for use in Japan, China, South Korea and Thailand. Analyses of its clinical performance have been submitted to WHO for usage approval.

Mediastinal TB is a form of extrapulmonary TB that is often seen in pediatric, HIV-infected patients and adult immigrants in developed countries. The number of reported cases of extra pulmonary TB in the UK has increased slightly in the past decade, a trend that is worrying.[101] Diagnosis of mediastinal tuberculous lymphadenitis is challenging due to its nonspecific clinical presentation, normal chest X-ray examination result and low-yield sputum culture.[102,103] These vague presentations require to be differentiated with other similar presenting diseases such as lymphoma, carcinoma and sarcoidosis.[102] **Onn Min Kon** (Imperial College London, UK) emphasized that mediastinal TB should not be overlooked especially when normal chest X-ray is presented in patients with suspected isolated mediastinal lymphadenitis. A follow up thoracic PET/CT scan is compulsory to confirm a positive lymphadenopathy.[104] Sampling of mediastinal lymph node tissue is obligatory to identify etiology of the disease, previously mostly done using techniques such as cervical mediastinoscopy. However, this invasive technique is associated with significant morbidity and mortality hence endoscopic ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), commonly used in diagnosing lung cancer, is recommended as a minimally invasive alternative for mediastinal TB diagnosis.[105-107] Prof Kon informed that this widely used microbiological diagnostic technique combined with GeneXpert® MTB/RIF could enhance the sensitivity and specificity of mediastinal TB diagnosis, quicken the suspected cases' assessment and enable rapid detection of resistant *M. tuberculosis* strains.[108]

*M. bovis* is a member of the MTB complex and is promiscuous in host specificity. It can infect alpacas, llamas, dogs and cats that can then transmit to the animal owners. The UK has not been able to contain the disease despite several radical measures being carried out to control the natural reservoir populations. Three months are required to diagnose *M. bovis* infection and it costs $140 per sample. Differentiation between *M. bovis* and *M.*

*tuberculosis* infections are necessary as the former is inherently resistant to pyrazinamide.

Traditional approaches to generate serological response tests either use non-pathogenic BCG strains or heat-killed/chemically-inactivated pathogens. These harsh conditions damage the important cell surface conformational epitopes which are not represented or maintained during detection. The novel approach tried by **Linda Stewart** (University of Belfast, UK) was to use gamma-irradiated pathogenic strains to develop *M. bovis* specific binders.[109] The whole-cell or ethanol extracted surface antigens were used and both polyclonal as well as monoclonal antibodies were raised using phage display technology. The outcome of this was an array of binders that were characterized by ELISA and immunofluorescent staining. The main aim of their efforts was to develop a diagnostic assay that would use the binders for disease detection that could differentiate between *M. bovis* and *M. tuberculosis* infections unlike the commercially available kits in the market at present.

**Gustavo Moreira** (Technische Universität Braunschweig, Germany), discussed the use of phage display for biomarker identification and generation of human antibodies for diagnostics and therapy. Antibody phage display involves an *in vitro* process, which does not involve the immune system. The process begins with insertion of the antibody genes into phage genome, which is then expressed as a fusion protein displayed on the outer surface of the phage. The next stage is called panning, where the antigen is interrogated with the library of antibodies that has been created. Identification of positively binding antibodies is performed by ELISA, immunoblot or flow cytometry.[110]

*M. tuberculosis* secretes a protein called Antigen 85 (Ag85). Antibody genes from a naïve library were selected and were cloned into yeast display systems. This combination of phage and yeast display was used to produce antibodies against Ag85. Clones were screened for the highest antigen binding revealing 7 antibody pairs that can detect Ag85 at nanomolar concentrations, however none of the clones were strictly specific for one of the antigen 85 subunits of the complex.[111] Another study used 5 human antibodies against Ag85B from the naive libraries, where 3 of them bound specifically to the 85B proteins as tested by ELISA.[112]

Monitoring host response to TB treatment can identify the efficacy of treatment and predict the likelihood of relapse. The current method to validate treatment success is through bacteriological culture conversion at 2 months from the onset of therapy. Bacterial transcriptomics offers a new avenue to study elimination of the pathogen from the patient. **Dimitrios Evangelopoulos** discussed molecular methods to detect the decline of bacterial load during treatment as a biomarker of infection progression. The method utilizing detection of mycobacterial RNA has been developed from using an artificial matrix resembling sputum samples and applied to a clinical case concerning a 12 y old patient.[113] In this case, staining methods detected the presence of acid-fast bacilli however liquid culture assays could not detect any live bacilli. On using the molecular bacterial load (MBL) assay, the patient was shown to have a high bacterial load and the treatment regimen was modified to include moxifloxacin. After a year of treatment, both MBL and staining assays did not detect any bacilli and the patient was confirmed as cured.

A pilot study with mice established that this assay can also be used with tissues from animals. MBL and colony forming unit (CFU) assays on the tissues of mice undergoing different treatments revealed that both the assays agreed with each other, with the former consistently detecting higher numbers of cells probably as a result of detecting the non-culturable cell population.[113] Therefore, the MBL assays can serve as a rapid and sensitive diagnostic and treatment monitoring tool, offer insight on drug efficacy and may be used in drug discovery.

## Drug discovery: Moving past the roadblocks

### *The search for novel therapeutic targets*

Since the landmark announcement of the complete genome sequence of *M. tuberculosis*, there has been a focused drive to identify important genes and visualize their protein products enabling target-based drug design projects to take-off. **Tom Blundell** (University of Cambridge, UK) is interested in exploring the biological space-going from genome to proteome. The traditional techniques of wet-lab research to understand the proteome of the pathogen need to be underpinned by systems biology approaches. To this end Prof Blundell's group has been working on Chopin, which inherits the information of the known structures for around 400 proteins and uses it to predict models for the remaining 3,500 protein structures.[114] Based on the information in publicly available databases; interactions, ligands and the oligomeric states of many of the proteins can be deciphered. This information can then be fed into structure-guided, fragment-based approaches in drug discovery. The rationale of this strategy is to use small molecules with promiscuous binding affinities to interrogate the substructures present with the protein targets using standard biophysical ligand binding assays. The hits are then optimised to yield a molecule which binds to the target protein at nanomolar concentrations. Using this approach they were successful in developing inhibitors against EthR, the transcriptional

repressor for ethionamide activating protein.[115] As development of mutations is a major disincentive for anti-TB drug development industry, Prof Blundell also discussed the use of second generation sequencing technologies to develop a knowledge-based approach to understand evolutionary dynamics. The effects of single nucleotide polymorphisms on protein stability, protein-protein/nucleic acid/ligand affinity can now be ascertained by using mCSM, another useful web-service tool developed by the group.[116]

Identifying drug targets that are druggable is an essential step toward rational drug design. The mycobacterial cell wall is unique from Gram-positive and Gram-negative organisms and is a good source for anti-mycobacterial drug targets as discussed by **Sanjib Bhakta** (Birkbeck, University of London, UK) and **Dirk Schnappinger** (Weill Cornell Medical College, USA). It has a mycolic acid-arabinogalactan-peptidoglycan complex forming the cell wall core, a characteristic feature that has caught the attention of several researchers over the years.

Disrupting *M. tuberculosis*' mycolic acid has proved to be an excellent strategy to target the pathogen as illustrated by the success of drugs such as isoniazid, ethambutol and pyrazinamide, all of which target mycolic acid synthesis. However, the increasing emergence of resistance in *M. tuberculosis* strains to these drugs has heightened the need for novel targets. Biotin is an essential co-factor for all acyl-CoA carboxylases (ACCases), which play a role in providing building blocks for the synthesis of the fatty acid component in *M. tuberculosis* cell envelope. Mammalian cells acquire biotin from the external environment and thus lack these enzymes, therefore validating this pathway for developing intrinsically selective anti-TB drugs.[117] Furthermore, mutants in the biotin synthesis pathway can cause only attenuated forms of infections in mice, suggesting the pathogen is unable to obtain exogenous biotin.[118] Prof Schnappinger and his group found that mutating one of the enzymes, BioA (biotin ligase) in *M. tuberculosis* made it incapable of surviving *in vitro* and in mice indicating that the *de novo* biotin synthesis is critical for the pathogen's survival and persistence.[119] BioA is susceptible to inhibition, as validated by its inhibition using the antibiotic, amiclenomycin.[120,121] However, this antibiotic is too polar and unsuitable for use as a part of the lengthy TB treatment regimen. A target-based approach undertaken by Prof Schnappinger's group has identified a potent bisubstrate inhibitor of biotin protein ligases (termed as Bio-AMS) from *M. tuberculosis* which acts by interacting at both the biotin and ATP binding pockets.[122] This competitive inhibitor was found to be very selective and potent against TB strains showing that genetic approaches to facilitate TB drug discovery are still worth pursuing.

Dr Bhakta (Birkbeck, University of London, UK) has pioneered research in the metabolism of peptidoglycan (PG) in *M. tuberculosis* for over a decade.[123] The ATP dependent Mur ligase genes are involved in the synthesis of PG precursors. These genes are restricted to bacteria and have been found to be essential in mycobacteria by transposon site hybridization (TraSH). The structure of murE has been resolved to 3Å and shows an accessible active pocket site that can be exploited for drug design.[124,125] Enzymatic inhibition of MurE from *M. tuberculosis* was observed with the extracts from Columbian plants and chemically synthesized quinolone compounds thereby demonstrating that the ligases can serve as drug targets.[126-129] Additionally, as cell division and cell wall biogenesis are stringently controlled, co-operative processes, it is no surprise to find the mur ligase genes clustered in the division cell-wall (*dcw*) operon, further indicating that targeting this pathway will have knock-on effects on co-regulated pathways.

The mycobacterial divisome is a multi-factorial complex that assembles at mid-cell and brings about cleavage of the bacteria resulting in the formation of 2 daughter cells. Disruption of the cell division process can severely impact the infectivity of the pathogen. With this in view, **Tim A. Cross** (Florida State University, USA) investigated 2 recruits of the divisome, CrgA and ChiZ about whom little is known. The structure of mycobacterial CrgA has been modeled with and without the division protein FtsQ. Using solid-state NMR they found CrgA to comprise of 2 transmembrane helices with very precise rotation axes. The exposed glycine residues on the structure are expected to be involved in binding to the proteins in the divisome.[130] The *N*–terminus of CrgA is intrinsically disordered, however, Dr Cross suggests that in the lipid interface it would probably take up the conformation of a $\beta$-sheet.

In contrast to expectations, rational drug design has not yielded the number of potential lead drug candidates that were obtained from whole-cell phenotypic screening methods such as the one discussed by Dr Bhakta. SPOTi is a solid-culture based technique wherein an optimised number of bacteria are spotted on to agar containing a series of dilutions of inhibitor molecules.[131-133] This method has been adapted to 96-well microplate format thereby increasing the throughput.

However, once a potent inhibitor of mycobacterial growth is found it is essential to identify the endogenous target of the molecule. There are several available technologies for target identification such as genomic-, affinity- and knowledge-based approaches. **Cristiano V. Bizarro** (PUCRS, Brazil) spoke about pulse proteolysis

and precipitation for target identification (PePTID).[134] It is an energetics-based method where protein extracts are incubated with or without a ligand followed by the application of a brief proteolytic pulse and trichloroacetic acid precipitation. Mass spectrometry is used to analyze the results and as a proof-of-concept they applied the methodology to identify ATP-binding proteins using non-hydrolysable ATP$\gamma$ in *M. smegmatis*.

### The promising molecules and natural products

**Daniele Castagnolo** (King's College London, UK), **Jaroslav Roh** (Charles University Prague, Czech Republic) and **Ill Y. Lee** (Korea Research Institute of Chemical Technology, South Korea) spoke about promising potential anti-TB specific drugs in different stages of development as a result of their research efforts.

Dr Castagnolo mentioned that using molecules that are similar to candidates that fail in clinical trials and optimising them to improve on their characteristics could be a winning strategy. He noted that 2 potent anti-mycobacterial molecules, BM212 and SQ109 have similar topological distribution of chemical features. However, they had limitations with respect to pharmacokinetic parameters, solubility, cytotoxicity profile and efficacy against MDR *M. tuberculosis* strains. The group's strategy was to hybridize the 2 to generate new derivatives to achieve improved anti-TB activity or possibly reduced cytotoxicity. Two generations of compounds were synthesized and interestingly the first generation of compounds turned out to be potent multidrug efflux pump inhibitors, meaning that these molecules could potentiate standard chemotherapy.[135]

Dr Lee investigated derivatives such as phenyl ether and carbonate derivatives of nitroimidazole as the molecule has space to accommodate reactive groups that could improve its anti-mycobacterial profile.[136,137]

Dr Roh investigated 5-substituted 2-[(3,5-dinitrobenzyl)sulfanyl]-1,3,4-oxadiazoles and 1,3,4-thiadiazoles for their excellent anti-mycobacterial activity against both susceptible and drug resistant forms of *M. tuberculosis* with MIC values as low as 0.03$\mu$M. The compounds exhibited selective anti-mycobacterial activity with no activity against Gram-positive, Gram-negative and fungi. There was no cross resistance observed with other first- and second-line TB drugs. Structure activity relationship studies demonstrated that the 3,5 dinitro phenyl fragment to be the crucial in conferring anti-mycobacterial activity.[138-140] Any changes in position of the dinitro group led to the loss or several fold decrease of anti-TB activity. An AMES test indicated these nitro group-containing compounds have no mutagenic activity.[140] They also exhibited low cellular toxicity *in vitro* on 3 mammalian cell lines. The compounds share structural similarities with the inhibitors of DprE1, such as dinitrobenzamides or benzothiazinone. However, radiolabeling experiments showed reduction in the incorporation of uracil thereby indicating that the bacterial cells suffer from DNA synthesis defect on treatment. Further studies on optimization of compounds to improve solubility and replace the sulfur atom are ongoing as its presence in the molecule makes it highly susceptible to metabolic degradation.

Natural products play an important role in drug discovery and have been a rich source of antimicrobials. It is estimated that one-third of the world's population rely on traditional medicine for their healthcare needs. The screening of natural products from higher plants constitutes one avenue in the search for new lead antitubercular agents. **Maria del Rayo Camacho-Corona** (Universidad Autónoma de Nuevo León, Mexico) evaluated the antimycobacterial activity of 9 plants used in Mexican traditional medicine. They selected the plants based on an extensive search of Mexican ethnobotanical literature. Some of the plants included *Citrus sinensis, Citrus aurantifolia, Foeniculum vulgare, Larrea tridentata, Musa acuminata* and *Olea europaea*.[141] Extracts from various parts of the plants were prepared by maceration using hexane, chloroform, methanol and water. The plant extracts were tested using the microplate alamar blue assay and tested against *M. tuberculosis* and its MDR isolates. The hexane extract of the fruit peels of *Citrus aurantiifolia* were fractionated by column chromatography and the major compounds were elucidated by NMR spectroscopy and GC-MS. Several compounds such as coumarins, linoleic, palmitic and oleic acids were identified, many of which were volatile and found to inhibit the growth of the pathogen.

Anti-microbial peptides (AMP) are essential part of innate immunity that helps in combating various pathogens. These generally act through disruption of the bacterial cell membranes. In aqueous environments, AMPs exists as single molecules. On contact with membranes they get ordered and form pores in the membranes. Their action is rapid and effective even against highly drug-resistant strains and hence, they have potential to be used as adjunctive treatment. Some of them are currently under clinical development for treating several bacterial infections. However natural AMPs are not good drug candidates due to their large size leading to higher production costs. Drawing inspiration from nature to draw out new AMPs, **Jasmeet S. Khara** (King's College London, UK) adopted rational design to produce peptides with less likelihood to induce resistance and those that demonstrate synergism with standard antibiotics.[142] There are various strategies to modify AMPs such as synthesising peptide conjugates, peptidomimetics and producing hybrid

peptides. They focused on *de novo* design and came up with a primary sequence containing 4 amino acids (xxyy) n where 'x' and 'y' are hydrophobic and cationic residues respectively with 'n' number of repeats. Sequences containing repeating leucine and lysine residues such as $(LLKK)_2$ as the backbone with short amphipathic $\alpha$-helices were found to be anti-mycobacterial. On addition of methionine residues to the $\alpha$-helical part, the hydrophobicity of the peptide increased which made its insertion into the cell membrane more efficient. The peptides exhibited synergism with first line anti-TB drugs and were effective against drug-susceptible and drug-resistant strains of *M. tuberculosis*. The mechanism of action of these peptides was analyzed by scanning electron microscopy and live-cell imaging of treated mycobacteria. In the presence of peptides, the membrane of bacterial cells was more permeable to dye.

## Drug repositioning: A promising alternative strategy for drug discovery?

Repurposing has emerged as an attractive strategy to discover drugs that can be directly added to the arsenal of drugs used to treat TB infections. **Susanne Brighenti** (Karolinska Institute, Sweden) is interested in enhancing the host's innate defense system as an alternative strategy for the treatment of infectious diseases. A combination of vitamin D3 and 4-phenylbutyrate (PBA) exhibit a synergistic effect on the production of LL-37 in different cell types. LL-37 is an AMP which induces autophagy in *M. tuberculosis* infected human macrophages.[143,144] Increased production of LL-37 could potentially enhance killing of both extra-cellular and intra-cellular bacteria as reported earlier. There is no significant clinical or microbiological data of the effects of treatment with these drugs. Therefore, based on a study carried out in Bangladesh on shigellosis, clinical trial studies were designed for execution in Ethiopia and Bangladesh.[145] The serum baseline level of vitamin D3 of the population in Ethiopia was found to be low and supplementation was recommended. The randomized double-blinded clinical trial using daily supplementation of vitamin D and PBA as adjunct treatment for standard chemotherapy was conducted to examine the combined effects on production of LL-37. Both increased production of LL-37 in human macrophages and LL-37 dependent autophagy in infected macrophages. They also observed reduced clinical symptoms with enhanced recovery and rapid sputum culture conversion on adjunct therapy with vitD3 alone or in combination with PBA. Therefore, vitD3 and PBA can counteract downregulation of LL-37 during infection which can have positive impact on reducing antibiotic requirements for cure.[145-147]

Dr Bhakta and his research group reported the common, over-the-counter non-steroidal anti-inflammatory drugs (NSAIDs), ibuprofen and carprofen to be anti-tubercular specific.[131,148,149] They were found to be active against drug resistant strains and stationary phase mycobacteria. The endogenous mechanism of action of these drugs is still under debate, however, transcriptomic analyses reveal that the drugs may have pleiotropic effects on the pathogen. Additionally, the immunomodulatory effects of NSAIDs and the protection they offer from tissue inflammation and damage is the leading argument to include this class of drugs as host-directed adjunct therapies to complement the standard anti-TB treatment in an effort to improve treatment outcomes.

*M. tuberculosis* possess a 2-regulatory system PhoPR, that is important in sensing and responding to the acidic environment found in macrophages and using it to its own advantage. **Robert Abramovitch** (Michigan State University, USA) strived to find a compound that inhibits the regulatory system making the pathogen susceptible to the host's immune responses. Whole-cell phenotypic screens were employed to identify inhibitors of the regulon. High-throughput screens of nearly 220,000 compound libraries identified ethoxzolamide to inhibit the regulon while having no effect on the growth of the pathogen. Ethoxzolamide is a carbonic anhydrase inhibitor which is used to treat glaucoma and duodenal ulcers. Transcriptional studies show ethoxzolamide to down-regulate 45 genes in *M. tuberculosis*. These genes are known to control lipid synthesis, carbon metabolism and virulence. As a main finding of this study, they state that ethoxzolamide does not change *M. tuberculosis*' pH homeostasis but inhibits its pH-dependent adaption and virulence.[150]

**Gerry Davies** (University of Liverpool, UK) revealed that the problem with development of new treatment is the lack of integrated approach in pharmacokinetic and pharmacodynamic analyses of clinical outcomes, together with poorly designed animal studies leading to failures in clinical trials. This is an exciting time for the field clinical development of drugs for TB as several new and repurposed drugs are under trial. Phase II B trials of one of the few promising candidates, the fluoroquinolones gatifloxacin and moxifloxacin, suggested that the drugs were working well and accelerating the rate of culture conversion in early clinical development giving hope for shortening therapy and reducing relapse rates. Phase III trials, where moxifloxacin was incorporated into first line drug regimens as a mean of shortening treatment down to 4 months were carried out ended up to be a disappointment. Dr Davies suggests a seamless

trial design concept with a core outcome set. This would enable outcomes are to be assessed at several intermediate points and the arms that perform poorly, discontinued. Hence resources and patients can be channelled to the arms that perform well at carefully selected junctures to minimise losses of funds and time.[151]

## Conclusion

There is significant evidence to support the fact that developing cheap and reliable diagnostics would be pivotal in the control this disease. Diagnostic tests with low turnaround times will reduce the number of patients lost to treatment; additionally, those that can profile drug susceptibility will improve treatment outcomes. Lateral flow assays and molecular diagnostics offer a lot of hope in this regard. Their initial successes will hopefully garner interest and funding for improving on the respective technologies.

Early and accurate detection of the disease can only be an effective means of control if an arsenal of robust, effective drugs against a spectrum of resistance phenotypes exists. To overcome hurdles of developing novel chemical entities from scratch, researchers are now making informed decisions and focusing their efforts in alternative approaches including strategies such as repositioning drugs, host-directed therapy and improving on previously unsuccessful drug candidates. These strategies have now populated the clinical trials pipeline with more molecules and possibilities than seen since the golden age of antibiotic discovery.

## Abbreviations

| | |
|---|---|
| ACCases | Acyl-CoA carboxylases |
| Ag85 | Antigen 85 |
| AMP | Antimicrobial peptides |
| AMR | Antimicrobial resistance |
| ART | Antiretroviral therapy |
| BCG | Bacillus Calmette-Guerin |
| BTK | Bruton's Tyrosine Kinase |
| cART | combined antiretroviral therapy |
| CFU | Colony forming unit |
| dcw | division cell-wall |
| DRAM1 | Damage related autophagy modulator1 |
| eATP | Extracellular ATP |
| EBUS-TBNA | Endoscopic ultrasound-guided transbronchial needle aspiration |
| ESAT-6 | Early secretory antigen-6 |
| FDA | Food and Drug Administration |
| HCW | Healthcare workers |
| HEWs | Health extension workers |
| Hif | Hypoxia inducible factor |
| HIV/AIDS | Human immunodeficiency/Acquired immune deficiency syndrome |
| HRM | High-resolution melt analyses |
| IFN$\gamma$ | Interferon-$\gamma$ |
| IFITM | Interferon induced transmembrane |
| IGRA | Interferon-$\gamma$, release assays |
| IPT | Isoniazid preventive therapy |
| LAMP | Loop-mediated isothermal amplification |
| LTBI | Latent TB infection |
| MBL | Molecular bacterial loads |
| MDR | Multi-drug resistant |
| MN | Methyl nicotinate |
| MPA | Methyl phenylacetate |
| mycoHIT | mycobacterial homolog investigation tool |
| NA | Nicotinic acid |
| NAATs | Nucleic acid amplification tests |
| NGS | Next generation sequencing |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| PBA | 4-phenylbutyrate |
| PePTID | Precipitation for target identification |
| PG | Peptidoglycan |
| p-MA | Methyl p-anisate |
| RNAP | RNA polymerase |
| Rpfs | Resuscitation promoting factors |
| SNPs | Single nucleotide polymorphisms |
| TA | Toxin-antitoxin |
| TAC | TaqMan array card |
| TB | Tuberculosis |
| TLR | Toll-like receptor |
| TNF$\alpha$ | Tumor necrosis factor-$\alpha$ |
| TraSH | Transposon site hybridization |
| TST | Tuberculin skin test |
| WHO | World Health Organization |
| XDR | Extensively drug-resistant |

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