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An Analytical Study to Understand the Mechanism Behind Derailing The Aspartate Pathway of Mycobacterium Tuberculosis To Eradicate Persistent Infection

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Abstract*: Tuberculosis, the infectious disease caused by Mycobacterium tuberculosis, is a global health problem with approximately two million deaths annually. About one-third of the world population is infected with Mycobacterium tuberculosis, among them 10% are asymptomatic although there is always a risk to develop into active disease, representing an unmanageable pool of tuberculosis. In latent tuberculosis, Mycobacterium tuberculosis is located within the granulomatous lesions in the host body and is resistant to currently available antibacterial drugs. As per the current narration, the treatment regimens for tuberculosis are not efficient to eliminate dormant bacteria in the lungs of patients. This article leads to evaluation of the therapeutic potential of a drug by inhibiting a selected metabolic pathway which is required for elimination of both active and dormant Mtb. The purpose of this review paper is to identify the essential enzymes that act jointly in derailing this metabolic pathway (aspartate), with the goal of developing them into highly active combination therapy against tuberculosis.*

Key Words: *Mycobacterium tuberculosis, Tuberculosis, Aspartate pathway, Metabolomics, Persistent infection,*

1. INTRODUCTION:

Metabolomics is an emerging field of 'omics' research. The metabolome is defined as a set of small molecules such as intermediates, products formed due to metabolism and endogenous metabolites found in an organism. It can provide an instantaneous snapshot of the entire physiology of a living being. With its potential to provide a comprehensive snapshot of the biochemistry of a biological system to be used for life science research in areas such as disease and biomarker discovery. It can be combined with transcriptomics, genomics, and proteomics studies which are known as multi-omics in biological research world.

In 1999, Professor Jeremy Nicholson first proposed the concept of metabolomics. By using the same concept as genomics and proteomics, metabolomics is a way to quantitatively analyze all metabolites in organisms and to find the relative relationship between metabolites and pathological, physiological changes. It is an integral part of systems biology, where most of the research objects are small molecules with a molecular weight of less than 1000. The methods of metabolomics research are advanced analytical techniques combined with expert systems and pattern recognition. The research process includes sample preparation, sample analysis, data analysis and metabolic pathway analysis. Biological samples analyzed mainly include urine, blood, feces, microbial cultures etc. Sample preparation is generally composed of sample extraction, compound pre-treatment and separation. The quality of the obtained sample is critical to the success of the experiment. Analytical tools for metabolomicsresearch include nuclear magnetic resonance spectroscopy (NMR), gas chromatography mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS). The study of metabolome within cells, biofluids, tissues, or organisms enables to identify and quantify all endogenous and exogenous low-molecular-weight (<1 kDa) small metabolites in a biological system in a high-throughput manner. The various applications of metabolomics are found to be in health and disease including metabolic phenotyping, precision metabolomics, single cell, epidemiologic population studies, precision/personalized medicine and metabolome-wide association studies (MWAS). They also have applications in combination with other omics disciplines as biotechnology, bioengineering and integrative omics. Mass spectrometry (MS) based metabolomics provide an approach for encompassing classification or characterization of disease or treatment, associated with molecular patterns generated from metabolites identification of disease-related metabolites in biofluids or tissue.

Microbial metabolism is regulated by a wide range of mechanisms that act on different cellular layers and together control the abundance and activity of enzymes. The reaction rates of metabolic pathways can be determined by both enzyme activity and enzyme abundance. There have been several significant efforts to identify new drug targets in Mtb focussing on the metabolism of essential nutrients. Mtb is a prototroph of an amino acid which has developed a conventional machinery to obtain nutrients from the host cell. It appears to be susceptible to inhibit amino acid biosynthesis. Moreover, these biosynthetic enzymes are absent in mammalian cells including humans. Thereby, making these pathways in attractive drug targetsof Mtb.

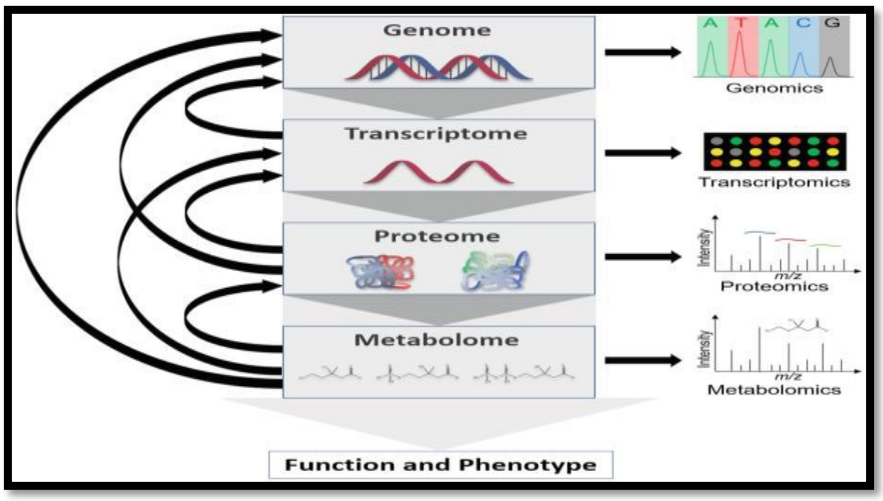

Figure 1: A General Overview of Microbial Metabolomics (Source Methods of Molecular Biology E-book)

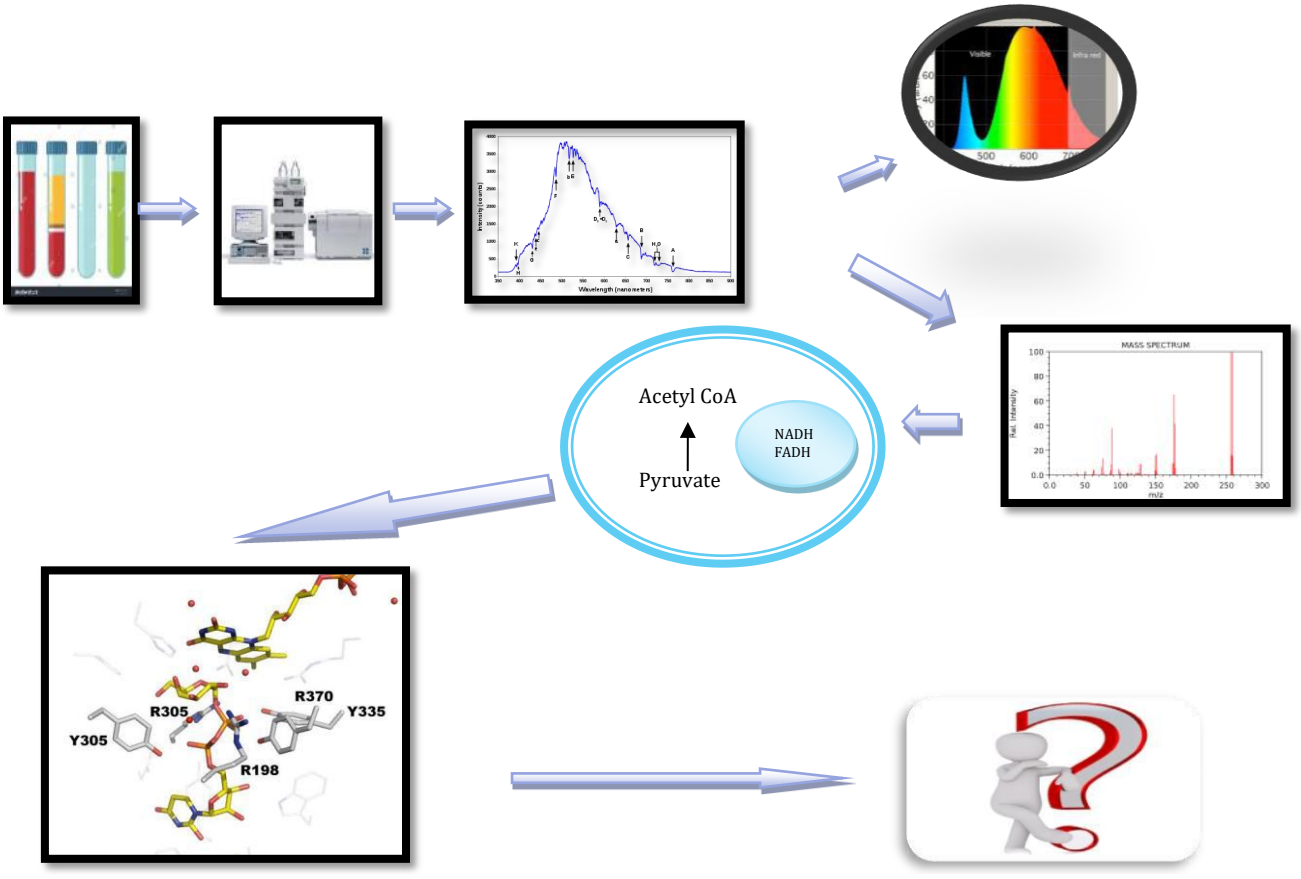

Figure 2: A Flowchart of Metabolomics Analysis Technology

2. TUBERCULOSIS: A POTENTIAL SERIOUS BACTERIAL INFECTION:

Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis, which commonly affects the lungs but also can affect other parts of our body that could define as pulmonary TB and extra-pulmonary TB, respectively. In case of pulmonary tuberculosis, vascular areas such as spine, eye, kidneys, bones, lymph nodes are commonly affected. The World Health Organization (WHO) estimates that one third of word's total population is currently infected with Mtb and approximately 10% of these people are expected to develop active TB at some point in their lifetime. It remains one of the top infectious causes of mortality, with 8.8 million incident cases and 1.4 million deaths worldwide in the year 2015. A new individual is infected every second whilst another one dies from the disease every 15 seconds. Mycobacterium tuberculosis mainly infects the lungs and causes a bad coughing. The clinical manifestations of tuberculosis can vary depending on the stage of tuberculosis, which include latency, primary disease, primary progressive (active) disease, and extra pulmonary disease. It is an obligate aerobe. For this reason Mtb complexes are always found in the well-aerated upper lobes of the lungs in patient with TB. The bacterium is a facultative intracellular parasite, usually of macrophages, and has generation time of 15-20 hours, which is extremely slow compared to other bacteria with division times measured in minutes.

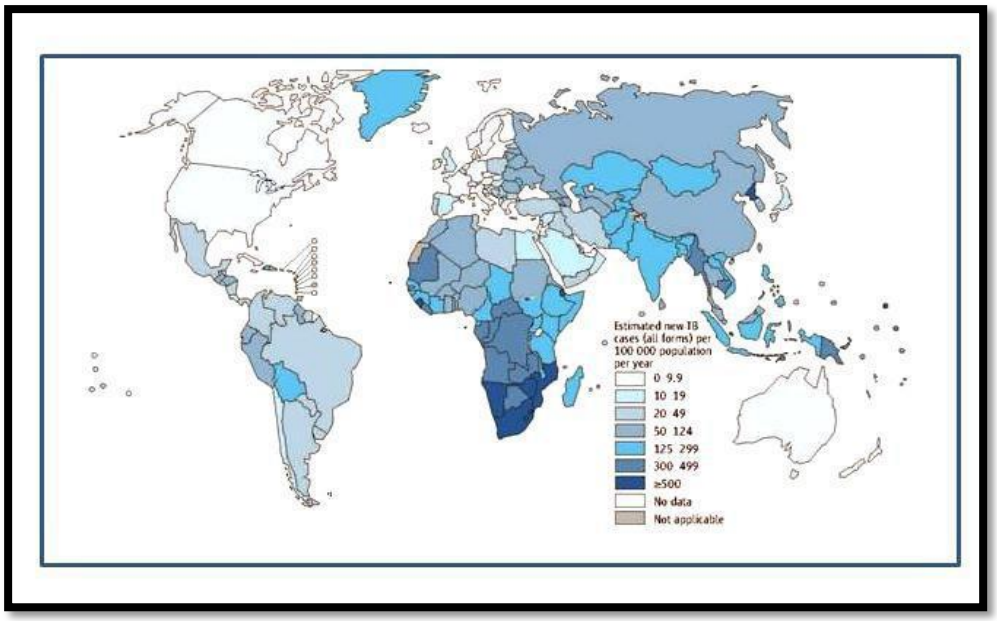

Figure 3: An Estimation of TB Incident Rates Throughout the World, 2014 (WHO, 2015).

3. TREATMENT:

Anti tubercular drugs can be divided into first-line drugs, those that generally have the greatest bactericidal activity when used for TB treatment, the second-line therapeutic-drugs, which are less effective, more expensive and have higher toxicities, and experimental drugs. First-line drugs include isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin. Second-line drugs contain p- aminosalicylicacid, ethionamide, cycloserine, kanamycin, capreomycin, amikacin, ciproflaxin, ofloxacin and viomycin. The current treatment for tuberculosis is a combination of four antibiotics administered over a course of 6 months. Noncompliance or abandonment of treatment is the major impediment to effective therapy due to the long and sometimes unpleasant regimen required to cure tuberculosis.

4. MULTI-DRUG RESISTANT TUBERCULOSIS (MDR-TB):

Multi-drug resistant tuberculosis (MDR-TB) is defined as resistance to the first-line drugs isoniazid and rifampin. The development of drug-resistance is a growing problem for tuberculosis control with the spread of risk factors such as human immunodeficiency virus (HIV), acquired immunodeficiency syndrome (AIDS) and diabetes. This make Mtb a health concern in developed countries and strengthened the urge to develop new treatment infection strategies. Multidrug-resistant TB (MDR-TB) and extensively drug- resistant TB (XDR-TB) are forms of tuberculosis that are even more difficult and expensive to treat because they fail to respond to standard first- and second-line therapy. There are an estimated 290,000 cases of MDR-TB in 2015 making antibiotic resistant tuberculosis a global health concern. Extensively drug resistant TB (XDR-TB) is defined as MDR-TB plus resistance to a fluoroquinolones, and at least one of three injectable second-line drugs. Treating XDR-TB is difficult and requires tailored individual

care. There are many governmental, multi-lateral and also non-governmental organizations dedicated to TB research. Their role is to accelerate progress on access to TB diagnosis and treatment, research and development for new TB diagnostics, drugs and vaccines, and tackling drug resistant- and HIV-associated. WHO carries global leadership on matters critical to TB by monitoring TB situation in the world, including new strategies and standard implementation and measuring progress in TB care and control. WHO shapes the TB research agenda and stimulates the generation, translation and dissemination of valuable knowledge

5. MYCOBACTERIM TUBERCULOSIS: THE CAUSATIVE AGENT: TAXONOMIC CLASSIFICATION

Kingdom	:	Bacteria
Genus	:	*Mycobacterium*
Phylum	:	*Actinobacteria*
Order	:	*Actinomycetales*
Suborder	:	*Corynebacterineae*
Family	:	*Mycobacteriaceae*

MORPHOLOGY:

Mycobacteriumis typically a rod-shaped, non–spore forming, aerobic bacteria, classified as acid-fast bacilli. The dimensions of the bacilli have been reported to be 1-10 µm in length (usually 3-5 µm), and 0.2 -0.6 µm width. Morphological variations can be observed when grown on solid media and some species exist as shorter cocci-bacilli or curved rods on artificial media.It is a facultative intracellular pathogen which multiplies inside macrophages, and resides within a specialized compartment such as phagosome, where there is a limited nutrient source.

MORPHOLOGICAL VARIATIONS

- **Rod, V, Y-shape, branched or buds** (frequently seen at exponential phase of growth)
- **Round, oval, ultra- virus, spore like, and cell wall defiant or L-forms**(occasionallyseen under stress or environmental

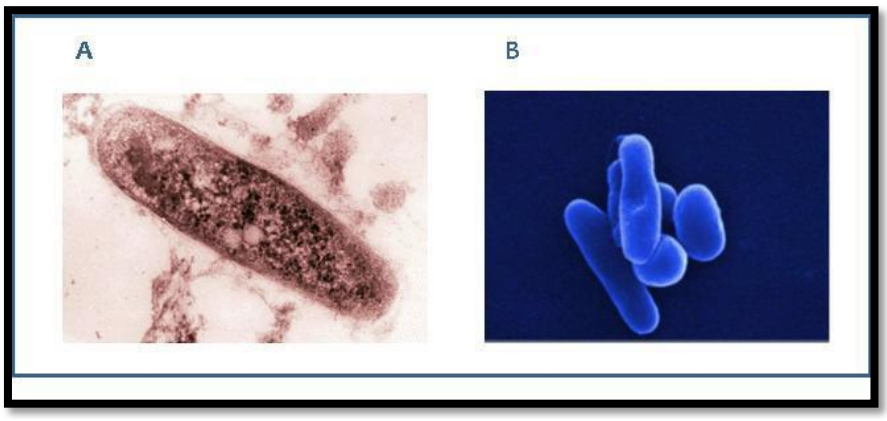

Figure 4: Morphological Variations in *M. tuberculosis*

- Thin section transmission electron micrograph of *Mtb* (extracted from www.wadsworth.org/databank/mycotubr.htm).
- Scanning electron microscope shows shape variation in Mtb at exponential phase of growth (Ali Akbar Velayati & Parissa Farnia, 2012)

CELL WALL:

- The cell wall structure of Mtb, unique among prokaryotes, is associated with the pathogenicity of Mtb. The richness in high molecular weight lipids represents the complexity of the cell wall. Unusual impermeable properties of Mtb cell wall are thought to be advantageous for the bacilli in stressful conditions of osmotic shock and the polymers, covalently linked with peptidoglycan and trehalose dimycolate, provide a thick layer involved in Mtb resistance to antibiotics and the host defense mechanisms.
- The mycobacterial cell wall consists of an inner layer and an outer layer that surround the plasma membrane. The outer compartment consists of both lipids and proteins. The inner compartment is made up of arabinogalactan (AG), mycolic acids (MA) and peptidoglycan (PG) covalently joined together to form an insoluble complex referred to as the essential core of the mycobacterial cell wall. PG, or murein, external to the bacterial cell membrane, is a major determinant of bacteria shape maintenance protecting bacilli from osmotic turgor pressure. The structure of PG is unique to bacteria and thus an excellent target for therapeutics. AG is important for cell wall integrity and for anchoring the impermeable MA layer to the PG layer.
- Another important component of the cell wall is lipoarabinomannan, a major lipoglycan involved in virulence of Mtb and in modulating the host response during infection. Mycolic acids are strong hydrophobic molecules which form a lipid shell around the organism and can influence the permeability properties at the cell surface, have been shown to be critical for the survival of Mtb.

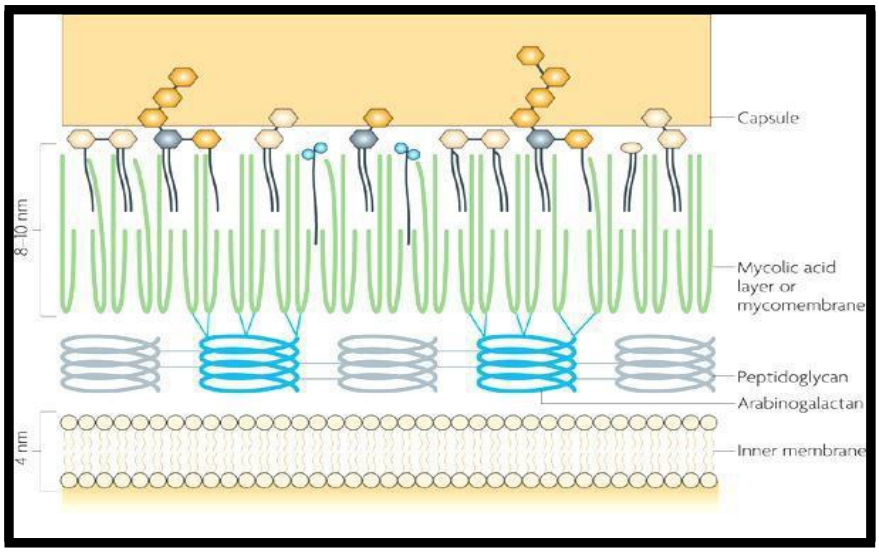

Figure 5: Schematic of the Bacterial Cell Wall (Abdallah et al., 2017)

- The cell wall is a key to the survival of mycobacteria and serves as an understanding of the biosynthetic pathways, gene functions and for the development of antibiotics in order to prevent cell wall formation as areas of great interest.
- Cord Factor, a glycolipid most abundantly produced in virulent strains of Mtb, is responsible for:

  - Inducing animal granulomas similar to those characteristic of TB infection.
  - Increasing cytokine production.
  - Inhibiting the transfer of phagocytosed bacteria to acidic compartments in macrophages.
  - Influencing the morphology of mycobacterial. colonies.

Table 1: Virulence Factors of *Mycobacterium tuberculosis*

6. VIRULENCE FACTORS:

Virulence Factor	Category	Role	Virulence Characterization	Reference
Antigen 85 complex	Exported protein	Fibronectin-binding	Possible impact in walling off bacteria from the immune system and facilitation of tubercle formation	Belisle *et al.*, 1997
HbhA	Adhesin	Heparin-binding hemagglutin	Involvement in binding *Mtb* to epithelial cells	Delogu & Brennan, 1999
ESX-1	Secretion-associated proteins	Virulence proteins delivery during infection	Avoidance of excessive virulence by maintaining ESX-1 activity that leads to long term survival of *Mtb*	Raghavan *et al.*, 2008
WhiB3 protein	Putative transcription regulator	Implication in sensing oxygen tension and redox state	Adaptation of mycobacteria to changes in oxygen tension	Banaiee *et al.*, 2006
Acr1 (hspX)	α-crystalline protein homolog	Dormancy-associated protein	Inhibition of antimicrobial effectors of the macrophage	Yuan *et al.*,2008
19-kDa protein	Lipoprotein antigen	Blockage of IFN-γ signaling through a TLR-2 dependent mechanism	Inhibition of MHC-II antigen processing and presentation in macrophages	Tobian *et al.*, 2003
Sigma factors	Gene expression regulators	Help in regulation of expression of specific genes during stress or morphological development	Adaptation to the changing environment within the host	Browning *et al.*, 2004
STPKs	Serine-threonine protein kinases	Regulation of cell shape; macrophages modulation	Regulation of host-pathogen interactions and developmental changes through signal transduction using reversible phosphorylation of proteins	Av-Gay & Everett, 2000; Leonard *et al.*, 1998

Inter-Relationship of *Mycobacterium tuberculosis* with Host Immune System:

When MTB infects host, it leads to a local inflammatory response which may be condemnatory to the pathological process of TB generation. Once inflammation is induced, it initiates the secretion of pro-inflammatory cytokines, such as: interleukin 1-beta (IL-1β), tumor necrosis factor alpha (TNF-α), gamma interferon (IFN- ϒ) and interleukin-12 (IL-12) etc. A major pro-inflammatory cytokine IL-1 β plays a major role by participating in host defense mechanism against infection by increasing antimicrobial properties of phagocytes and initiating Th1 and Th17 adaptive immune responses. IL-1β is activated by processing upon gathering of the inflammasome, an exclusive inflammatory caspases-activating complex of the proteins. After stabilizing itself, MTB is also responsible for secretion of some anti-inflammatory cytokines such as IL-10 and TGF-β which adds in the survival of bacilli inside host cellular environment. Anti- inflammatory cytokines such as IL-10 and TGF-β are also produced by macrophages during MTB infection which down regulate the pro-inflammatory cytokines and T-cell multiplication and activation. These pro-inflammatory and anti-inflammatory cytokines stabilize the reciprocation between removal and proliferation of bacterium.

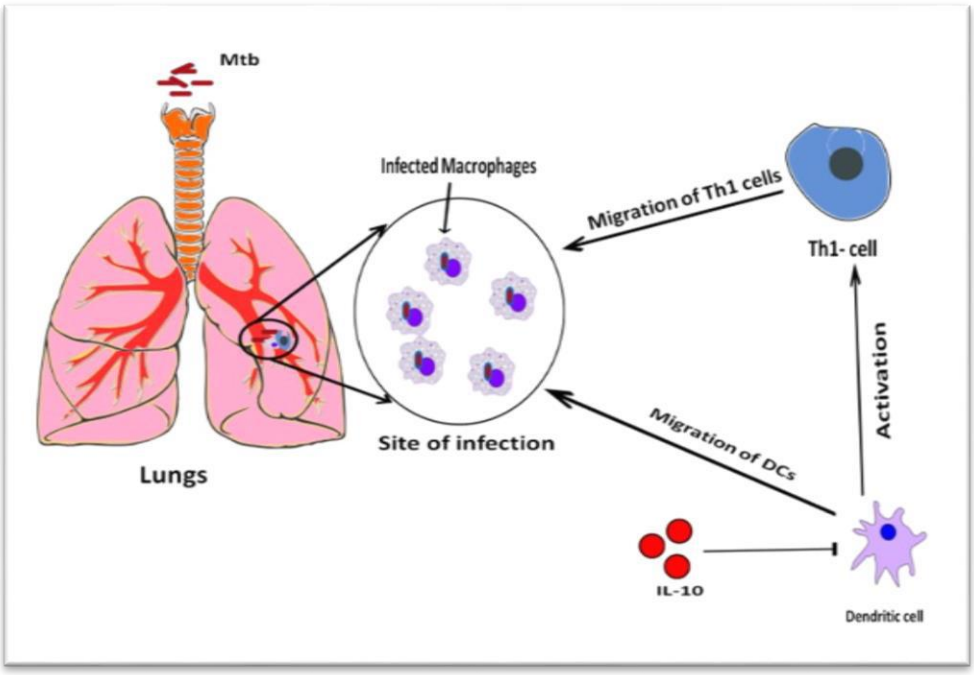

Figure 6: Role of IL-10 in Th 1 and DC migration: Immune system in response to MTB infection, secrete IL-10 as anti-inflammatory response which inhibits macrophage killing and dendritic cells (DC) uptake, processing, presentation, Th1 migration and trafficking of cells from the draining lymph node back to the lungs. IL-10 achieved this effect by inhibiting the dendritic cells (DCs) to further inactivate Th1 cells.

An Unsuccessful Host Immune Response : The Granuloma

Granuloma is an organized structure (size-1-2 mm) formed by highly differentiated immune cells, to provide shelter for bacterium. The central part of the granuloma is enclosed by infected and frothy macrophages, other mononuclear phagocytes and surrounded by lymphocytes. It provides an unsuccessful host immune response which restricts but unable to control MTB infection. Granuloma considered as a classical pathological hallmark of TB and it also provide an immunologic microenvironment in which the bacterium can grow or preserve as latent state therefore attain success to establish a mutual relationship.

Sequential Process of Granuloma Formation

- Antigen presenting cells such as macrophages and dendritic cells (DC) trigger the activation of T-cells
- Macrophages, dendritic cells and activated lymphocytes start to release cytokines and chemokines (small proteins secreted by cells that influence the immune system)
- Secreted cytokines and chemokines stabilize the progressive accumulation of immunocompetent cells (mature B and T cells) and emergence of organized structure known as granuloma;

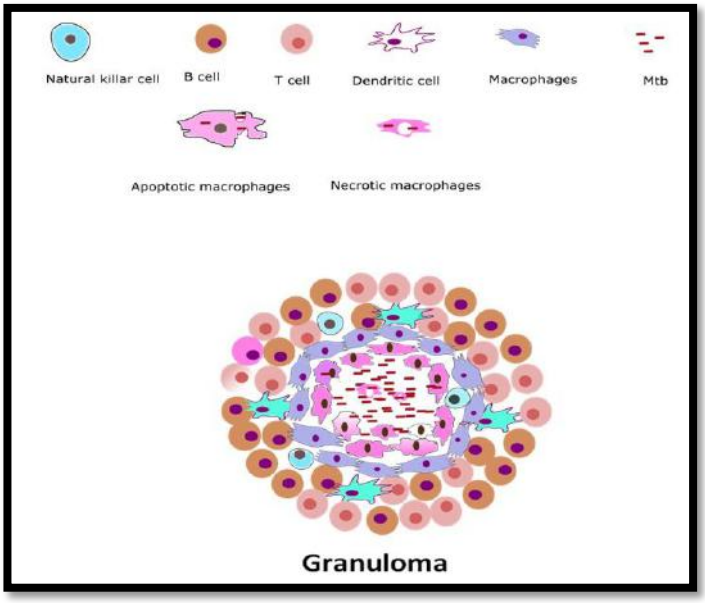

Figure 7: Granulomatous lesion: Granuloma is a self-protective structure that mediates foreign invaders such as fungi or bacteria to keep them away from spreading. On invasion, macrophages, dendritic cells and activated lymphocytes start to release cytokines and these secreted cytokines and chemokines stabilize the progressive accumulation of immunocompetent cells on the site of infection forming granuloma.

7. INHIBITION OF MATURATION OF PHAGOSOME IS A DIVERSION PATHWAY EMPLOYED BY MTB TO SAVE ITSELF FROM HOST IMMUNE SYSTEM

Inhibition of phagosome maturation is a diversion pathway apart from granuloma and inflammatory responses employed by Mtb to save itself from host immune system. Maturation of phagosome is manipulated by hindering phagosome – lysosome fusion which is an important mechanism for survival of Mtb inside host macrophages. Hindering of phagosome-lysosome fusion is mediated by IL-10, is an anti-inflammatory cytokine reaction resulting in the deactivation of macrophages. Apart from anti-inflammatory effect, IL-10 also blocks the migration of T helper type 1 (Th1) cells and dendritic cells to the site of infection that secrete cytokines such as IFN- gamma, IL-2, and TNF-alpha/beta. These cytokines promote macrophage activation, nitric oxide production and cytotoxic T lymphocyte proliferation leading to phagocytosis of pathogens.

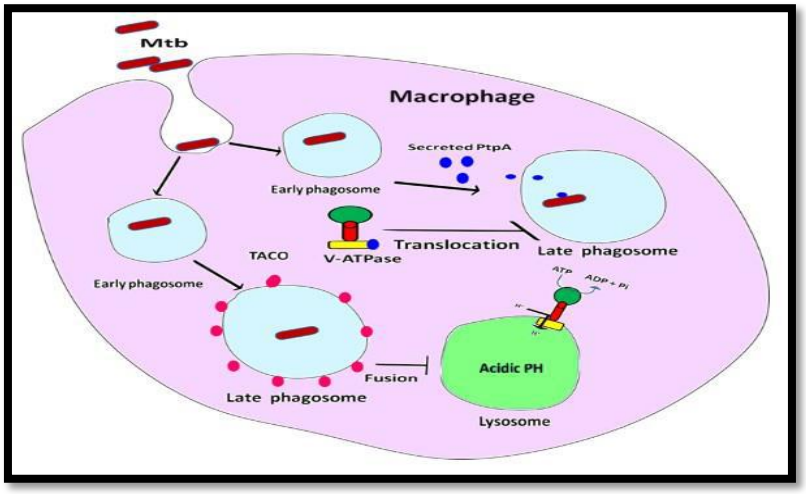

Figure 8: Hampering of phagosome maturation: MTB inside macrophage, inhibits phagosome acidification by secreting PtpA protein in cell cytosol which binds with V-ATPase and inhibits V-ATPase transportation towards phagosome membrane. TACO/ coronin-1 is an actin binding protein which retains on the phagosome membrane and inhibits phagosome–lysosome fusion.

8. NEW DRUG TARGET OF MTB BY INHIBITING THE METABOLIC PATHWAYS

Recently, efforts have been made to target Mtb during persistent infection and also made to focus on inhibiting those metabolic pathways which have been previously ignored. Several studies have demonstrated that central carbon metabolism (CCM) is required during persistence, and several targets have been identified as essential in both acute and chronic infections in mice. Importantly, biosynthetic pathways that utilize TCA cycle intermediates, such as amino acid biosynthesis, are also required for Mtb survival. However, till date none of the amino acid biosynthetic pathways of Mtb have yet shown to be essential during chronic infection and it is merely unknown if such building blocks can be removed from the host cells during persistent infection.

Michael Berney, Albert Einstein College of Medicine, Bronx, NY, USA, and colleagues have shown that a metabolic pathway, such as the aspartate pathway which is not present in animals or humans, is a vulnerable target in the metabolism of Mtb. The utilisation of the amino acid aspartate from the environment and its metabolism are important for the existence of Mtb. Various essential amino acids, such as threonine, are produced via the aspartate pathway, as well as other building blocks such as diaminopimelate, which is necessary for the formation of bacterial cell walls. The researchers have genetically modified Mtb strains in such a way that they are not able to manufacture threonine or homoserine by help of the aspartate pathway. The experiments conducted by them have proved that these genetically modified strains of bacteria are unable to exist without the supply of these compounds. Therefore, derailing the aspartate pathway can be a constructive approach towards the discovery of anti-tuberculosis drugs.

Various methods have been developed to predict the essentialities of these genes and making them suitable for target selection in drug discovery. Among such methods are in-vitro transposons sequencing, selective growth medium enriched with antibiotics, gene expression analysis. These screening methods have limitations as they cannot predict the in-vivo essentiality and cannot differentiate lethal and non-lethal variants. They also cannot predict the slow growth, phenotypes, bacteriostatistics and in formations required for assessing the ability of the drug.

M. tuberculosis is an intracellular pathogen so the essentiality and drug ability of the target must be tested during chronic infections in order to eradicate false targets being taken forward for drug development.

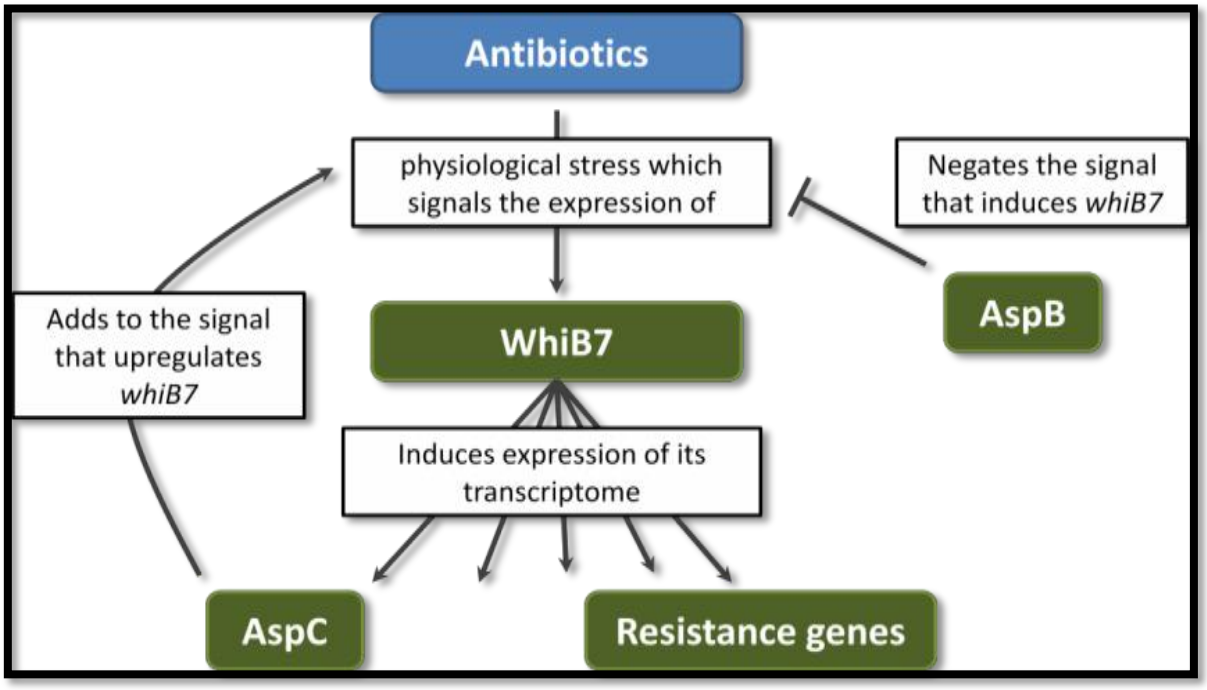

Figure 9: Schematic representation of the proposed antibiotic induced regulatory network of whiB7, aspB, and aspC. Antibiotic treatment results in physiological stress which up regulates expression of whiB7. WhiB7 induces expression of the transcriptome which includes aspC. AspC acts to enhance the whiB7-inducing signals, which further amplifies whiB7 expression. AspB neutralizes/suppresses the signal, so that whiB7 and aspC are not upregulated when aspB is expressed.

9. THE ASPARTATE BIOSYNTHETIC PATHWAY IN MICROORGANISMS

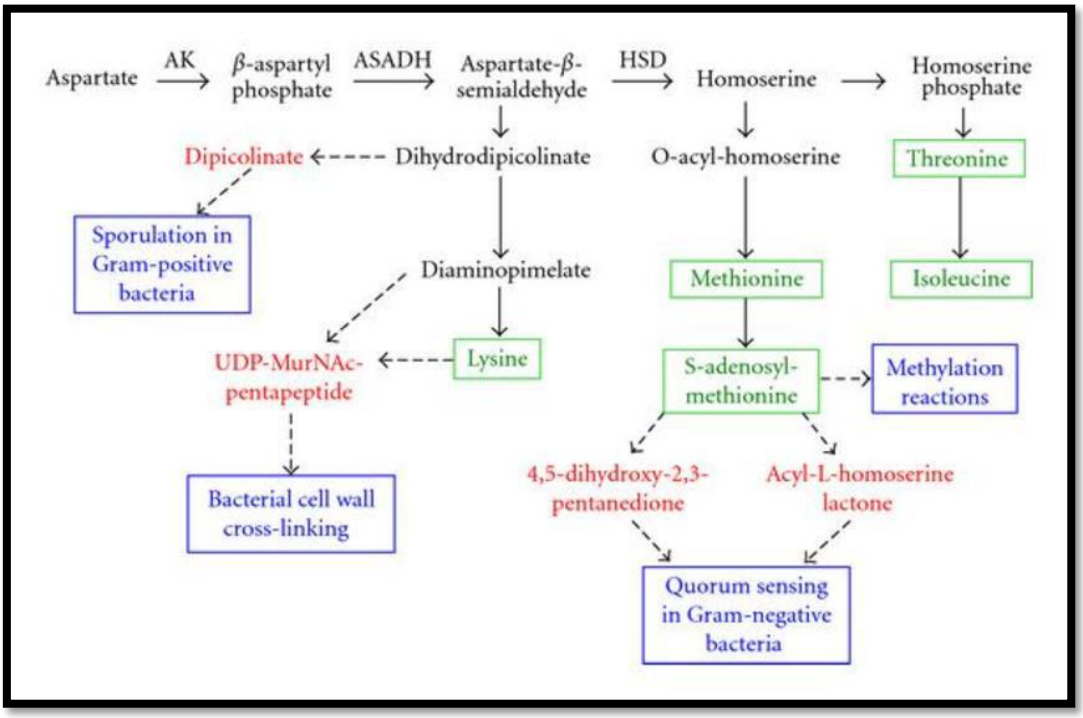

Figure 10: The aspartate biosynthetic pathway in microorganisms.
The end product amino acids produced by this pathway are shown in green.
Pathway-specific metabolites (shown in red) play crucial roles in microbial life cycle functions (shown in blue).

The Role of Aspartate in the Physiology and Virulence of Mycobacterium Tuberculosis

The vital question is about the origin and rise of mechanism of transport which allows mycobacterium to access the aspartate pathway inside host cells. TB lesions which are enriched in aspartate basically relies on global metabolic changes within immune cells during granulation. How nutrients, such as aspartate, access the M. tuberculosis phagosome during infection is an intriguing issue. Using mass spectrometry imaging, it was further showed that aspartate can access the mycobacterial phagosome, at least in vitro. In mammalian cells, aspartate uptake relies on transporters of the solute carriers (SLC) super family, neutral amino acid (SLC1) and cationic amino acid (SLC7) transporter families. Among these transporters, SLC1 have been reported to mediate aspartate transport inside macrophage. Interestingly, we reported the expression of the slc1a2 gene is increased in humanmacrophages upon M. tuberculosis infection. Increased expression of this transporter could impact considerably the ability of M. tuberculosis to multiply inside macrophages. Another issue raised by various review literature survey is: How nitrogen is transferred from aspartate to other nitrogen-containing molecules in M. tuberculosis?

Once acquired by the bacillus through its unique aspartate importer AnsP1, it reflected that this amino acid species serve as provider of nitrogen and hardly enters into carbon metabolism through the Krebs cycle. Assimilation of nitrogen from aspartate relies on transamination steps which allow the shifting of aspartate-derived nitrogen to glutamate, which in turn forms glutamine, and this provides nitrogen to most of biosynthesis pathways. In M. tuberculosis, two aspartate transaminase enzymes called AspB and AspC are predicted to mediate nitrogen transfer from aspartate to glutamate. Interestingly, the aspC gene is thought to be essential in M. tuberculosis, which may indicate that Asp C is involved mostly in aspartate biosynthesis, rather than in aspartate catabolism and glutamate synthesis. Genetic inactivation of aspC may thus result in aspartate auxotrophy. The aspB gene, on the opposite, is not essential in vitro. Interestingly, aspB is expressed at higher level in bacteria withstanding conditions mimicking the intracellular environment. AspB might be involved in aspartate-derived nitrogen assimilation during infection, and may be required for M. tuberculosis virulence. It has been noted that AspB and AspC owns two homologous proteins, called Rv0858c and Rv1178, which have been predicted to act as aspartate aminotransferase, and this might provide alternative pathways for aspartate-derived nitrogen assimilation in the TB bacillus.

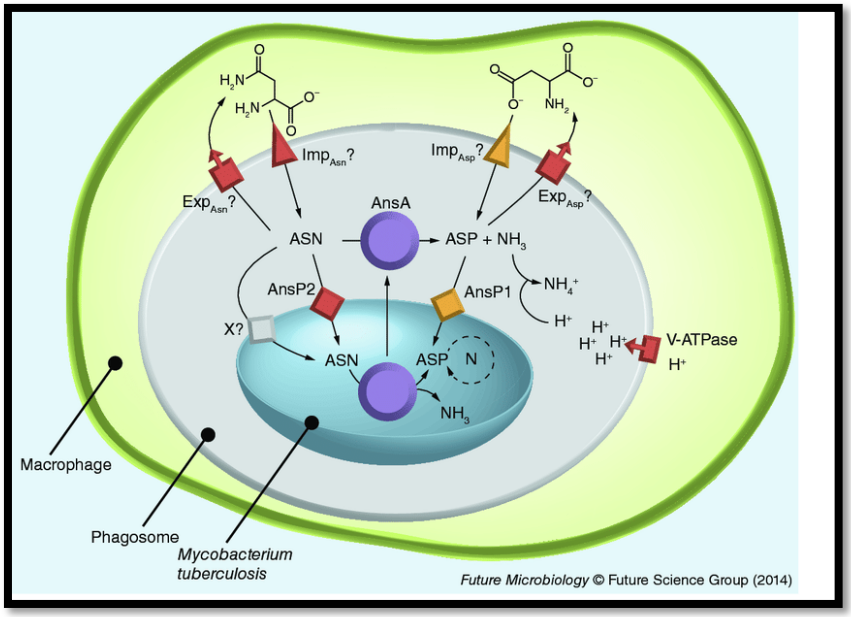

Figure 11: Aspartate metabolism in intracellular *Mycobacterium tuberculosis* (Source : Future Microbiology)

Inside macrophages, aspartate enter the *M. tuberculosis* phagosome through unknown transporters (Imp Asp). Asparagine is captured by *M. tuberculosis* through AnsP2 and one or more other yet to be identified transporter(s) (X), and hydrolyzed by cytosolic AnsA resulting in aspartate production, nitrogen assimilation and release of ammonia. AnsA is secreted in the lumen of the phagosome where it can hydrolyze asparagine. Aspartate is imported by AnsP1 for nitrogen assimilation. In the phagosomal lumen, ammonia reacts with protons transported by the V-ATPase to form ammonium ions allowing phagosomal pH buffering. Aspartate and/or asparagine export systems (Exp Asp and Exp Asn) might constrain intracellular multiplication through amino acid starvation.

10. CONCLUSION:

This review paper focuses on the aspartate pathway because it produces essential building blocks that are vital for cellular functions in *Mtb* such as biosynthesis of cell wall, translation and carbon metabolism. This paper elucidates the study of the auxotrophs threonine, homoserine and methionine which are essential for the inhibition of the pathway. By studying these metabolic responses it has been discovered that *Mtb* employs a combination of feedback inhibition, overflow metabolism and catabolic action to prevent flux imbalances and also to ensure a balance in the production of these precursors. Various studies and literature review reveals that the committed step of the aspartate pathway in Mtb is the action of the enzyme aspartate kinase in controlling the allosteric feedback mechanism of threonine and not by lysine. Universally, Mtb lacks the lysine-AK (aspartate kinase) feedback machinery, so it must employ other regulatory mechanism to control the production of lysine and cell wall building blocks.

11. FUTURE PROSPECTS:

Since the birth of metabolomics, its research has made great progress. As an emerging research technique, it also faces the challenges of methods and applications. Methodologically, the analytical techniques, analytical instruments, data acquisition and data analysis require further improvement. From the application point of view, although a large number of important landmark metabolites related to genetic variation or physiological and pathological changes have been obtained, it is a huge challenge to establish a clinical predictive diagnostic expert system to achieve diagnostic rationalization. A challenge is accompanied by opportunities, so the challenges currently encountered in metabolomics are opportunities for future development. Target selection should focus on genes coding for essential proteins, with emphasis on the first enzymes in critical pathways, which usually are the enzymes most tightly regulated. However, auxiliary or complementary proteins should also be considered. Our opinion is that more work should be done on transporters through the *Mtb* membrane. Inhibitors found or developed in the way that we have adopted now will, after all, face the barrier of the *Mtb* membrane, which is very complex and not permeable to many potential drugs.

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I pay tribute to My Parents for lifting me up till this phase of life. I thank them for their love, trust, patience, support and bearing all kind of stress to make me what I am.

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