3. REVIEW OF LITERATURE

The genus *Mycobacterium* is responsible for more misery and suffering than other known bacteria. As a result of their clinical importance a lot of attention has been focused on them. Mycobacterial diseases, tuberculosis and leprosy, prevail prominently among the tropical diseases prevalent in developing world.

3.1. *Pathogenesis of tuberculosis*

Tuberculosis has been described as an infectious, granulomatous disease caused by *M.tuberculosis*. It has been well established *M.tuberculosis* possesses various unique properties that help in the spreading of disease. The tubercle bacillus has the ability to survive within the infected host by inhibiting phagolysosome biogenesis in macrophages (Glickman and Jacobs Jr 2001, Malik *et al.* 2001) A unique characteristic of *M.tuberculosis* is the ability to exist in a dormant state within infected tissue, where it can persist for several decades (Karakousis, Yoshimatsu, *et al.* 2004, Ehrt and Schnappinger 2009) without onset of disease. This has been in part explained by the presence of the *M.tuberculosis* distinctive complex cell wall, which also plays a role in the survival of the organism, low permeability to hydrophobic antibiotics, resistance to chemical injury, acid-base staining (Bhatt *et al.* 2007) and biofilm formation (Ojha *et al.* 2005) of the bacterium (Jarlier and Nikaido 1994, Karakousis, Bishai, *et al.* 2004).

3.1.1. *Route of Infection*

Source of TB infection is usually an open case of pulmonary tuberculosis. The germs are usually transmitted through the air when TB patients sneeze, cough, speak, and germs spread in the form of tiny droplets. These droplets are about 1.5 microns in diameter. The infection
may also occur less commonly through ingestion e.g. infected milk Oral transmission has also been reported (American Thoracic Society and others 2000).

3.1.2. Site of Infection (Pathogenicity)

In man the lung is the most common site of infection, although other areas like bones, brain, joints, lymph nodes, meninges, peritoneum, genitourinary tract and skin may also get infected. On the basis of site of infection the tuberculosis is classified into two types -

a) Pulmonary tuberculosis – It refers to disease involving lung parenchyma. It constitutes about 85% of the tuberculosis cases.

b) Extra-pulmonary tuberculosis – Tuberculosis of organs other than lung is called extra pulmonary tuberculosis. It accounts for 10-15% of total cases of tuberculosis in India and includes lymph nodes, bones, genitourinary tract, skin and other organs.

(a) Some of the common symptoms of pulmonary tuberculosis:

- Prolonged, productive Cough (< 3 weeks duration).
- Pain in chest
- Loss of weight
- Loss of appetite
- Fever; which may be low grade and usually at night
- Night sweats and chills
- Haemoptysis (blood in sputum)
- Increased fatigue

(b) Symptoms of Extra pulmonary tuberculosis:
• Lymph node tuberculosis-Swelling in the neck

• Tuberculous meningitis-Headache, fever, drowsiness, confusion and neck rigidity.

• Spinal tuberculosis-Back pain, Fever and in some cases swelling of back bone.

### 3.2. Global Picture of Tuberculosis

Tuberculosis remains a major infectious disease and causes high morbidity and mortality worldwide. According to an estimate *M. tuberculosis* infects about 32% of the world’s population (more than 40% in India). Every year, approximately 8 million of these infected people develop active tuberculosis (TB) and almost 2 million of these will die from the disease (WHO 2010, p. 200). TB hinders socioeconomic development: 75% of people with TB are within the economically productive age group of 15-54 years. Ninety-five% of all cases and 99% of deaths occur in developing countries, with the greatest burden in sub-Saharan Africa and South East Asia. Household costs of TB are substantial (Lönnroth *et al.* 2009).

In several countries, TB is on the increase in recent years, largely owing to HIV co-infection, immigration, increased trade, and globalization (Deivanayagam *et al.* 2002). The emergence of AIDS has increased the incidence of infections with other mycobacteria that live in the environment and are resistant to anti-tuberculosis drugs. It accounts for about 13% of AIDS deaths worldwide.

In India, out of a total population of over 1 billion, each year about 2 million develop active disease and up to half a million die (Thorpe 2013). It implies that every minute, a death occurs due to tuberculosis in our country. It also imposes a cost on our economy in terms of current and future output losses because of premature deaths and ill health. To add to the existing burden, the situation is compounded by the large scale increase of new TB cases.
associated with increasing HIV infection (Chauhan 2008). India is estimated to have 3.5 million HIV patients and about 1.8 million of these are co-infected with TB (Thorpe 2013).

### 3.2.1. History of antituberculosis treatment

Streptomycin, the first effective antituberculosis drug, was introduced into experimental clinical use in 1945 (Hinshaw et al. 1946, Heifets 1991). Soon thereafter it was observed that although there was striking initial improvement in patients who received streptomycin, their condition subsequently worsened, and the organisms isolated from these patients were found to be resistant to streptomycin (Crofton and Mitchison 1948, Mitchison 1985). The findings of clinical failure caused by drug-resistant organisms identified the major bacteriologic principle on which successful chemotherapy for tuberculosis depends: wild strain populations of *Mycobacterium tuberculosis* are not uniform in their susceptibility to antimycobacterial agents, thus, it is always necessary to treat with more than one drug to which the organisms are susceptible. The effectiveness of multiple-drug chemotherapy was first demonstrated in a British Medical Research Council study in which streptomycin was given in combination with *p*-aminosalicylic acid (PAS) (Tripathy et al. 1969, Fox et al. 1999). Antituberculosis chemotherapy that was both effective and well tolerated became a reality in 1952 with the introduction of Isoniazid; an effective, well-tolerated, and cheap drug (Joiner et al. 1952, Offe 1988). Again, however, it was found that single drug treatment with isoniazid was inadequate and that resistance developed quickly. Thus, the combination of isoniazid and PAS with or without streptomycin came to be the standard therapy for tuberculosis (Tucker and Livings 1955).

In 1967 the effectiveness of ethambutol as a substitute for PAS was documented (Bobrowitz and Robins 1967). Ethambutol was found to be much more tolerable and less toxic as a companion drug for isoniazid than PAS. Subsequently, it was demonstrated that the
combination of isoniazid and rifampin (introduced in the early 1970s), generally with ethambutol or streptomycin, could shorten the necessary duration of treatment from the standard 18–24 months to 6–9 months (Somoskovi et al. 2001). Investigators then began to focus on the differential effects of antituberculosis drugs and especially on the potential role of pyrazinamide (Timmins and Deretic 2006, Vilchèze et al. 2006). Dickinson and coworkers (Trnka et al. 1988) demonstrated that streptomycin, rifampin, and isoniazid are quickly bactericidal for rapidly growing *M. tuberculosis* in vitro. The *in vitro* conditions could be likened to the *in vivo* conditions under which the extracellular organisms in tuberculous lesions are living. Although both rifampin and isoniazid are rapidly bactericidal, Mitchison and Dickinson (Awaness and Mitchison 1973) demonstrated that rifampin is more effective in killing organisms that grow in spurts rather than continuously. Although both isoniazid and rifampin are effective in killing intracellular organisms, pyrazinamide is especially effective in this regard, suggesting that the addition of pyrazinamide would strengthen the isoniazid–rifampin combination. Two studies have substantiated that the addition of pyrazinamide for two months to a regimen of isoniazid and rifampin improves the effectiveness of a six-month regimen (Peloquin, Namdar, *et al.* 1999, Peloquin *et al.* 2008). Thus, a core regimen of isoniazid and rifampin, supplemented by pyrazinamide and ethambutol for the initial two months, is now recommended as standard treatment for both pulmonary and extrapulmonary tuberculosis (Heifets 1991, McEvoy 2006, Peloquin *et al.* 2008).

**3.2.2. Control measures against tuberculosis**

Prompt, accurate diagnosis and effective treatment for tuberculosis are the key elements in the public health response to tuberculosis and are the cornerstones of tuberculosis control (Peloquin 2002). Effective treatment not only restores the health of the individual with the disease but, also, quickly renders the patient noninfectious and no longer a threat to the community. Thus, all programmes that undertake treatment of patients with tuberculosis must
recognize that not only an individual is being treated but they are assuming an important public health function that also entails a high level of responsibility to the community as well as to the individual patient (Peloquin 2001).

### 3.3. RNTCP (Revised National Tuberculosis Control Programme)

To control the menace of TB, Government of India launched the Revised National Tuberculosis Control Programme (RNTCP), which is achieving remarkable success. National tuberculosis programme (NTP) has been in operation since 1962. RNTCP strategy was implemented in phased manner. RNTCP recommended DOTS and it was implemented in 1993, which had proven to be effective in controlling TB. In 1998 only 2% of total population was under RNTCP coverage, RNTCP covered complete country in 2006 (Park, 2013a).

Under RNTCP, standardized treatment regimens are prescribed for different treatment categories. The RNTCP was initially implemented in a population of 2.35 million in 5 pilot sites in diverse settings to document effectiveness and feasibility of scaling up. Following on from the success at these pilot sites, the programme was expanded to a population of 13.85 million in 1995 and 20 million in 1996, full coverage of the population was targeted in the 2005 (Jawahar 1999). RNTCP consist following components:

- Diagnosis of pulmonary TB by sputum microscopy.
- Regular drug supply to be ensured
- Treatment to be given under direct observation, at least during the intensive phase. Treatment is given thrice –weekly both during the intensive phase and the continuation phase (Chauhan 2008).
3.3.1.1. Objectives of RNTCP

- To reduce mortality and morbidity from TB.
- To interrupt the chain of transmission of infection.
- To cure at least 85% of all newly detected cases of pulmonary TB with supervised short course chemotherapy.
- To detect at least 70% of the estimated incident cases of smear positive pulmonary tuberculosis cases.

3.3.1.1. Achievements of RNTCP

The treatment rate is 88% in 2010 as compared to 25% in 1998. Death rate is 4% which is seven folds less than previous 29%. More than 15 million patients have been initiated in the treatment (Park, 2013b).

3.3.2. Chemotherapy

The factors affecting response to chemotherapy are:

- The tubercle bacillus and its metabolic activity.
- The size of population in the lesion.
- The pharmacodynamics-pharmacokinetics of the anti-tubercular agent.
- The location of the disease.
- The compliance of the patient.

Chemotherapeutic agents used in the treatment of tuberculosis should satisfy the following goals:

- Early bactericidal activity
Drugs can be grouped based on the spectrum of their activity:

- **Broad spectrum agents**
  - Ansamycin: Rifampicin, Rifabutin, Rifapentine
  - Aminoglycosides: Streptomycin, Kanamycin, Amikacin
  - Macrolides: Roxithromycin, Clarithromycin
  - Fluoroquinolones: Ciprofloxacin, Ofloxacin, Moxifloxacin, Levofloxacin, Gatifloxacin and Sparfloxacin

- **Narrow spectrum agents**
  - Capreomycin, Dapsone, Ethambutol, Ethionamide, Isoniazid, PAS, Pyrazinamide and Thioacetazone.

### 3.3.4 DOTS (Directly Observed Treatment, Short Course)

For eradication of TB from the world, currently the WHO recommends that the most effective way to combat tuberculosis is by further implementing the Direct Observed Therapy Short Course (DOTS). This strategy works by ensuring that patients are diagnosed and monitored during treatments, which stops TB at the source preventing the spread of the bacteria (Raviglione and Uplekar 2006). DOTS is an inexpensive strategy that could prevent millions of TB cases and deaths over coming decades. By guaranteeing that treatment
regimes are completed, DOTS prevents the further spread of infection, the development of MDR-TB.

3.3.5. Obstacles to Overcome for DOTS to Expand

The main obstacles to overcome for DOTS to expand are:

- Shortages of trained staff.
- Lack of political commitment.
- Weak laboratory services
- Lack of management of drug resistant TB.

To address these challenges, tuberculosis needs to be a high priority with national policy makers and governments. Practices that would employ DOTS expansion:

- Providing financial support for DOTS implementation
- To increase the manufacturing of drug.
- Supporting an increase of manpower to supervise and sustain DOTS.
- Enhancing inter country collaboration to maximize benefits from private donors and grants.
- Promoting operational research to continually advance strategies.

Implementation of the DOTS program can save millions of lives over the next 20 years in an extremely cost effective manner (Raviglione and Uplekar 2006). This target has already been achieved as 100% of Indian population was covered under DOTS on 24\textsuperscript{th} March 2006 under revised national tuberculosis control programme (RNTCP) (Chauhan 2008).
3.3.6. Drug Resistance / Drug Susceptibility Testing (DST)

Effective management of TB relies on accurate diagnosis. Culture and drug sensitivity testing (DST) on *M. tuberculosis* is an expensive and slow diagnostic technique, which costs about ~10.62 USD (~600 INR) per sensitivity test and takes on average 41 days to establish the resistance phenotype. The period of time taken to determine drug susceptibility by initially growing the specimen on culture (3 weeks) as well as the ensuing DST (3 to 6 weeks), is approximately 6 – 9 weeks. These drawn out procedures can contribute to the transmission of MDR-TB (Victor *et al.* 2002) since initiating treatment during this crucial period is delayed. This is especially true in lower income countries where DST facilities are not part of routine diagnostic procedures and where current drug-sensitive strains may develop resistance over time (Van Rie *et al.* 2000, Victor *et al.* 2002). Identification of MDR strains can be established by means of DST. Thus, after an initial positive culture is obtained, DST is done on a subsequent subculture. DST is done by conventional methods (absolute concentration method, the resistance ratio (RR) method and the proportional method) or by means of sensitive methods such as the BACTEC 3D method. The proportion method is frequently done to determine drug susceptibility of *M. tuberculosis*. The results obtained from this method are reported as the percentage of the total bacterial population resistant to a specific drug, which is defined as the amount of growth on a drug-containing medium compared with growth on a drug-free control medium (Chauhan 2008).

3.4. Targets and Mode of Action of Current TB Drugs

Current chemotherapy for TB largely relies on drugs that inhibit bacterial metabolism with a heavy emphasis on inhibitors of the cell wall synthesis (Zhang 2005). According to their mode of action, first and second line TB drugs can be grouped as cell wall inhibitors (isoniazid, ethambutol, ethionammide and cycloserine), nucleic acid synthesis inhibitors
(rifampicin, quinolones), protein synthesis inhibitors (streptomycin, kanamycin) and inhibitors of membrane energy metabolism (pyrazinamide). The current TB drugs, on the basis of mode of action, can be divided into two categories: bacteriostatic (inhibit the growth of bacteria) and bactericidal (kill the susceptible bacteria) drugs. The static drugs include EMB and PAS, whereas the cidal drugs include INH, RIF, SM and FQ (fluoroquinolones). However, the distinction between static and cidal drugs is only relative, because some static drugs can be cidal under some conditions (such as with higher drug concentrations, smaller inoculum, or change in bacterial physiological status) (Zhang 2005).
Table 3.1. Targets and known mechanisms of action of current TB drugs

<table>
<thead>
<tr>
<th>Drug (year of discovery)</th>
<th>MIC (µg/ml)</th>
<th>Effect on bacterial cell</th>
<th>Mechanisms of action</th>
<th>Important Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (1992)</td>
<td>0.01-0.2</td>
<td>Bactericidal</td>
<td>Inhibition of cell wall mycolic acid and other multiple effects on DNA, lipids, carbohydrates and NAD metabolism</td>
<td>Primarily acyl carrier protein reductase (InhA)</td>
</tr>
<tr>
<td>Rifampicin (1966)</td>
<td>0.05-0.5</td>
<td>Bactericidal</td>
<td>Inhibition of RNA synthesis</td>
<td>RNA polymerase β subunit</td>
</tr>
<tr>
<td>Pyrazinamide (1952)</td>
<td>20-100 Ph 5.5 or 6.0</td>
<td>Bactericidal</td>
<td>Disruption of membrane transport and energy depletion</td>
<td>Membrane energy metabolism</td>
</tr>
<tr>
<td>Ethambutol (1961)</td>
<td>1-5</td>
<td>Bacteriostatic/Bactericidal</td>
<td>Inhibition of cell wall arabinogalactan synthesis</td>
<td>Arabinosyl transferase</td>
</tr>
<tr>
<td>Streptomycin (1944)</td>
<td>2-8</td>
<td>Bacteriostatic</td>
<td>Inhibition of protein synthesis</td>
<td>Ribosomal S 12 protein and 16S rRNA</td>
</tr>
<tr>
<td>Kanamycin (1957)</td>
<td>1-8</td>
<td>Bactericidal</td>
<td>Inhibition of protein synthesis</td>
<td>Ribosomal S 12 protein and 16S rRNA</td>
</tr>
<tr>
<td>Quinolones (1965)</td>
<td>0.2-4</td>
<td>Bactericidal</td>
<td>Inhibition of DNA replication and transcription</td>
<td>DNA gyrase</td>
</tr>
<tr>
<td>Ethionamide (1956)</td>
<td>0.6-2.5</td>
<td>Bacteriostatic</td>
<td>Inhibition of mycolic acid synthesis</td>
<td>Acyl carrier protein reductase (InhA)</td>
</tr>
<tr>
<td>PAS (1946)</td>
<td>1-8</td>
<td>Bacteriostatic</td>
<td>Inhibition of folic acid and iron metabolism?</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cycloserine (1952)</td>
<td>5-20</td>
<td>Bacteriostatic</td>
<td>Inhibition of peptidoglycan synthesis</td>
<td>D-alanine racemes</td>
</tr>
<tr>
<td>Polypeptides Capreomycin</td>
<td>10</td>
<td>Bactericidal</td>
<td>Inhibit prokaryotic protein synthesis</td>
<td>16SrRNA</td>
</tr>
</tbody>
</table>

The varying types of lesions determine different metabolic status of tubercle bacilli in vivo and are the basis for diverse bacterial population. According to Mitchison (Mitchison 1985), tubercle bacilli in lesions consist of at least four different populations:

1. Bacteria that are actively growing killed primarily by isoniazid (INH).
2. Bacteria that have spurts of metabolism, killed by rifampicin (RIF).
3. Bacteria that are characterized by low metabolic activity and reside in acid pH environment, killed by pyrazinamide (PZA).
4. Bacteria that are “dormant” or “persisters”, not killed by any current TB drug.

3.4.1 Isoniazid mode of action

INH was discovered in 1952, and still remains one of the forefront chemotherapeutic agents to fight TB (Middlebrook 1954). This is mainly due to its high activity against dividing M.tuberculosis (Zhang et al. 1992). Isoniazid (INH) used for treatment of TB, as it is used for treatment of latent M.tuberculosis infection and prevention of active disease and subsequent transmission (Dooley and Sterling 2005, Vilchèze et al. 2006). INH enters the mycobacterial cells by means of passive diffusion across the mycobacterial cell envelope (Whitney and Wainberg 2002), a superpolymer of covalently attached subunits (Slayden and Barry III 2000). These covalent structures terminate in a lipophilic layer of extraordinarily long-chain α- branched β-hydroxylated fatty acids, which are called the mycolic acids. The biosynthesis of mycolic acids is essential for cell structure and once the cellular integrity is disrupted, the bacterium dies (Barry III et al. 1998, Slayden and Barry III 2000). INH enters the tubercle bacillus by passive diffusion and primarily targets mycolic acid biosynthesis (Bardou et al. 1998, Mdluli, Slayden, et al. 1998, Paramasivan et al. 2005). It is then activated by catalase peroxidase encoded by katG gene to produce acyl radical-INH-NAD adduct (Heym et al. 1993). This in turn blocks the InhA (NADH-dependent enoyl-[acyl-carrier-protein) reductase located in FAS-II complex (Rawat et al. 2003),
resulting in the accumulation of saturated fatty acids and disruption of lipophilic layered cell wall which leads to killing of *M. tuberculosis* (Vilchèze *et al.* 2000).

**3.5 History of Drug Resistance**

STR, an aminoglycoside, was administered for the first time in 1944 (Pfuetze *et al.* 1955), but as with most bacterial infections, resistance against STR soon occurred and the administration of two or more drugs proved to be more effective. An even greater advance against TB came when INH (isonicotinyl hydrazine) was introduced. It was first synthesised in 1912 but its therapeutic effect against tuberculosis in humans was only detected in 1952 (Robitzek *et al.* 1952). These discoveries started the era of modern chemotherapy. Within approximately ten years after the introduction of INH, cycloserine (CS), PZA, EMB, and RIF were introduced as anti-TB drugs. INH remained the most important single agent for the treatment of tuberculosis (Patil *et al.* 1991, Hoffman *et al.* 1996). Globally, TB control efforts are seriously threatened by the increasing rate of drug resistance (Pablos-Méndez *et al.* 1998, Espinal *et al.* 2001, Sharma *et al.* 2003). The emergence of drug-resistant strains of *M. tuberculosis*, especially MDR strains, defined as resistant to at least INH and RIF, poses a threat to the success of TB control programmes. Drug surveillance studies by the WHO in 64 countries indicated that 3.4% of TB cases are MDR and that there were an estimated 237 000 new cases of MDR-TB world-wide in the year 2000 (Dye *et al.* 2002). Acquired drug resistant strains arise when treatment is intermittent or otherwise inadequate and primary resistance occurs when a patient is infected with a resistant strain. Furthermore, the rising HIV epidemic in many countries, especially in sub-Saharan Africa where the prevalence of TB infection is high, further complicates the control of drug susceptible and resistant TB (Harries *et al.* 2001). Approximately 98% of all TB deaths occur in developing countries where there are limited resources and surveillance for drug resistance is irregular (Raviglione *et al.* 1995). Several factors may influence the measure of success of treatment programs. The
main factors responsible for the increase in the global TB burden are poverty, poor programme management (inadequate case detection, diagnosis or cure), and population increase (Small and Katoch 2012). Outbreaks of MDR-TB in institutions such as hospitals (Edlin et al. 1992, Fischl et al. 1992, Coronado et al. 1993, Bifani et al. 1996) and prisons (Casal et al. 2005), and among health care workers (Beck-Sagué et al. 1992) (Beck-Sagué et al. 1992, Pearson et al. 1992, Jereb et al. 1995) in the developed world have focused attention on MDR-TB as a major health issue.

3.6. Drug Resistance in M. tuberculosis

The drug resistance may be defined as the response of the patient to chemotherapy with the drug concerned. As it is difficult to establish a precise clinical definition of resistance, a definition in purely bacteriological terms has been adopted for the purposes of these genera. Accordingly, Mitchison in 1962 proposed the following statement that could be adequate (Canetti et al. 1969): “Resistance is defined as a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with the drug”. The use of inadequate regimens and the absence, or inappropriate application, of directly observed treatment can lead to the development of drug resistance and potentially to an increase in drug resistance levels amongst the community (Singh and Saha 2007).

3.6.1. Types of Drug Resistance

The various definitions used in drug resistance are as follows:

I. Primary drug resistance
Primary resistance is defined as the resistance in tuberculosis patients who have not been exposed to anti-tuberculosis drug. It is caused by infection with organism from other patients having drug resistant organisms.

II. Acquired drug resistance

Secondary or acquired resistance is defined as resistance that arises during or after course of treatment due to non-adherence to the recommended drug regimen or wrong prescription.

III. Transient or transitional drug resistance

It occurs in patients, who respond to therapy just before sputum conversion, and usually occurs in the form of a few colonies, and doesn’t warrant change of treatment.

IV. Multi Drug Resistance (MDR)

Multidrug resistant tuberculosis strains are generally considered to be those that are resistant to at least isoniazid and rifampicin. These strains have been described worldwide. The frequency of acquired resistance is more common than primary resistance in MDR-TB.

V. Extensively Drug Resistant TB: XDR-TB or extremely drug resistant TB is a variety of MDR-TB (rifampicin and isoniazid) in which the isolate is also resistant to three or more of the six classes of second-line drugs (a fluoroquinolones and an injectible drug i.e. kanamycin, amikacin or capreomycin). XDR TB was first described in early 2006, following a joint survey by the US Centre for disease control and prevention (CDC), WHO and the Supranational Reference Laboratory Network (SRLN) as agreed upon by the WHO Global Task Force on XDR-TB (Singh 2007).

VI. Combined drug resistance
It is the prevalence of drug resistance among all the patients with TB (regardless of treatment history) in a given year and country.

### 3.6.2. The Recent Global Drug Resistance Scenario

The 4th report of global project (WHO 2008) on anti-tuberculosis drug resistance surveillance revealed that the proportion of resistance in new cases to at least one anti tuberculosis drug (any resistance) ranged from 0% to 56.3%. In India MDR TB among new cases were 2.8% in 2008 and 2.2% (1.9-2.6%) in 2012, among previously treated cases 17.7% in 2008 and 15% (11-19%) in 2012. The highest proportions of MDR were reported 0-62.5% among previously treated cases in 2008 in India.

In Rwanda (landlocked country in East Africa), mono-resistance was observed in 5.5% cases; non-MDR polyresistance in 1.1% and 3.9% MDR cases was reported in new cases, among the previously treated cases, 22.4% showed resistance; with 11.85% mono resistance cases and 9.4 % MDR cases in previously treated cases (Umubyeyi et al. 2007). According to Global Project, the proportion of resistance in various cases is as follows:

A. Among new cases are: any resistance 17.0%, isoniazid resistance 10.3%

B. Total MDR cases reported 2.9%. Global population of resistance among previously treated cases: any resistance 35.0%, isoniazid resistance 27.7%, MDR 15.3%.

C. Global population of resistance among all TB cases: any resistance 20.0%, isoniazid resistance 13.3%, and MDR 5.3%.

D. In India, Estimates of MDR-TB among all TB cases are 4.9%. Global report estimated by epidemiological region that the total percentage of MDR-TB cases estimated to have occurred in 2006 among newly diagnosed TB cases was 3.1%.

E. The MDR-TB among previously treated cases was estimated to be 19.3% in 2006 in the 175 countries.
The global estimated percentage of incident MDR-TB cases in 2006 is 4.8% in 2006. Two high TB burden countries, China and India are estimated to carry 50% of the global burden of cases (25% in India alone), and the Russian Federation is estimated to carry a further 7% (WHO 2008). The WHO report 4 (2008) estimate that the high proportion of XDR-TB among MDR-TB was ranging from 0% to over 30.9% as well as the large underlying burden of MDR-TB suggests a significant problem within the countries of available in most of the countries of the former. The proportion of XDR-TB among MDR-TB was highest in Japan, 30.9%, and Hong Kong, 14.6%, respectively (WHO 2008).

There have been some well conducted surveys, the prevalence of resistance in new TB cases to at least one drug (any resistance) from 55,779 patients ranged from 0 % in some Western European countries to 57.1 % in Kazakhstan (median = 10.2 %). Median prevalence of resistance to individual drugs was: streptomycin (SM), 6.3 %; INH, 5.9 %; RIF, 1.4 %; and ethambutol (EMB), 0.8 %. Prevalence of MDR-TB ranged from 0 % in eight countries to 14.2 % in Kazakhstan and Israel (median = 1.1 %) (WHO 2008).

Among previously treated cases with data available from 66 settings (8,405 patients) the median prevalence of resistance to at least one drug (any resistance) was 18.4 %, with the highest prevalence being 82.1 % in Kazakhstan. Median prevalence of resistance to individual drugs was: INH, 14.4 %; SM, 11.4 %; RIF, 8.7 %; and EMB, 3.5 %. The median prevalence of MDR-TB was 7.0 %. (Martin et al. 2007, WHO 2008). According to third Global Report of WHO total the prevalence of acquired drug ranged from 25 to 100% in several studies. The acquired multi-drug resistance rates also varied from 6 - 100%. The WHO–IUATLD Global Drug Resistance Surveillance carried out in between 1996-1999 reported the median prevalence of primary and acquired MDR-TB to be 3.4% (1.8-5.7%) and 25% (7.3-52.3%) respectively (Prasad 2005, WHO 2008). Multidrug-resistant *M. tuberculosis* is an emerging problem of great importance to public health, with higher mortality rates than
drug-sensitive TB, particularly in immunocompromised patients. MDR-TB patients require treatment with more toxic second line drugs and remain infectious for longer than patients infected with drug-sensitive strains, incurring higher costs due to prolonged hospitalization. The frequency of resistance to multi drugs varies geographically, and acquired (secondary) resistance is more common than primary resistance. High rates of acquired MDR-TB have been reported in Nepal (48%), India (33.8%) and New York City (30%) in the early 1900s (Pablos-Mendez 2001).

3.6.3. Drug Resistance in India

During the 1980s, the level of MDRTB in initial drug resistance cases was observed to be less than 5%. A study done at New Delhi showed a high level of initial drug resistance to isoniazid (18.5%) and a low level of resistance to rifampicin (Paramasivan and Venkataraman 2004).

The Tuberculosis Research Centre, Chennai, undertook several studies during 1997-99, these studies showed initial resistance to rifampicin to range from 2.5-4.4% while the prevalence of MDR-TB was around 3%. Studies on DRS have also been undertaken by the National Tuberculosis Institute (NTI) in the districts of Mysore (2001), Hoogly (2003), Mayurghanj (2003) and Naogaon (2003). Trivedi and Desai, 1988 showed that in chronically ill, treatment failure or relapsed patients, resistance to rifampicin increased from 2.8% in 1980 to 37.3% in 1986 and to isoniazid from 34.5 to 55.8% (Trivedi and Desai 1988). In this study MDR-TB was 30%. A study from Chennai showed that acquired resistance was 63%, out of which 23.5% was resistance to single drug and 39.5% to more than one drug. Resistance to isoniazid and rifampicin (MDR-TB) was reported in 20.3%. Further, studies from TRC, Chennai during 1997-2000 revealed the incidence of MDR-TB to vary from 25-100%. However, DRS data obtained from Tamil Nadu (1999-2003) revealed the incidence of MDR TB to be 11.8%
and in the HIV, TB study, MDR TB was 5.9%. (2000-2002 year) (Paramasivan and Venkataraman 2004). The prevalence of primary /initial drug resistance in India is 0-3.4% in studies from Gujarat (0% during 1983-89), Bangalore (1.2% during 1985-86), Kolar (3.2% during 1987-89), Jaipur (0.7% during 1988-91), New Delhi (0.6% during 1990-91), Tamil Nadu (3.4% during 1997) Raichur (2.5% during 1999). Slightly higher initial drug resistance has been reported from Wardha (5.3% during 1982-83), North Arcot (4.4% during 1989-98) and Jaipur (4.5% during 1997-99).

In acquired drug resistance, the prevalence of resistance to drugs like INH and STR is quite high and even MDR levels are high (30.2% in Gujarat during 1983-86, 10.9% in Wardha during 1988-89, 17.1% in Raichur during 1988-89 and 33.3% in New Delhi during 1990-91). This might be due to misuse of the anti TB drug like INH and EMB. Very high prevalence of acquired MDR has been observed in some of the studies, some of them based on limited number (less than 20) of isolates (69% in North Arcot during 1999, 78% in Wardha during 2000, 100% in Raichur during 1999). These are very important from clinical point of view for instituting the most appropriate and individualized treatment at the earliest (Singh and Katoch 2006). Indian data from Gujarat State, on previously treated cases shows 17.2% MDR-TB. Data from India show that drug resistance among new cases is relatively low. However, the burden is high, it is estimated that 110,132 MDR-TB cases emerged in India in 2006, representing over 20% of the global burden (WHO Report-4., 2008).

The rate of anti tubercular drug resistance in the study was found in 56.64% resistance to INH plus RIF was found in 65.22% of the total resistant cases and resistance to all four drugs was 25-93% (Shah et al. 2002).

In India, the overall prevalence of secondary drug resistance in cat II cases was observed to be about 18.8% (7.9% -27.1%) and initial multi-drug resistance varied between 0-5% (Prasad 2005, WHO and others 2005). Drug resistance surveillance (DRS) survey in Gujarat
and various districts level DRS showed a prevalence of MDR-TB in new smear positive pulmonary tuberculosis (PTB) cases around ~ 3% and 12 to 17% amongst smear positive previously treated PTB cases (Singh and Saha 2007). A study conducted by Tuberculosis Research Centre, Chennai in collaboration with the National Tuberculosis Institute, Banglore using WHO/IUATLD guidelines in between 1999-2002 including 6 districts in India showed that incidence of primary MDR ranged from 0.7-2.8%. Study conducted in two districts of south India showed that acquired drug resistance ranged from 69 –100% (Prasad 2005, WHO and others 2005). A survey, performed by 14 supra-national laboratories, on drug susceptibility testing results from 48 countries confirmed this. From 19.9 % of identified MDR-TB isolates, 9.9 % met the criteria for XDR-TB (Shah et al. 2002). The team, led by Dr Sushil Jain, examined 3,904 lab samples and found 1,274 were positive for TB. Of these, 32% were multiple drug resistance (MDR-TB), of which 8% were XDR-TB cases. Mortality rate of XDR-TB patients in the study was as high as 42%. What's worse, majority of patients in the study with XDR-TB were from a younger age group, their average age being 30 years (Jain 2007).

3.6.4. Epidemiology of XDR-TB

Global data on XDR-TB are limited; however, an article reported that the problem of XDR TB is worldwide reported from countries i.e. Republic of Korea, South Africa, India etc (Shah et al. 2002, Mondal and Jain 2007). Mondal and Jain (2007) have reported that a total of 5 (7.4%) of 68 MDR TB strains met criteria for XDR TB. The numbers of MDR cases tested for the appropriate second-line anti-TB drugs are used as a denominator. In total, data were reported on MDR-TB cases, and among those 7.0% XDR-TB cases were detected (Shah et al. 2002). The results of the CDC and the World Health Organization survey, to assess the frequency and distribution of XDR-TB, determined that during 2000-2004, of 17,690 TB isolates, 20% were MDR 2% were XDR. XDR -TB has emerged world wide as a threat to
public health and TB control, raising concerns of a future epidemic of virtually untreatable TB. New anti TB drug regimens, better diagnostic tests and international standards for second line drugs (SLD) susceptibility testing are needed for effective detection and treatment of drug resistant TB (Centers for Disease Control et al. 2007).

3.7 Mechanisms of Drug Resistance

Various studies have shown that mycobacteria can acquire drug resistance due to the following mechanisms:

- Mutation in the target site rendering the DNA inefficient to govern the proper drug interaction within the cell.
- Drug efflux system for transporting out the drug.
- Permeability barrier by the cell wall.

3.7.1. Emergence of Drug Resistance

In *M.tuberculosis* spontaneous mutations occur at a frequency of approximately 10^-5 to 10^-8. Since resistance to various drugs arises independently, the likelihood of spontaneous mutation to isoniazid and rifampicin for instance, is 1 in 10^{14}. Hence resistance of *M. tuberculosis* to anti-TB drugs is the result of a spontaneous genetic event and worse, “a man made amplification of the natural phenomenon”. Drug resistance in *M. tuberculosis* occurs by random, single step, spontaneous mutation at low but predictable frequency in large bacterial populations.

It has been estimated that the probability of incidence of drug resistant mutants in a population of *M. tuberculosis* is depicted by the following formula:

\[ P=1-(1-r)^n \]
Where $P$ = probability of incidence of drug resistance cases.

$r$ = probability of incidence of drug resistant mutants.

$n$ = number of bacilli in the lesions.

The value of $r$ for rifampicin is equal to $10^{-8}$, that for isoniazid, streptomycin, kanamycin, ethambutol and para-amino salicylic acid $10^{-6}$.

### 3.7.2. Factors Contributing to the Emergence of Drug Resistance

- Deficiency of adequate tuberculosis control programs.
- Improper prescription of treatment regimens.
- Non-adherence of patient to prescribed drug therapy.
- The epidemic of HIV infection.
- Inadequate training of health care workers regarding epidemiology, treatment and control.
- Laboratory delays in identification and susceptibility testing of *M. tuberculosis*.

### 3.7.3. Mutational changes conferring resistance in *M. tuberculosis*

Resistance associated point mutations, deletions or insertions and their targets implicated in resistance to anti-mycobacterial drugs are summarised in Table 3.2 Cellular targets for anti-mycobacterial drugs and gene mutations responsible for resistance in *M. tuberculosis* (Victor *et al.* 2002, Ramaswamy *et al.* 2003).
<table>
<thead>
<tr>
<th>Drug</th>
<th>Cellular target</th>
<th>Gene locus</th>
<th>Enzyme/functional role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>Cell wall</td>
<td>$katG$</td>
<td>Catalase-peroxidase/activation of prodrug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$inhA$</td>
<td>Enoyl reductase/mycolic acid biosynthesis.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$ahpC$</td>
<td>Alkyl hydro-peroxidase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$kasA$</td>
<td>β-ketoacyl synthase/mycolic acid biosynthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$ndh$</td>
<td>biosynthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$oxyR-ahpC$</td>
<td>NADH dehydrogenase/regulation of NADH/NAD+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>detoxifying enzymes/regulatory protein</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Nucleic acids</td>
<td>$rpoB$</td>
<td>β subunit of RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>polymerase/transcription</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Protein synthesis</td>
<td>$rpsL$</td>
<td>Ribosomal protein S12/translation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$rrs$</td>
<td>16S rRNA/translation</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Cell wall</td>
<td>$embB$</td>
<td>Arabinosyl transferase/arabinan polymerization.</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>Unknown</td>
<td>$pncA$</td>
<td>Pyrazinamidase/activation of prodrug.</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Nucleic acids</td>
<td>$gyrA$</td>
<td>DNA gyrase subunit/DNA replication</td>
</tr>
</tbody>
</table>
Table 3.2. Cellular targets for anti-mycobacterial drugs and gene mutations responsible for resistance in *M. tuberculosis*
3.8 Isoniazid (INH) resistance

The natural mutation rate of INH is higher than other anti-TB drugs, thus resistance of *M. tuberculosis* to INH occurs more frequently when compared to other drugs (Rattan et al. 1998). As a result, global increase in the incidence of INH resistant *M. tuberculosis* strains have been observed over the years, with estimates of 10% in new cases and 27% in previously treated cases (Cattamanchi et al. 2009). In addition, INH resistance has been associated with high level mutations in the *katG* gene and four other genes (*inhA, kasA, ndh* and *ahpC*) of *M. tuberculosis* (Chen et al. 2005). However, only 70-80% of INH resistant clinical isolates are phenotypically resistant (Musser et al. 1996). The inability to genotypically detect INH resistance in these isolates could be due to the presence of mutations in other unidentified genes or mechanisms.

3.8.1 Permeability of the Cell Wall and Isoniazid Resistance

The basic innate drug resistance mechanism in mycobacteria is that of drug efflux and this contributes to natural or acquired resistance (Li and Nikaido 2004). Efflux pumps are proteins that transport antibiotics to the outside of the bacterium, generating low levels of resistance to antibiotics and have been identified in all bacteria (Viveiros et al. 2002). Mycobacteria possess innate resistance to most antibiotics as a consequence of the gradual uptake of drugs across the hydrophobic cell wall envelope. It is believed that considerable contributions to acquiring resistance are made from efflux transport proteins, such as the efflux protein *efpA*, by reducing the cytoplasmic drug concentration to sub-inhibitory levels (Viveiros et al. 2003). There is however no evidence of strain dependence for efflux proteins since it is common in all *M. tuberculosis* strains. INH is further neutralised within *M. tuberculosis* by the up-regulation of arylamine N-acetyltransferase, by means of inhibiting NAD+-binding proteins (Viveiros et al. 2002), as well as the upregulation of enzymes which act as antioxidants, when compensating for the loss of the catalase-peroxidase protein. Isolates of *M. smegmatis* deficient in the energy-dependent NADH dehydrogenase efflux pump exhibit resistance to INH (Choudhuri et al. 1999).
and it has been shown that the combination of the absence of mycolic acids with the stabilizing
consequence of glycopeptidolipids confers innate resistance to *M. avium* (Mdluli, Swanson, *et al.*
1998, Choudhuri *et al.* 1999). During ineffective TB management and non-compliance, it has
been shown that overexpression of existing efflux pumps in reaction to prolonged exposure to
sub-effective concentrations of antimycobacterials, lead to increasing levels of resistance to the
drugs used in chemotherapy (Viveiros *et al.* 2003). The accumulation of INH in *M. smegmatis* is
known to be modulated by active extrusion systems (Choudhuri *et al.* 1999). Furthermore, the
role of efflux pumps in acquiring drug resistance might be elaborated by the recent discovery that
INH-sensitive *M. tuberculosis* isolates, possess a reserpine-sensitive efflux mechanism (reserpine
being a pump inhibitor), and that this is induced by step-wise exposure to INH, generating high-
level resistance to INH (Viveiros *et al.* 2002). One particular gene which is implicated in INH
resistance, and hence drug tolerance, is iniA. Very recently it was found that a deletion in iniA
results in an increased susceptibility to INH (Colangeli *et al.* 2005) and thus the role of iniA was
assigned as being part of an efflux pump. However, the authors could not show that iniA directly
transports INH from the cell. In addition, it was found that the *whiB7* gene (which confers
resistance to antibiotics having specific targets) in mycobacteria may act synergistically with the
cell wall to provide high levels of innate resistance (Morris *et al.* 2005).

### 3.8.2 INH Drug Resistance Conferring Mutations

Strains develop drug resistance by means of mutations in genes of which the gene products either
activate the drug or in turn are the drug target (Somoskovi *et al.* 2001). However, while many
mutations in a number of different genes have been found to be associated with INH resistance,
about 25-30% of INH resistant clinical isolates lack a known INH resistance gene mutation
(Victor *et al.* 2002). Several mechanisms towards resistance against anti-tubercular drugs are
employed by *M. tuberculosis*. Presently, a total of 12 loci responsible for drug-resistance to first-
line drugs in *M. tuberculosis* have been identified (Victor *et al.* 1997), and six of these (katG,
inhA, aphC, kasA, ndh and oxyR-ahpC) are associated with resistance to INH. Most 3 prominent
of these mutations is the S315T found in katG gene which is the major mechanism of INH resistance in *M. tuberculosis* and mutation in *inhA* gene or its promoter region found at position 15(C-T) (Heym *et al.* 1993, Musser *et al.* 1996). However these resistance mutations have been found to be absent in approximately 20-30% of INH resistance clinical isolates (Ramaswamy and Musser 1998). INH mono-resistance or resistance in combination with other drugs is now the second most common type of resistance globally, accounting for 13% of all new and previously treated TB cases (Cattamanchi *et al.* 2009, WHO 2009).

![Figure 3.1. Globally 13.3% of all TB cases involve isoniazid mono-resistance, vs. 0.6% rifampicin mono-resistance and 5.3% MDR-TB cases (WHO 2007)](image)

Isoniazid monoresistant cases respond poorly in comparison with the susceptible patients (Deepa *et al.* 2013).

### 3.8.2.1 Catalase-Peroxidase Enzyme and INH Resistance

INH is a prodrug which needs to be activated by the mycobacterial katG gene product, catalase peroxidase, by producing a reactive antimicrobial intermediate. The physiological
role of KatG is that of protection, by resisting the low pH found within the human macrophages during the oxidative burst where free oxygen radicals are converted to peroxides (H₂O₂). KatG activity counteracts this harmful reaction in the following process: (Whitney and Wainberg 2002)

\[2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2\]

It thereby protects the bacilli from oxidative peroxidation. INH resistance in *M. tuberculosis* may also be due to the loss of catalase-peroxidase activity within the mycobacterial genome (Middlebrook 1954, Zhang *et al.* 1992). Genetic studies revealed that when a functional *katG* gene was introduced into INH resistant strains of *M.smegmatis* and *M.tuberculosis*, deficient of catalase-peroxidase, these strains were capable of restoring their susceptibility to INH and thus *katG* deletions are key to understanding INH resistance (Zhang *et al.* 1992, 1993). However, the complete deletion of the *katG* gene only occurs in a small fraction of highly INH resistant strains (Heym *et al.* 1993).

### 3.8.2.2. Mutations within the *katG* gene

The genome of *M. tuberculosis* possesses repetitive DNA sequences, and the region within which *katG* lies, exhibits relative instability (Zhang and Young 1994, Slayden and Barry III 2000). The instability within the *katG* region may contribute to the relatively elevated rates of INH-resistant mutants, which are approximately 1 in $10^5$ – 1 in $10^6$ organisms generated during every *in vitro* selection of INH (Versalovic *et al.* 1991, van Soolingen *et al.* 1993, Mdluli, Swanson, *et al.* 1998) which may further account for mono- or multidrug- resistance to INH. The mutations involved are located either on the N-terminal or C-terminal of the *katG* gene product (Heym *et al.* 1999). The N-terminal of the katG protein is associated with the active site of the catalase-peroxidase enzyme and most of the mutations conferring a high level of INH resistance are located between codons 138 and 350. The most frequent mutation
however, is a point mutation at 315-codon of the katG gene (Slayden and Barry III 2000) which results in an amino acid substitution by replacing serine with threonine (Ser315Thr). The most frequent secondary polymorphism associated with the katG 315 mutation is that of the katG 463-codon where the Arginine residue is replaced by Leucine. This codon is situated at the C-terminal of the katG gene and is not readily associated with the catalase-peroxidase active site, and hence it is considered as a natural polymorphism (Heym et al. 1999, Victor et al. 2002). It is estimated that the katG 315 mutation and katG 463 polymorphism occur in 55 – 75% of all INH resistant isolates (Musser et al. 1996, Ramaswamy and Musser 1998, Slayden and Barry III 2000, Victor et al. 2002) and are characteristic of the Beijing/W family (Ramaswamy and Musser 1998). The majority of mutations in clinical isolates are missense mutations, and this indicates that even though the activity of KatG is reduced in vivo, the reduced activity still confers a selective advantage since nonsense mutations results in truncated proteins (Whitney and Wainberg 2002).

3.8.2.3. Increased Expression of Target Proteins

The loss of catalase-peroxidase is linked to the increased expression of compensatory M.tuberculosis genes such as ahpC, InhA, KasA and AcpM (Heym et al. 1999, Slayden and Barry III 2000). Mutations can occur at several sites in the promoter regions of the genes which result in altered transcriptional activity (Heym et al. 1999, Slayden and Barry III 2000). Mutations in the coding regions of these genes, however, are less frequently observed. Nonetheless, mutations in these promoter sequences do not completely elucidate INH resistance in clinical isolates and recently it has been found that mutations in the ndh gene, which encode for NADH dehydrogenase, may be an additional marker for INH resistance in M.tuberculosis (Lee et al. 2001).

3.8.2.4. The Enoyl Reductase Enzyme and INH Resistance
After initial activation of INH by catalase-peroxidase, an ensuing target is the Enoyl acyl carrier protein (ACP) reductase, encoded by the \textit{inhA} gene (Wilson \textit{et al.} 1995, Ramaswamy \textit{et al.} 2003). The activated INH derivative binds to the \textit{inhA}-NADH complex, forming a ternary complex, which results in INH resistance due to inhibition of mycolic acid biosynthesis. Point mutations in the structural \textit{inhA} gene result in amino acid substitutions, resulting in the decreased affinity of \textit{inhA} for NADH, and presently only six mutations have been reported so far. However, mutations in the promoter region of this gene cause the over-expression of \textit{inhA}, with the resulting effect of low levels of INH resistance (Ramaswamy and Musser 1998, Slayden and Barry III 2000).

\textbf{3.8.2.5. The Alkyl hydro-Peroxidase Enzyme and INH Resistance}

Alkyl hydro-peroxidase, encoded by the \textit{ahpC} gene, functions as a detoxifying agent and has its effects on organic peroxides (Sherman \textit{et al.} 1996). Promoter mutations in this gene in INH resistant isolates also result in its over-expression and function as a compensatory mechanism for the loss of catalase-peroxidase activity due to mutations in the \textit{katG} gene (Ramaswamy \textit{et al.} 2003). High levels of peroxides within the cell result in the over-expression of \textit{ahpC} to combat oxidative damage. However, the increased expression does not permit any effect on INH and results in the emergence of INH resistance (Dhandayuthapani \textit{et al.} 1996).

\textbf{3.8.2.6. The $\beta$-ketoacyl Synthase Enzyme and INH Resistance}

In addition to the Enoyl acyl carrier protein (ACP) reductase, another target after initial activation of INH is $\beta$-ketoacyl ACP synthase (KasA), encoded by \textit{kasA} (Slayden and Barry III 2000, Ramaswamy \textit{et al.} 2003). This enzyme is also involved in the mycolic acid biosynthesis and low levels of INH resistance have also been attributed to mutations in this gene (Mdluli, Slayden, \textit{et al.} 1998, Ramaswamy \textit{et al.} 2003).
3.8.2.7. The NADH Dehydrogenase Enzyme and INH Resistance

NADH dehydrogenase, an essential respiratory chain enzyme, is responsible for the regulation of NADH/NAD+ in the cell (Ramaswamy et al. 2003). It is encoded by ndh and it has been found that missense mutation in this gene results in INH resistance due to reduced enzymatic activity. The increased NADH accumulation and decreased NAD depletion result in the inhibition of the biosynthetic pathway which is crucial for metabolic flux (Lee et al. 2001).

3.8.2.8. The OxyR Enzyme and INH Resistance

The OxyR enzyme, encoded by oxyR, controls the expression of katG and ahpC during times of oxidative stress. It is a regulatory protein that functions as both an oxidative stress sensor and activator of gene transcription (Sreevatsan et al. 1997). Mutations in this intergenic region lead to impaired gene transcription of katG and ahpC and result in INH resistance (Ramaswamy et al. 2003).

3.9 Pharmacogenetics and Pharmacogenomics

Each human being carries its own unique genetic makeup. In concert with the environmental factors to which he / she is exposed, this may result in differing reactions to external treatments including drugs.

The first case of a genetic factor influencing drug response was reported back in the 1950s with the case of metabolising the muscle relaxant, suxamethonium chloride, by serum cholinesterase (Lehmann and Ryan 1956). The recessive allele resulted in a serum cholinesterase peptide which has less efficient enzymatic activity and thus prolongs the drug’s effect which in turn causes slower recovery from surgical paralysis.
Pharmacogenetics was initially used to refer to the study of inherited differences in genes responsible for drug metabolism i.e. the pharmacokinetics. The term comes from the words pharmacology and genetics. With the decoding of the human genome sequence and the subsequent systematic study of human genome variations, the term pharmacogenomics (pharmacology and genomics) has emerged to refer to a broader study of many different genes which together may determine drug behavior (covers both pharmacokinetics and pharmacodynamics). More precisely, pharmacogenetics is the study of differential responses in patients to drug compounds based on their genetic polymorphisms, and it mainly involves the study of patient sample collections (Paul and Roses 2003).

Pharmacogenomics is to identify disease-relevant drug targets at the molecular level and to target drugs to clinical populations with specified genotypes/haplotypes, and it often entails using large scale and high-throughput technologies to identify genes for tractable or screenable targets that are not yet known to be genetically related to diseases (Paul and Roses 2003).

3.9.1 Pharmacogenomics of Isoniazid (INH)

INH is a synthetic agent and its pyridine nucleus and carboxylic acid hydrizide side chain both are key structural features (Offe 1988). INH is a pro-drug, activated within \textit{M. tuberculosis} by the enzyme, katG (Blanchard 1996, Somoskovi \textit{et al.} 2001, Timmins and Deretic 2006). INH-derived reactive intermediates that form adducts with NAD\(^+\) (nicotinamide adenine dinucleotide) and NADP\(^+\) (phosphate form), leading to a blockade of mycolic acid synthesis (Timmins and Deretic 2006). The \textit{katG} gene encodes for mycobacterial catalase peroxidase and organisms lacking this gene do not synthesize catalase or peroxidase, and generally show INH resistance. InhA, an enoyl acyl carrier protein reductase, appears to be the primary target for the INH-NAD product described above
(Vilchèze et al. 2006). Resistance occurs at a rate of about 1 in $10^7$ organisms. INH has an MIC against *M. tuberculosis* of 0.01–0.25 μg/mL (Trnka et al. 1988). *M.kansasii* and *M.xenopi* are also susceptible to INH, but most other non-tuberculous mycobacteria (NTM) are resistant. INH is considered bactericidal, with minimal bactericidal concentrations (MBCs) close to its MIC. *In vitro*, INH’s maximum bactericidal effect is seen around 1 μg/mL (Trnka et al. 1988). With long periods of exposure, INH can produce a prolonged post-antibiotic effect (PAE), lasting up to 5 days (Awaness and Mitchison 1973). However, in humans, INH is relatively short-lived, so the clinical relevance of this finding is not clear. In animal models, INH-resistant isolates may show reduced virulence, but the clinical relevance of this observation also remains debatable (Trnka et al. 1988).

**Figure 3.2. Pathways of metabolism for isoniazid.** Bold lines: main biotransformation pathways in mammals including man; hatched lines with a question mark: metabolic pathway not clearly documented in mammals including man (Preziosi 2007)
INH appears to lack clinically significant cross-resistance with the other TB drugs. INH is not stable in blood left at room temperature, so PK studies must provide for rapid centrifugation, harvesting of plasma and prompt freezing. INH shows good absorption from the gastrointestinal (GI) tract and from intramuscular (i.m.) injection sites. Food, including high-fat food, reduces oral absorption, so INH is best given on an empty stomach (Peloquin, Namdar, et al. 1999). INH reacts with reducing sugars, thus limiting the choices of sweeteners for oral solutions to non-reducing sugars, such as sorbitol (Offe 1988). Antacids have demonstrated variable effects on INH’s absorption (Peloquin, Namdar, et al. 1999). The time of maximum plasma concentrations (T_{max}) ranges from 0.5 to 2 hours after oral doses (Peloquin, Namdar, et al. 1999) C_{max} of 3–5 \mu g/mL typically are achieved after 300 mg doses and 9–15 \mu g/mL after 900 mg doses. The C_{max} may be somewhat lower in fast acetylators, due to greater first-pass metabolism. The absorption of INH is unaffected by formulation with other TB drugs in combination products (McEvoy 2006). INH is widely distributed into most body tissues and fluids, with a volume of distribution (V_{d}) of roughly 0.7 L/kg (Peloquin et al. 2008). INH crosses the placenta and is excreted in human breast milk. INH enters macrophages and displays intracellular activity against \textit{M. tuberculosis} (Trnka et al. 1988).

INH is primarily cleared through acetylation by N-acetyltransferase 2 (NAT2) in the liver, resulting in acetylisoniazid which is then hydrolyzed to isonicotinic acid and monoacetylhydrazine (MAH) (Figure 3.3.). INH also undergoes hydrolysis catalyzed by isoniazid hydrolase and forms hydrazine, which is then metabolized to MAH by NAT2. MAH can be acetylated to diacetylhydrazine which is non-toxic, or oxidized by cytochrome P4502E1 (CYP2E1) into hepatotoxic intermediates (Ryan et al. 1985). Acetyl hydrazine can be hydrolyzed to hydrazine which may further induce CYP2E1, increasing the production of toxic metabolites. Hydrazine, MAH and isonicotinic acid are potentially hepatotoxic.
metabolites of INH and accumulation of these reactive metabolites in patients may cause serious adverse drug reactions. The enzyme responsible for INH hydrolysis has still not been well characterized. It is usually described as an amidase but in a recent report (Tafazoli et al. 2008), it was found that hydrazine formation from INH in rat hepatocytes could be inhibited by treatment with bis-p-nitrophenylphosphate, which was originally described as a carboxylesterase inhibitor (Heymann et al. 1969). There is increasing evidence that the hydrolysis of INH is catalyzed by a carboxylesterase but the precise isoform responsible is still unclear (Yamada et al. 2009). In human liver, two carboxylesterase isoforms CES1A1 and CES2 are expressed at detectable levels and show different substrate specificities (Yang et al. 2009). The gene encoding NAT2 is polymorphic, thus resulting in rapid and slow acetylator phenotype. Early NAT2 phenotyping study has shown that the fast acetylator phenotype with detectable NAT2 is associated with an increased risk of INH-induced hepatotoxicity (Mitchell et al. 1975). However, later studies have revealed that individuals with the slow acetylator phenotype lacking functional NAT2 catalytic activity are more susceptible to developing INH-induced hepatotoxicity due to accumulation of the toxic metabolite acetylhydrazine (Timbrell et al. 1980, Dickinson et al. 1981, Lauterburg et al. 1985). The slow acetylator phenotype results from nonsynonymous SNPs in the NAT2 coding region. These SNPs have been identified and characterized for their functional and structural effects (Walraven et al. 2008).
3.9.2. Pharmacogenomics of other Anti-Tb drugs

3.9.2.1. Rifampicin

RIF inhibits DNA-dependent RNA polymerase, blocking transcription (Somoskovi et al. 2001). RIF resistance results from single amino acid substitutions in the β-subunit of RNA polymerase (Somoskovi et al. 2001). This alters the binding of RIF, with the degree of resistance depending upon the location and nature of the amino acid substitution. The mutations leading to this resistance occur at a rate of about 1 in 10^8 (Blanchard 1996). RIF use leads to a small risk of hepatotoxicity and this risk is additive to that of INH (Girling 1982). Risk factors include advanced age, alcohol consumption, diabetes and concomitant hepatotoxic agents. Other adverse effects associated with intermittent RIF dosing include the ‘flu-like’ syndrome, which generally occurs after 3 months of treatment with doses of 900–1800 mg given once or twice weekly (Peloquin et al. 2008). More severe effects, including thrombocytopenia, haemolytic anemia and acute renal failure, may also occur, and these require permanent discontinuation of RIF (Girling 1982). These reactions appear to be immunologically related, as they are associated with the presence of RIF dependent IgM or IgG antibodies. RIF has variable effects on cellular and humoral immunity. Suppression of in vitro lymphocyte responses in cells collected from TB patients have been reported, but clinically evident immunosuppression has not been demonstrated (Humber 1980). RIF is a profound inducer of CYP3A4 and other hepatic P450 enzymes (Burman et al. 1999). However, RIF is not a substrate for these enzymes, so other agents, such as HIV protease inhibitors, do not affect RIF’s clearance (Burman et al. 1999). A simple rule of thumb is that most heptically metabolized drugs will have shorter half-lives in the presence of RIF, especially if they are substrates for CYP3A4, and to a lesser degree, 2C9, 2C19 and 2D6.
Enzyme activity and the pharmacodynamic effects of the affected drug generally return to baseline levels within 2 weeks after discontinuing RIF therapy (Burman et al. 2001).

**3.9.2.2. Pyrazinamide**

PZA initially was used at daily doses approaching 50 mg/kg and this led to unacceptable rates of hepatotoxicity (McDermott et al. 1954). After years on the shelf, PZA was rediscovered for clinical use as the third most important TB drug, after INH and RIF. PZA contributes important sterilizing activity to the treatment regimens during the first 2 months of therapy. It is also used for longer durations in the face of resistance to INH or RIF. Pyrazinoic acid appears to be the active moiety, although it is only the pyrazinoic acid created within tubercle bacilli that appears to be active, since the organisms do not appear to take up significant amounts of the acid from their surroundings. (Boshoff et al. 2002, Zhang and Mitchison 2003, Zhang et al. 2003) Some reports have suggested that PZA acts against fatty acid synthesis, as does 5-chloro-PZA, by inhibiting fatty acid synthase I (FAS-I) and this debate continues (Zimhony et al. 2000, 2007). It may be that the accumulation of inorganic acids within the organisms produces a stress that they cannot withstand (Boshoff et al. 2002, Zhang and Mitchison 2003, Zhang et al. 2003). Mutations in the *pncA* gene are associated with PZA resistance (Somoskovi et al. 2007). The main toxicities of PZA are GI upset and arthralgias (Peloquin et al. 2008). PZA routinely causes an increase in plasma uric acid concentrations, but not frank gout. In fact, normal uric acid concentrations during treatment with PZA generally indicate non-compliance. Hepatotoxicity is the most important PZA-associated toxicity (McDermott et al. 1954). High daily doses dramatically increase the incidence of this toxicity and they are no longer used. PZA has been associated with an unexpected number of severe liver injuries when used daily with RIF for the treatment of latent infection (Centers for Disease Control et al. 2001). Because the true incidence is not known, currently it is not possible to say if this rate is particularly high. When given b.i.w, PZA plus RIF apparently
has not caused significant liver injury, based upon available reports (Chaisson et al. 2002). PZA is not associated with significant drug interactions. When co-formulated with RIF and INH, the absorption of RIF is decreased by about 13 per cent. This is overcome by the somewhat higher RIF dose used in Rifater (combination formulation of RIF & INH).

3.9.2.3. Ethambutol

EMB inhibits arabinotransferases involved in the biosynthetic pathway of the mycobacterial cell wall (McNeil and Brennan 1991). Arabinotransferase is involved in the polymerization of arabinofuranose, required for synthesis of arabinogalactan (AG), a structural component of mycobacterial cell wall (Mikusová et al. 1995). Ethambutol also has an inhibitory effect on the polymerization of arabinofuranose into lipoarabinomannan (LAM), but this occurs more slowly, requiring longer exposure to the drug. Mutations in the embB region, specifically codon 306, appear to be the most common source of EMB resistance (Srivastava et al. 2006). Optic neuritis, specifically retrobulbar neuropathy, is the most important EMB toxicity (Peloquin et al. 2008). It is uncommon with standard doses when given to otherwise healthy individuals. However, some patients with pre-existing ocular problems, such as those resulting from diabetes, may be at greater risk of toxicity. Patients should be tested routinely for visual acuity (Snellen charts) and red–green color discrimination (Ishihara color plates) at baseline and throughout treatment (Blumberg et al. 2003). Daily doses of 30 mg/kg or more increase the likelihood of this toxicity, as does the administration of standard q.d. doses (15–25 mg/kg) to patients with impaired renal function (Girling 1982). Should the toxicity develop, the drug should be stopped promptly. When this is done, gradual improvement in vision generally is achieved. Other adverse reactions include elevations in plasma uric acid, and uncommonly, cholestatic jaundice, interstitial nephritis, thrombocytopenia, neutropenia and skin reactions (Peloquin et al. 2008). It is possible to desensitize patients to EMB (Matz et al. 1994). EMB should not be given with antacids and presumably sucralphate, iron or
other drugs or supplements containing di- or trivalent cations (Peloquin, Bulpitt, et al. 1999). EMB does not have other significant drug interactions.

### 3.10. Human N-acetyltransferase gene

Arylamine N-acetyltransferase (NAT) is a phase II enzyme involved in the detoxification of aromatic and heterocyclic amines and hydroxylamine, arylhydrazines and arylhydrazides in the liver (Westwood et al. 2006). About half of the population of white North Americans carry an autosomal recessive allele for N-acetyltransferase (Evans and White 1964, Drayer et al. 1977), a liver enzyme that metabolises drugs such as isoniazid, phenelzine, hydralazine and salicylazosulfapyridine. Drugs such as Isoniazid (Hughes et al. 1954, Devadatta et al. 1960), which are metabolised by this enzyme, will remain in the body longer and reach a higher blood level in slow acetylators. NAT also plays an important role in carcinogen metabolism. The N-acetylation metabolizing pathway is a major route for the conjugative metabolism of many drugs and chemicals (Weber and Hein 1985). Based on the substrate, individuals can be phenotyped as either “fast” or “slow” acetylators. The relationship between these polymorphisms and the resulting phenotypes is well established (Upton et al. 2001). Individuals with the slow phenotype are homozygotes for the slow allele, whereas subjects with the fast phenotype are either heterozygotes or homozygotes for the fast allele. The frequency of slow acetylator varies worldwide, ranging from 5% to 10% in Asia and reaching 90% frequency in certain European populations (Puga et al. 1997).

In contrast to other xenobiotic biotransforming enzymes, the number of N-acetyltransferases is limited (Vatsis et al. 1995). Human genome consists of two acrylamine N-acetyltransferase enzymes 1 (NAT1) and 2 (NAT2), which catalyze the metabolism of N-acetylation of arylamines, arylhydroxylamines and arylhydrazines (Hein 2002, Garcia-Martin 2008). NAT1 is expressed in most tissues of the body, whereas NAT2 appears to be expressed only in the
liver and the gut. The genes encoding both the isoforms, NAT1 and NAT2 were mapped to chromosome 8p21.3-23.1 by Hickman et al. (Hickman et al. 1994). NAT1 has a single, intronless protein-coding exon with an open reading frame of 870 bp. The nucleotide homology between NAT1 and NAT2 is 87% in the coding region. The pseudogene NATP has a high sequence homology to NAT1 and NAT2 but contains multiple frameshifts and stop codons and does not encode a protein (Blum et al. 1990). Although they have high sequence similarity, human NAT1 and NAT2 show different substrate specificities and behaviors in carcinogen bioactivation (Hein 2002, Kim and Guengerich 2005). No substrate is exclusively N-acetylated by one enzyme or the other. Substrates preferentially N-acetylated by NAT1 include para-aminosalicylic acid, para-aminobenzoic acid, sulfamethoxazole, and sulfanilamide, while substrates preferentially N-acetylated by NAT2 include isoniazid, hydralazine, procainamide, dapsone, aminoglutethimide, and sulfamethazine. Some xenobiotics, such as the carcinogenic aromatic amine, 2-aminofluorene, are acetylated equally well by NAT1 and NAT2 (Vatsis et al. 1995). The interindividual variation in NAT2 function is associated with the classical isoniazid acetylation polymorphism, which was discovered about fifty years ago by Bönicke and Reif (Bönicke and Reif 1953). However, the exact biological functions of each isozyme are still unclear. N-acetylation is the major route of biotransformation for xenobiotics containing an aromatic amine (R-NH2) or a hydrazine group (R-NH-NH2), which are converted to aromatic amides (RNH- COCH3) and hydrazides (R-NH-NH-COCH3), respectively (Vatsis et al. 1995). Like methylation, N-acetylation masks the amine with a nonionizable group, so that many N-acetylated metabolites are less water soluble than the parent compounds. Nevertheless, N-acetylation of certain xenobiotics such as isoniazid facilitates their urinary excretion. Upon acetylation of INH to acetylisoniazid, by N-acetyltransferase, INH becomes therapeutically inactive. As shown by the study of Evans et al. (Evans et al. 1960), the «slow inactivator» person is homozygous for
a slow inactivator allele, whereas the «rapid inactivator» is either homozygous or heterozygous for a rapid acetylator allele. Population studies show a distinct bimodal distribution of NAT2 phenotypes, as shown by Cascorbi et al. (Cascorbi et al. 1995), The slow acetylator phenotype was shown to be associated with a higher risk of bladder cancer (Cartwright et al. 1982, Risch et al. 1995, Brockmöller et al. 1996), whereas the rapid acetylator phenotype is at a higher risk of developing colorectal cancer (Lang et al. 1986, Probst-Hensch et al. 1995).

3.10.1 Arylamine N-acetyltransferase 2

Human arylamine N-acetyltransferase 2 (NAT2) (EC 2.3.1.5) is one of two functional NAT isoforms expressed in human tissues. The NAT2 gene was first described by Blum et al. (Blum et al. 1990). Since then eight different point mutations have been characterized (Vatsis et al. 1991, Bell et al. 1993, Lin et al. 1994, Leff et al. 1999). NAT2 consists of a noncoding exon at the 5′ end, separated by a 9kb intron from an uninterrupted coding region of 873 bp that encodes a 290 amino acid protein. Among the eight point mutations in the coding region are five which cause amino acid changes: 191G>A (Arg to Glu), 341T>C (Ile to Thr), 590G>A (Arg to Gln), 803A>G (Lys to Arg), 857G>A (Gly to Glu), and three silent ones: 111T>C, 282C>T and 481C>T (Figure 3.4.) Among these, the most studied alleles were NAT2*5, NAT2*6 and NAT2*7 at positions 341, 590 and 857, respectively.

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NAT2 is a highly polymorphic enzyme, and individuals can be divided into rapid, intermediate, and slow acetylator phenotypes based on the genetic polymorphisms of this gene. $NAT2^*4$ is defined as wild-type, and major variant haplotypes $NAT2^*5$, $^*6$, and $^*7$ bear 341T>C (I114T), 590G>A (R197Q) and 857G>A (G286E) substitutions, respectively (Hein 2002). Acetylation activities in vitro are ranked for several substrates as follows: $NAT2^*4>NAT2^*7>NAT2^*6>NAT2^*5$. (Hein et al. 2000, Hein 2002). The I114T substitution in $NAT2^*5$ yields a 90% reduction of acetylation activity for sulfamethazine (Zang et al. 2004). In addition, $^*14$ (with 191G>A, R64Q) is associated with reduced acetylation activities, but its frequency is relatively high only in Africans (Sabbagh et al. 2008). Recently the effects of various SNP on structure of NAT2 have been studied by in silico molecular modelling studies (Rajasekaran et al. 2011).
3.10.1.1 Possible role of the Human N-acetyltransferase-2 gene in the development of Isoniazid resistance

It was observed that urinary excretion of unchanged INH, after repeated administration, was constant in any individual, but the inter-individual excretion was clearly different for each individual (Meyer 2004). It was later found that this phenomenon was due to differences in the individual’s ability to metabolise INH to acetylisoniazid (Meyer 2004). The ability to inactivate INH was shown to be inherited in a Mendelian fashion by family pedigree analysis (Evans et al. 1960) and diminished elimination of INH, an autosomal recessive trait, was found to be the result of reduced enzyme activity (Evans and White 1964).

The antituberculosis regimen was standardized based on the dose-response relationship of INH from Caucasian subjects, in which population the frequency of the RA phenotype is approximately 5% (Cascorbi et al. 1995). During the first 2 to 5 days of the multidrug therapy, the bactericidal activity is derived mainly from INH (Jindani et al. 1980, 2003). Therefore, the currently recommended daily dose (5 mg/kg) is likely to be determined without concern for the RA type. As a correlation between low plasma concentrations of INH and therapeutic failure has been reported (Kimerling et al. 1998, Weiner et al. 2003), RA-type patients might be administered an increased dosage of INH to achieve sufficient antituberculotic effects. It is important to determine the optimal dosage for RA-type subjects because half of all Japanese are RA type (Ohno et al. 2000). Thus, the international pharmacogenomics trial, based on NAT2 genotype, is required to examine the outcome of INH dosage for RA type (Kubota et al. 2007). Reduction in early treatment failures was observed by increasing dosage (1.5 times) in rapid acetyltators (Azuma et al. 2013).

3.10.1.2 Molecular mechanism of Inactivation of Isoniazid by the Human N-Acetyltransferase Enzyme
Enzymes involved in xenobiotic metabolism are categorized according to their roles: either by changing functional moieties (Phase I enzymes), or by conjugating with an endogenous compound (Phase II enzymes) (Tribut et al. 2002).

![Diagram showing Phase I and Phase II enzymes](image)

*(Genetically polymorphic enzymes that contribute significantly to the metabolism of therapeutic drugs are sliced off the pie charts)*

*Figure 3.5. Schematic representation of various phase I and II enzymes (W. E. Evans & Relling, 1999)*

Isoniazid is principally metabolised by acetylation of a hydrazino or amino group. This reaction is catalysed by Arylamine Nacetyltransferase 2 (NAT2), a cytosolic polymorphic Phase II conjugating enzyme (Grant 1993, Delomenie et al. 1997, Payton et al. 1999), capable of N-acetylation (usually deactivation), O-acetylation (usually activation) and N,O-acetylation (Brockton et al. 2000). Upon acetylation of INH to acetylisoniazid, by Nacetyltransferase, INH becomes therapeutically inactive. NATs use acetyl Coenzyme A (Ac-CoA) as a cofactor in conjugating an acetyl group onto the amine, hydroxylamine or hydrazine moiety of an aromatic compound (Vatsis et al. 1995). The metabolic pathway of NAT2 is shown in Figure 3.5. (Ohno et al. 2000). The acetylated metabolite is usually
pharmacologically inactive and less lipid soluble than its precursor, and is excreted (Johnson 2001).

**Figure 3.6. Metabolic pathway of NAT2 Interactions with Rifampicin.**

### 3.10.2.3 Inactivation of Isoniazid by N-Acetyltransferase

The activation of the pro-drug INH is altered when the hydrazine moiety has undergone acetylation of INH to acetylisoniazid (AcINH), and this is responsible for the reduction of therapeutic concentrations of INH (Evans et al. 1960, Upton et al. 2001). Recently it was suggested that impaired INH metabolism might be associated with polymorphisms within the *NAT2* gene, in a Japanese population (Kita et al. 2001). It is therefore possible that a fast acetylator genotype (Parkin et al. 1997) can indirectly contribute to the INH resistant phenotype in *M.tuberculosis* by lowering the serum concentration of the drug, since the fast acetylator may not respond to INH in the management of TB (Price Evans 1989).
3.11. Hepatocytes and xenobiotic metabolism

Hepatocytes express a variety of metabolic enzymes, which are responsible for different types of reactions. Two main processes usually occur sequentially called phase 1 and phase 2. The former leads to an activation of the compound by introducing functional groups into the compound by oxidation, reduction or hydrolysis reactions. This is followed by phase 2 reactions, the conjugation of the active metabolite with a highly polar ligand like glucuronic acid or glutathione, leading to more hydrophilic products. The directed transport of metabolites out of the cells by specialized transporters is often referred to as phase 3 of xenobiotic metabolism. Typical phase 1 enzymes are shown in Figure 3.5. The CYP enzymes, the predominant group of phase 1 enzymes in mammals, consist of at least 17 gene families with 50-60 individual isoforms (Waxman 1999, Guengerich 2003). The major human CYP enzymes involved in metabolism of drugs or exogenous toxins are Cyp3A4, Cyp1A1, Cyp1A2, Cyp2D6 and Cyp2C. The amount of each of these enzymes present in the liver reflects their importance in drug metabolism (Jackson and Garrison 1996). Depending on the chemical properties of the introduced functional groups, phase 1 products can be classified as electrophilic or nucleophilic metabolites. Strong electrophilic metabolites are able to covalently bind to biological molecules like DNA, RNA or proteins and therefore have inherent cytotoxic or mutagenic potential (Besaratinia and Pfeifer 2005). In contrast to this, nucleophiles can show biological activity by binding to cellular receptors and activating downstream reactions. Thus, the metabolic activity of cells can lead not only to a detoxification but also, in certain cases, to a toxification of compounds. The activation reaction is in most cases followed by a detoxifying phase 2 conjugation reaction. Thereby, the water solubility is increased allowing the cells to excrete the conjugates into the bile canaliculi and/or the blood plasma. Enzymes catalyzing phase 2 reactions are e.g. sulfotransferases (SULT), acetyltransferases (AT), glucoronyltransferases and Glutathione-S-
Transferases (GST). Several factors can influence the efficiency of xenobiotic metabolism. The activation and inhibition of enzyme activity and the induction and repression of gene expression are the main elements of regulation. Inducers usually affect multiple enzymes from different steps of xenobiotic metabolism. Thereby, an entire metabolism cascade can be activated leading to the detoxification of the compound (Xu et al. 2005, Elias and Mills 2007).

3.12. Primary Hepatocytes as a model to study Drug Metabolizing Enzymes

*In vitro* tests have the advantage of allowing multiple testing of different compounds, doses and/or time points simultaneously under well-defined conditions. The simplicity of some *in vitro* systems, besides saving time, money and animals used for experimentation, provides the ability to specifically manipulate and analyze a small number of well-defined parameters. The most commonly used test systems include, the isolated perfused liver, liver slices, primary hepatocytes in suspension or culture, cell lines, transgenic cells and sub-cellular fractions such as S9 mix, microsomes, supersomes or cytosol (Brandon et al. 2003). Primary hepatocytes are well suited for pharmacogenomic studies because they display a certain level of metabolic activity and the liver is a major stage for toxic events (Waring et al. 2001). In addition, the use of cell culture models reduces the animal utilization and need for the compounds on a large scale (Boess et al. 2003). However, it is also clear that there are a number of limitations in using *in vitro* approaches such as the functional differences observed in primary hepatocytes relative to the intact liver, the absence of interactions with biological entities (e.g., organs, blood) under *in vitro* conditions, and the difficulty to select doses and time points which are representative of an *in vivo* situation (Schwab and Tuschl 2003).
3.13. Whole Genome High throughput transcriptomics studies

The new and developing field of microarray technology evolved from E.M. Southern’s realization that labelled nucleic acid molecules can be hybridized to their counterparts and therefore be used to detect their existence and amount in the original sample (Southern 1975). The sequencing of whole genomes from human, as well as of many “laboratory” animal species, quickened the development of new technologies for the measurement of several thousand genes in a single experiment (Schena 1996, Brown and Botstein 1999). Meanwhile, these microarray technologies are used for a wide spectrum of issues, like drug discovery, basic research and target discovery, biomarker determination, pharmacology, toxicology, target selectivity, development of prognostic tests and disease-subclass determination (Butte 2002).

In the field of gene expression analysis, DNA microarray technology is having a major impact on many different areas including xenobiotic metabolism. A number of studies have shown that transcription profiling can generate the information needed to map a complete metabolic pathway of a drug (Fielden and Zacharewski 2001, Storck et al. 2002). Isoniazid is mainly metabolized in the hepatocytes. As many aspects of INH metabolism are still sketchy (Preziosi 2007) a wholesome transcriptomics picture may reveal many hidden aspects of INH metabolism. DNA microarrays consist of DNA fragments corresponding to genes. The use of high density microarrays containing thousands of DNA fragments has the main advantage that the expression level of a large number of genes can be studied simultaneously. However, the major drawbacks are related to the high cost and the time taken for analysis and interpretation of the data (Allison et al. 2006).
3.14. Spot test for Isoniazid

INH is a well-absorbed drug, distributes to all body compartments and 95% is metabolised and eliminated within 24 h. As discussed in the previous sections estimation of Isoniazid is most important for ascertaining actual phenotype of the patient. The liver metabolizing enzymes have a wide substrate and inducer range. Inducers may exist in numerous other sources, thus many factors contribute to variations between phenotype and genotype.

- Diet of the patient strictly vegetarian or non-vegetarian.
- Substance abuse: commercial products both legal and illegal.
- Additional medicines for some coexisting ailment
- Intake of highly caffeinated products/brews.
- Chemical adulterants in the food.

Due to such a variety of factors direct determination of acetylation statuses of the patient is most important. Numerous different analytical methods have been developed for quantitative determination in pure, pharmaceutical dosage forms and/or biological fluids. These methods are; liquid chromatography (Mawatari et al. 1990), thin layer chromatography (Guermouche and Guermouche 2004, Ali et al. 2007), gas chromatography (Khuhawar and Zardari 2008), capillary electrophoresis (Liu et al. 2011, Tsai et al. 2011, Faria et al. 2012), electrochemically (Atta et al. 2011), spectrophotometry (Al-Enizzi et al. 2012), spectrofluorimetry (Ioannou 1988, Garcia Bautista et al. 1998) and chemiluminescence methods (Zhang et al. 2001). These methods, suffer from their sophisticated instrumentation and high-analysis cost. Moreover, these instruments are not available in most quality control laboratories specially, in field settings.

In general, paper strip spot test is considered one of the most convenient analytical techniques, because of its inherent simplicity, low cost, and easy transport. To the best of our
knowledge, there were no reported Spot test methods for INH acetylated metabolite. For these reasons, in the present study we have attempted to formulate a simple, sensitive and economical biochemical paper strip spot test method for the analysis of acetylator types by detecting INH and its acetylated metabolite.