6. DISCUSSION

Isoniazid (INH) is one of the most important drugs for the prevention and treatment of tuberculosis. Isoniazid has been in the treatment of tuberculosis for more than 50 years and there has been emergence of resistance due to which prevalence of isoniazid (INH) resistance is quite common and is much higher than that of RIF (WHO 2014). Mono-resistance against INH ranges from 10-19% of TB patients globally (Cattamanchi et al. 2009) & 9-18% in India (Agarwal and Chauhan 2005). INH is metabolized differently from patient to patient. Upon acetylation of INH to acetylisoniazid, by N-acetyltransferase, INH becomes therapeutically inactive. Current regimen is designed to avoid toxic side effects observed more frequently in slow acetylators. INH has many metabolites that have varying degree of toxicity and elimination curves, but none of them are active against TB. The information regarding metabolic pathways for INH and its metabolites in the host as well as in M.tbc is not complete. The variable pharmacodynamic profile of INH is mostly based on N-acetyltransferases genetic polymorphisms, to determine the same accurately and inexpensively may enable us to rationalize the therapy at an individual level (Tripathy 1968). In this work we have attempted three distinct approaches to address INH differential therapy response, metabolism and field testing. In section 6.1 we have discussed results from genotyping association with INH mono-resistance in the study population of TB patients. In section 6.2 we have discussed microarray approach to elucidate whole genome picture of INH on cultured murine primary Hepatocytes. In section 6.3 the results of biochemical spot test to ascertain acetylator status of patients in the field settings.

6.1. NAT2 Genotyping

The calculated allele frequencies of NAT2 in the control group are in close agreement with those previously reported from North India (Pandey et al. 2007). The frequency for slow
alleles in the mixed ancestry population is shown in Table 5.1. To our knowledge, this is the first report that shows the impact of NAT2 genotypes on occurrence of INH mono-resistance. Systematic investigation of wild-type and three NAT2 genes functional SNPs (rs1799929, rs1799930 and rs1799931) in 104 INH mono-resistant patients and 100 controls, both of Indian origin (Ghatampur, Kanpur, UP), showed that wild type SNP of NAT2 is significantly associated with INH incidence of mono-resistance (Table 5.4.). When the wild type NAT2 haplotype is considered dominant a significant association with INH-monoresistance [p-Value < 0.05 (0.047933)] which assumes that both homozygous & heterozygous are at same risk to develop mono-resistance. If the multiplicative effect is considered i.e. subjects with one copy of the wild type NAT2 haplotype are at an intermediate risk the association is highly significant [p-Value < 0.01 (0.009691)]. Previous studies support these findings as intermediate acetylators exhibit a median phenotype as compared to rapid & slow (Parkin et al. 1997). Slow and intermediate acetylators combined were 53.8% among total control population, thus the INH dose is being administrated to a majority of population in the current drug regimen could be insufficient. The NAT2 individual deduced acetylator phenotypes have not yielded statistically significant ORs, when calculated in relation to NAT2*4/*4 as reference genotype. Not so significant armitage’s trend tests can be attributed to lack of effective sample size due to break down of population tested among different genotypes populations. Also deviations from Hardy Weinberg equilibrium were not significant as it is a test more effective on large populations with fewer alleles. Various other factors are also responsible for occurrence of INH mono-resistance like primary resistance i.e. infection by resistant strain to begin with, Improper following of therapy regimen overlooked by the providers and hyper-micturation conditions such as diabetes may cause elimination of the drug more rapidly. Also concomitant consumption of phytochemicals, caffeine (Fuhr et al. 1996), heterocyclic amines from fry-cooked animal proteins (Hein 2002)
and probably most commonly abused substances like gutkha and zarda (nicotine products with wide range of unknown chemicals and solvents) or their metabolites may influence expression of liver enzymes especially NAT2 modulating effective phenotype towards rapid-acetylator type. Thus a significant association of rapid inactivation of drug with occurrence of INH mono-resistance is further signified as the effect is not diminished by above mentioned numerous coexistent factors.

Individuals who are classified as intermediate acetylators are generally considered fast NAT2 acetylators when metabolically probed with caffeine during phenotypic testing (Le Marchand et al. 1996). This may be relevant in this study, since the findings suggest that the combination of fast and intermediate acetylator status is a risk factor for the development of INH mono-resistance. Before MDT during Isoniazid-streptomycin trials, it was well documented that fast inactivators respond less favorably to "marginal chemotherapy". Rapid inactivators, also responded less well to a once-weekly dosage regimen (Menon 1968), and develop isoniazid resistance sooner, than slow inactivators (Tripathy 1968). Initial clinical trials in South India have established that tuberculosis patients who are Fast and intermediate acetylator patients infected with TB have a significant increased risk of developing INH resistance (Tripathy 1968). Slow acetylators are able to inhibit a significant proportion of the INH resistant mutants, whereas the serum concentrations of fast acetylators cannot do this (Gangadharam et al. 1961). In addition, once the peak serum concentration of INH has been reached, no additional benefits are conferred since resistant mutants are capable of growing in much higher concentrations of INH (Mitchison 1973). It is hypothesized that a fast acetylator genotype may indirectly contribute to mycobacterial adaptation to INH during TB treatment, since the time of bacterial exposure to INH is limited and thus small systemic concentrations might lead to the gradual build-up of INH-resistance. Hence, these patients may indirectly contribute to the development of INH resistance in M.tuberculosis. The difference observed
in the rates of acetylation in patients with INH mono-resistance may be one of several possible contributing factors for increased susceptibility to developing INH resistance and later MDR-TB. Though the effect is less studied with MDT-DOTS programme this study sheds light on the significant pharmacogenomic variability towards treatment even in MDT scenario. However, \textit{M. tuberculosis}, the causative agent of tuberculosis (TB), also possesses a \textit{NAT} gene and exhibits high catalytic ability towards INH (Payton \textit{et al.} 1999) and may further contribute towards the development of INH resistance in \textit{M.tuberculosis}. Therefore the relationship between fast and intermediate acetylator status and INH-resistance is highly probable since patients infected with a drug resistant strain are predominantly fast and intermediate acetylators of INH. The implications of the findings in this study suggest that it might be possible to administer higher dosages of INH to TB patients who are either fast or intermediate acetylators of INH.

\textbf{6.2. Microarray}

In the present study microarray has proved to be a useful tool to identify various genes differentially expressed in mice primary Hepatocytes. Various statistical tests were performed which showed that the experimental conditions were comparable among control and INH induced groups. The samples were mostly similar except for differentially expressed genes. The false discovery rate was negated and differentially expressed genes were subjected to pathway analysis. Though many genes were not known to be belonging to a pathway, the genes that did were mapped. This data is suggestive that a number of high-level GO terms (functional annotations) were influenced by the corresponding mapped expression data. Of particular interest is the xenobiotic transformation which is mainly responsible for drugs inactivation and neutralization. Other observations indicate that INH may influence many cancer and mutation associated pathways such as base excision repair, basal cell carcinoma,
cell cycle and p53 signaling pathway (Figures 5.16. & 5.17.). These changes observed could be due to hydrazine toxic intermediate generated after hydrolysis of INH. Also INH is known to react with Vitamin B6 (pyridoxal) and thereby inactivating the later. Many genes involved in Vitamin B6 metabolism as well as genes depended on pyridoxal for their function are differentially expressed (Figures 5.16. & 5.17.). The genes found could be important leads for future investigations and human orthologous counterparts need to be subjected to Real time PCR analysis in human primary Hepatocytes. As the starting sample for Human primary Hepatocytes is always small a Real time PCR analysis targeting the genes found to be differentially expressed in mice could generate a better picture.

**6.2.1. Xenobiotic metabolism Pathways**

INH is one of the drugs that affect the metabolism of many other drugs. The observed differential expressing genes are scattered in different groups. INH has many metabolites such as hydrazine, acetyl hydrazine, di-acetyl hydrazine, Isonicotinic acid and AcINH etc. thus it may affect wide range of xenobiotic metabolizing enzymes. It was found that NAT8 is overexpressed which is homologous to human NAT2, NAT10 is under expressed whose function is little known. Cyp1a1 is overexpressed which is associated with Rifampicin metabolism (Preissner et al. 2010). As INH is one of the drugs that affect a many of other drug’s metabolism, the current findings are affirmative and explainatory to observed phenomenon. Role of genes like NAT8 and Cyp1a1 has been reported earlier (Ohno et al. 2000, Preissner et al. 2010). Our study shows that there are many other genes such as NAT10, other cytochrome P450 enzymes, methyltransferases, sulfotransferases and glutathione transferases which are needed to be investigated further.

The hydolysis of INH which yields hydrazine and isonicotinic acid is the main reason for the drugs toxicity. As hydrazine is a potential carcinogen and substrate for p450 oxidation
yielding various toxic intermediates. Still it is not clear which hydrolase brings about this reaction. 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. In the present study microarray results show that Ces1 was differentially expressed (Figure 5.19.) and could be involved in INH hydrolysis. The repression of Ces1 and Lipa gene products (Figure 5.19.) is probably indicative of protactive mechanism by which INH hydrolysis is controlled and explains why the majority of INH is acetylated instead of being hydrolyzed.

6.2.2. Insulin Pathway & Oxidative Damage Pathway

Inhibition of Insulin pathway is previously reported as INH mediated steatosis, which implies irresponsiveness towards insulin and lipid accumulation in the liver. Liver steatosis is a complex condition and is believed to be caused by both hydrazine and oxidative damage. By looking at the pathway most probably INH or its metabolites acts glucose export inhibitors by repressing vesicular transport and reducing glucose export transporters. Actin regulatory pathways are also affected thus a common underlying mechanism is needed to be further investigated. Induction of signaling, gene regulation and modulators of insulin action could be due to feedback up-regulation.

Oxidative stress pathway of the liver causing regeneration is also upregulated. It is well known that the alcohol induced hepatitis and later liver cirrhosis is the irreversible end result of fibrous scarring and hepatocellular regeneration (Døssing et al. 1996). The co-activation of the same pathway may cause synergic toxic effects of INH and alcohol. Alcoholism in TB patients has been reported previously to be associated with higher chance of
hepatotoxicity (Huang et al. 2003). Therefore alcohol related substance abuse should be closely monitored throughout in the patients receiving DOTS to avoid complications.

6.2.3. ABC transporters

Unusual drug accumulation is a common mechanism underlying serious drug-induced liver injury. The directed transport of metabolites out of the cells by specialized transporters is often referred to as phase 3 of xenobiotic metabolism. Previous workers have tried to associate ABC transporter polymorphisms with INH induced toxicity (Kim et al. 2012). Both drug metabolizing and detoxifying-transporting systems may work in synergy. But Abcc12 has not yet been investigated or associated with INH. Thus further expression analysis and polymorphism screenings may yield vital information regarding differential INH toxicity.

6.2.5. Vitamin B6

Vitamin B6 deficiency due to INH conjugation is cause of major side effects. More evident toxicity is seen due to decrease in GABA levels in brain resulting in Tonic-clonic seizures (Wason et al. 1981). Though effect of INH induced toxicity in brain is studied in detail the effect of Vitamin B6 deficiency in other organ and developmental function is little known. The microarray showed differential over expression of Phosphoserine aminotransferase1 (Psat1). Psat1 is depended upon pyridoxal-5-phosphate for its function. It’s over expression could be due to substrate accumulation because of pyridoxal deficiency. Psat1 mutations are known to cause Neu-Laxova syndrome which is characterized by severe intrauterine growth restriction and multiple congenital malformations (Acuna-Hidalgo et al. 2014). This is an important finding which shows that as a higher dose of Vitamin B6 could be required by pregnant women TB patients receiving INH.
6.2.6. Possible interactions of INH

It is known that if a drug is co-administered with another drug which have common metabolizing enzyme they compete and raise toxicity of each other. Also if one of them or its metabolite is an inhibitor then the toxicity is aggravated (Preissner et al. 2010).

6.2.6.1. Interactions with other anti-tuberculosis drugs

Cyp1a1 is overexpressed under the influence of INH which is also associated with Rifampicin metabolism (Preissner et al. 2010). Data regarding enzymes involved in metabolism of other anti-tuberculosis drugs is not thoroughly investigated. INH induces differential expression of 70% of genes of metabolic enzymes grouped according to involvement in the xenobiotic transformation of drugs (Figure 5.17.) and INH is inhibitor of 8 p450 (Cyp) genes (Preissner et al. 2010). The drug metabolic profiles of other anti-TB drugs thus need to be investigated in relation to genes / group of genes identified in detail. Such potential interactions should then be investigated and validated in humans.

6.2.6.2. Interactions with anti retroviral therapy

INH is commonly used as chemoprophylactic agent as Isoniazid preventive therapy (IPT) for TB and AIDS patients. Further INH is known inhibitor of Cyp2c8 and Cyp2c9 which are involved in metabolism of Lamivudine, Emtricitabine & Co-trimoxazole drugs given in ART regimen (Preissner et al. 2010). However these genes were not found to be differentially expressed in present study (Figure 5.18). A follow up detailed experimental validation in humans is required to study the metabolic interactions of these drugs.

6.2.6.3. Pregnancy

In the present study INH has been found to be interfering with Psat1 gene important for fetus intrauterine growth (6.2.5.). Cyp26a1 and Cyp26b1 were also found overexpressed (Figure
5.17.) which are involved in metabolism of steroid hormones especially progesterone (Preissner et al. 2010). Progesterone is responsible for maintenance of pregnancy and rapid metabolism of which may increase risk of miscarriage. Also progesterone is principle component of female contraceptive formulations and its rapid elimination may hinder their effectiveness.

**6.2.6.4. Cancer**

There were a number of cancer associated pathways induced by INH (Figures 5.16 & 5.17.). INH itself has a low Ames test toxicity score of 0.8557 (www.drugbank.ca) and is non-carcinogenic. Small fraction INH is hydrolysed to give hydrazine also many other metabolic intermediates are known (Figure 3.2.). It is difficult on the basis of current literature and findings which of the intermediates are carcinogenic except hydrazine (Nebert et al. 1999). In mammalian test systems where INH is partially metabolized unlike Ames test, INH and hydrazine both reported to be tested positive for mutagenicity (Röhrborn et al. 1972). But this is not apparent in clinical studies because only a fraction of INH is hydrolysed majority of it is acetylated. Slow acetylators acetylate INH slowly and possibly are at a greater risk of INH being hydrolyzed generating hydrazine. Slow acetylator phenotype has been shown to be associated with various cancers such as colorectal and renal (cancers arising from environmental carcinogens) (Jain et al. 2007). Thus a follow-up of slow acetylators who have received INH should be conducted to determine the actual risk over a period of time.

**6.3. Spot test**

The test developed to check the INH acetylation status (retention time) and follow-up during the course of INH therapy is simple with application to field settings. Due to simplicity of the testing procedure the patient can be trained to report the follow ups. The spot test strip eliminates the time bound degradation of INH in the samples and can be done in field
settings. This test is a simple aid for personalizing the medicine of the patients in the future. Also the same strips can be utilized to monitor compliance with the therapy among the patients. However, testing capability for AcINH could not be achieved in this study.