Monaldi Archives for Chest Disease

eISSN 2532-5264 https://www.monaldi-archives.org/

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Monaldi Arch Chest Dis 2024 [Online ahead of print]

To cite this Article:
Munshi R, Panchal F, Desai U, et al. **A study of N-acetyltransferase 2 gene polymorphisms in the Indian population and its relationship with serum isoniazid concentrations in a cohort of tuberculosis patients.** *Monaldi Arch Chest Dis* doi: 10.4081/monaldi.2024.3181

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A study of N-acetyltransferase 2 gene polymorphisms in the Indian population and its relationship with serum isoniazid concentrations in a cohort of tuberculosis patients

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Contributions: RM, conceptualization, validation, data analysis, manuscript writing - review & editing, supervision of the project, project administration and funding acquisition; FP, conceptualization, methodology, literature search, validation, formal analysis, manuscript writing original draft, visualization; UD, KU, recruitment and clinical management of patients, manuscript writing and reviewing; KR, performed the Investigations, data analysis, manuscript writing. All the authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors declare that there is no conflict of interest.

Ethics approval and consent to participate: the study protocol was reviewed and approved by the Institutional Ethics Committee (approval no. ECARP/2019/140 dated 23/01/20).

Patient consent for publication: written informed consent to participate was obtained from all the study participants.

Funding: the authors are thankful to our Institution for the financial support received from the Institutional Research Society.

Availability of data and materials: all data generated or analyzed during this study are included in this published article. The data used to support the findings of this study are available from the corresponding author upon request.

Acknowledgments: the authors would like to thank Nadiya Mirza, Alifiya Balsara, Susan martin, Aarti Nair, Sonia Kanojiya for their technical support.

Abstract

The N-acetyltransferase 2 (*NAT2*) gene exhibits substantial genetic diversity, leading to distinct acetylator phenotypes among individuals. In this study, we determine *NAT2* gene polymorphisms in tuberculosis (TB) patients and analyze serum isoniazid (INH) concentrations across the various genotypes. An observational prospective cohort study involving 217 patients with pulmonary or extrapulmonary TB was carried out. The *NAT2* genotypes were identified using real-time polymerase chain reaction technology. INH concentrations at baseline and 2 hours post-dosing were estimated using high-performance liquid chromatography. The association between the acetylator status and INH concentrations was evaluated using odds ratios (OR) and the occurrence of adverse events across the different patient genotypes was also assessed. The genotype frequency of fast, intermediate, and slow acetylators was 7.37%, 39.17%, and 53.46%, respectively, while allele frequency was 27% for fast acetylators and 73% for slow acetylators. All the alleles followed the Hardy-Weinberg equilibrium. Patients with slow acetylator status had significantly increased serum INH concentrations 2 hours post-drug administration, followed by intermediate acetylators as compared to fast acetylators. 69 (31.8%) patients developed adverse drug reactions post-therapy. Patients with slow acetylator status had the highest (OR: 9.66) risk of developing drug-induced hepatoxicity, especially those with raised serum INH concentrations (OR: 1.34). Understanding the correlation between genetics and serum antitubercular drug levels in antitubercular drug-induced hepatotoxicity will provide valuable information to the medical community, minimizing the risk of adverse reactions and hospitalizations.

Key words: tuberculosis, serum isoniazid concentrations, NAT2 genetic polymorphisms, adverse drug reactions, drug-induced hepatotoxicity.

Introduction

India faces a significant public health challenge with tuberculosis (TB), having one of the highest TB burdens worldwide. The annual incidence of TB in India is about 2.7 million cases, which constitutes a large portion of the global TB cases. The standard TB treatment regimen spans 6 months, starting with a 2-month intensive phase involving isoniazid (INH), rifampicin, ethambutol, and pyrazinamide, followed by a 4-month continuation phase with INH and rifampicin, sometimes including ethambutol [1]. Despite its effectiveness, treatment failures occur due to suboptimal drug levels, antibiotic resistance, and adverse events.

Common adverse drug reactions (ADRs) of antitubercular drugs (ATDs) include gastrointestinal issues like nausea and vomiting. Drug-induced hepatotoxicity is another risk, with incidence rates varying between 2-39% globally [2]. Studies indicate a higher incidence of drug-induced hepatotoxicity in the Indian population compared to Western countries. ADRs can reduce treatment adherence, leading to therapy failure, relapse, or drug resistance. INH and Rifampicin are major contributors to hepatotoxicity in TB treatment.

Isoniazid is bactericidal, reducing the bacterial load by 90-95% within the first 2 days of treatment [3]. Its therapeutic range is 3-6µg/ml. The NAT2 gene, located on chromosome 8p22, encodes the N-acetyltransferase 2 enzyme, crucial for the metabolism of drugs like isoniazid [4]. NAT2 gene polymorphisms result in different acetylator phenotypes: fast (NAT2*4), intermediate, and slow (NAT2*5, NAT2*6, and NAT2*7) acetylators. Fast acetylators metabolize INH rapidly, risking treatment failure, and may need higher doses [5]. Slow acetylators metabolize INH slowly, increasing serum INH concentrations and the risk of hepatotoxicity. Identifying NAT2 polymorphisms is essential for personalized medicine, influencing drug dosing, efficacy, and toxicity.

Studies have reported that comorbid conditions such as HIV, diabetes, hypertension, and demographic factors like age, sex, body weight, and alcohol use can affect serum ATD levels [6-9]. Thus, therapeutic drug monitoring (TDM) of ATDs 2 hours post-drug administration becomes as peak serum concentrations of ATDs are reached around this time [10]. Research has shown that individuals with slow acetylator status are more likely to experience isoniazid (INH)-induced liver disorders, particularly in Asian populations. Nevertheless, inconsistent findings across studies have led to ongoing debate regarding this association. The influence of genetic polymorphisms on the hepatotoxic effects of antitubercular drugs (ATDs) in the Indian population, which is at a heightened risk for such adverse reactions, remains underexplored.

Till date, there are very few studies that have explored the genotype- phenotype association in Indian TB patients. This study thus aimed to genotype TB patients from Mumbai for variations in the NAT2 gene. The study also compared serum INH concentrations across the different genotypes and evaluated the incidence of adverse events among the various genotypes following the initiation of ATD therapy.

Materials and Methods

Study design

This was a prospective observational cohort study.

Study participants

Patients with pulmonary or extrapulmonary TB of any gender aged between 18 to 65 years, who were already initiated or were to be initiated on antitubercular drugs as per National Tuberculosis Elimination Program (NTEP) program and whose liver functions tests (LFT) were within the normal ranges and who were willing to participate were recruited from the Revised National Tuberculosis Control Program (RNTCP) Centre and Pulmonary Medicine Department OPD of our hospital & allied municipal hospitals. Patients diagnosed with multidrug- resistant tuberculosis (MDR TB), abnormal liver function tests and pregnant or lactating women were not eligible to participate in the study.

Sample size estimation and sampling strategy

Considering that the drug-induced hepatotoxicity incidence rate ranges from 2% to 28% in TB patients receiving 1st line ATDs drugs, a midpoint incidence rate of 15% was used to calculate the sample size. This calculation resulted in a sample size of 196 patients. To account for an anticipated 10% dropout rate, 217 patients with pulmonary or extrapulmonary tuberculosis were enrolled in the study

Study procedures

The study was commenced after receiving the approval from the Institutional Ethics Committee (approval no. ECARP/2019/140) in accordance with Indian Good Clinical Practice guidelines (2001), Declaration of Helsinki principles (2018) and ICMR National Ethical Guidelines for Biomedical and Health Research Involving Human Participants (2017). Patients fulfilling the

eligibility criteria and ready to give written informed consent were requested to come to the Clinical Pharmacology department for the study related investigations. Detailed medical history along with co-morbid conditions, past history of TB, family history of TB, date of initiation of ATDs were documented. Also, patients were followed up till they completed the ATD course for the development of any adverse events if any. 10 ml blood was withdrawn from the patients who had consumed their ATDs doses for at least 15 days, at 0 hours (prior drug administration), of which 6 ml was collected in plain tubes for the estimation of the serum INH concentrations and LFT and remaining 4 ml was collected in EDTA tubes for the NAT2 genotyping. The compliance to ATD treatment was checked via telephonic follow ups with the study patients on a monthly basis after the initiation of therapy till completion of treatment duration. A second blood sample of 6 ml was collected 2 hours after drug consumption to estimate peak serum INH concentrations. The blood samples were centrifuged at 3000rpm for 15 minutes and serum obtained was separated and stored at -80^{0}C until the samples were processed for serum INH concentrations.

Determination of NAT2 genetic polymorphisms

Genomic DNA was isolated from whole blood using a Phenol chloroform method. Quantification of DNA was done using Eppendorf Nanodrop spectrophotometer and was stored at -80^{0}C until analysis. Three SNPs in the NAT2 gene NAT2*5 C481T (rs1799929), NAT2*6 G590A (rs1799930) and NAT2*7 G857T (rs1799931) were analyzed using Taqman SNP genotyping assay on Applied Biosystem Quantstudio3 Real Time PCR system [11]. The reaction mixture for the TaqMan genotyping assay included the TaqMan genotyping master mix, assay probes, and 20 ng of genomic DNA. The polymerase chain reaction (PCR) protocol consisted of the following stages: a pre-read stage at 60°C for 30 seconds, an initial holding stage at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 65°C for 1 minute. The post-PCR read stage was conducted at 60°C for 30 seconds. Alleles were determined using Quant Studio design and analysis software, which identifies the probe labels with either VIC or FAM reporter dyes.

Determination of serum concentrations of INH

Serum samples were analyzed for INH concentrations using high-performance liquid chromatography (HPLC). Stock solutions of Isoniazid and the internal standard (β-

Naphthoflavone) were prepared by dissolving each in methanol to achieve a concentration of 1 mg/ml, which were then diluted to create working standard solutions. Serum samples from both standards and patients were spiked with the internal standard. Subsequently, 450µl of acetonitrile was added, and the mixture was vortexed and centrifuged at 10,000 rpm for 15 minutes. The supernatant was then injected into the HPLC system, equipped with a C18 column and a mobile phase composed of acetonitrile, potassium dihydrogen phosphate buffer, and trifluoroacetic acid (TFA), monitored at a wavelength of 205 nm. The chromatographic run time was 8 minutes at a flow rate of 1.0 ml/min at room temperature. INH and the internal standard had retention times of 2.5 minutes and 7.9 minutes, respectively. This method was validated and exhibited linearity with a correlation coefficient (r) of 0.99 within the concentration range of 2-10µg/ml. The therapeutic range for INH is 3-6µg/ml.

Determination of serum liver enzymes

Serum obtained from the plain tube was used for Liver Function tests (Serum Bilirubin, SGOT and SGPT) using commercially available Meril kits on Fully Automated Biochemistry analyzer.

Statistical analysis

Data analysis was conducted using GraphPad InStat software (Version 3.06). For non-normally distributed data, the median and interquartile range (IQR) have been reported. The Kruskal–Wallis post hoc test was used to examine differences in serum INH concentrations at 0 and 2 hours across the various genotypes. Allelic and genotypic frequencies were determined through the counting method and assessed for Hardy-Weinberg equilibrium using the Chi-square test. The odds ratio for the relationship between genotype, drug-induced hepatotoxicity, and serum INH concentration at 2 hours was calculated.

Results

Clinical demographics of enrolled patients

Two hundred and seventeen patients with pulmonary and extrapulmonary TB with median age ranging from 20-37 years were enrolled in the cohort with a predominance of female patients.

Majority of the patients had consumed ATD for at least 15 days [75% (163 patients)] prior to study participation and the remaining 25% of patients (n=54) who were yet to be initiated on

ATD therapy were requested to come for participation in the study after the taking the ATD for minimum period of 15 days.

The median duration of treatment in patients with TB depended on the site of the infection. The duration of treatment was 6 months in case of Pulmonary TB & 12-18 months in cases of extrapulmonary TB like CNS TB or Pott's spine. If issues of compliance or resistance were detected in these patients, the duration was prolonged till there was clinical improvement and/or M.tb was not detected in their samples.

No difference in weight and age was recorded amongst the 3 acetylators groups. 69 (31.8%) TB patients exhibited adverse effects due to antitubercular drugs. Amongst the various adverse events documented, the majority of TB patients (n=26) with slow acetylators & intermediate acetylators (n=16) developed drug induced hepatotoxicity (deranged transaminases) followed by gastrointestinal disturbances in 11 and 6 patients with slow acetylators & intermediate acetylators. No significant difference in the median INH dose was observed across the acetylator status. Significant increase in serum INH concentrations at 0 hours and 2 hours was observed in the slow acetylators (SA) group in comparison to fast acetylators (FA) group and increased INH level at 2 hours was observed in the slow acetylators (SA) group when compared to intermediate acetylators (IA) (*Supplementary Table 1*).

Allelic and genotypic frequencies of NAT2 polymorphism

In the present cohort of TB patients residing in Mumbai, four alleles i.e. NAT2*4 (Wild allele), NAT2*5 C481T (rs1799929), NAT2*6 G590A (rs1799930), NAT2*7 G857A (rs1799931) of the NAT2 gene were studied. The minor allele frequency for all the alleles were calculated. (*Supplementary Table 2*). The allele frequency for wild and mutant allele of NAT2*5 C481T, NAT2*6 G590A and NAT2*7 G857A are 66%, 67%, 92% and 34%, 33%, 8% respectively. 10 different genotypes from the NAT2 four alleles were observed in the study. The genotype frequently observed in this cohort was NAT2*5/*6 followed by NAT2*4/*5, NAT2*4/*6, NAT2*6/*6 and NAT2*5/*5. Fast, intermediate and slow acetylators had genotype frequency of 7.37%, 39.17% and 53.46% respectively. The allele frequency for fast acetylators was 27% and 73% for slow acetylators. All the alleles followed the Hardy-Weinberg equilibrium. (*Supplementary Table 3*).

Association between serum INH concentration and NAT2 genotype

The patients with slow acetylator showed maximum and significantly increased serum INH concentrations post 2 hours of drug administration followed by intermediate acetylators. (Figure 1) Whereas patients with fast acetylators showed the lowest median serum INH concentration. Across the various genotypes of NAT2 gene NAT2*5/*7 showed maximum median INH concentration post 2 hours of drug administration (13.36µg/ml) followed by NAT2*5/*5 and NAT2*7/*7 (*Supplementary Table 3*).

Antitubercular drugs induced adverse drug reactions

Patients consuming ATDs were followed up till they completed therapy. Whenever any patient developed any adverse reaction/s during the study period, the details of the ADR were captured from the date of occurrence to resolution and the management of the same. In the present cohort the average duration for development of ADRs was between 15-45 days. Of the 217 TB patients enrolled in the study, 69 (31.8%) patients developed ADRs (Figure 2). Of these, 42 (61%) reported drug induced hepatotoxicity followed by gastrointestinal disturbances 19 (28%), skin related issues 5 (7%), blurred vision in 2 (3%) and 1(1%) patient developed convulsions. (Figure 3) These adverse effects were resolved on stoppage of medications. Resolution of adverse effect of ATD in our patients was confirmed by monitoring the liver enzymes in case of drug induced hepatitis and in case of other adverse effects, the patients were monitored clinically with reference to the respective specialty physician wherever needed.

Association between drug induced hepatotoxicity and NAT2 acetylator status

Drug-induced hepatotoxicity is defined according to the guidelines established by the American Thoracic Society [12,13]. The odds ratio shows that the risk of developing drug-induced hepatotoxicity was highest in patients with slow acetylator status (9.66) compared to fast acetylators followed by individuals with a combination of intermediate and slow acetylator status. Under the recessive model, slow acetylators demonstrated a 1.53 times greater risk for drug-induced hepatotoxicity compared to the combined group of fast and intermediate acetylators (*Supplementary Table 4*).

Association between INH concentration 2 hours post drug administration and NAT2 acetylator status

As per reported literature it is documented that the therapeutic range of serum INH is 3-6μg/ml. Thus, the association between NAT2 acetylators and serum INH concentrations at 2 hours post drug administration was evaluated by considering serum INH concentrations below and above the therapeutic range. While considering the recessive model, it was noted that patients with slow acetylators had 1.4 times risk of higher serum INH concentration in comparison to fast and intermediate acetylators. Similarly, when considering the dominant model, patients with slow acetylators also demonstrated 1.34 times the risk of raised serum INH concentrations when compared to fast acetylators (*Supplementary Table 4*).

Discussion

Isoniazid undergoes hepatic metabolism through the enzyme NAT2. Variations in the NAT2 gene can significantly affect the metabolic rate of INH, thereby influencing both its therapeutic efficacy and the likelihood of adverse effects. This study aimed to analyze the genotypic and allelic frequencies of NAT2 mutations in a group of TB patients undergoing antitubercular treatment. Additionally, we measured serum INH concentrations 2 hours post drug administration to investigate the correlation between NAT2 genotype and INH metabolism.

While standard antitubercular therapy is generally effective, managing adverse drug reactions remains a significant challenge. The role of genetic polymorphisms in ATD-induced hepatotoxicity is not fully established in the Indian population, who have a higher susceptibility to such adverse effects. Consequently, patients were monitored throughout the treatment period to detect any emerging toxicities.

The median dose of INH used in this study was 5.0 mg/kg of body weight, aligning with the World Health Organization (WHO) 2022 guidelines, which recommend a dosage range of 4-6 mg/kg/day for TB patients [14]. This study examined the genotypes NAT2*5 C481T, NAT2*6 G590A and NAT2*7 G857A in a group of TB patients using real-time polymerase chain reaction (RT-PCR) technology. The minor allele frequencies (MAF) observed for NAT2*5, NAT2*6, and NAT2*7 were 34%, 33%, and 8% respectively. These allele frequencies vary among different populations globally, including those in India and other countries.

Kumar et al. reported frequencies of 29%, 39%, and 7% for NAT2*5, NAT2*6, and NAT2*7, respectively, in the Indian population [15]. In North India, the frequencies were 27.6%, 42.8%,

and 8.4%, respectively, aligning with our study's minor allele frequencies (MAF). Internationally, frequencies for NAT2*6 and NAT2*7 were 22.4% and 13.2% in South Korea, 28.5% and 2.9% in Caucasians, and 26.0% and 2.8% in Egyptians [16-18].

In our study, the genotype frequencies of slow, intermediate, and fast acetylators were 53.46%, 39.17%, and 7.37%, respectively. These figures are similar to those reported in the South Indian population (58%, 35%, and 7%). Yadav et al. observed frequencies of 62% for slow, 34% for intermediate, and 4% for fast acetylators in Eastern Uttar Pradesh [19]. Conversely, Rana et al. found a higher prevalence of fast acetylators (37.05%) and a lower frequency of slow acetylators (19.52%) [20].Jain et al. also noted a higher prevalence of fast and intermediate acetylators and a lower prevalence of slow acetylators in North India, which differed from our study [21]. The frequency of slow acetylators observed in our study is comparable to American [22] (55.9%) and UK Caucasian (66.1%) populations [23]. These variations highlight the genetic heterogeneity among different populations, suggesting that results from one group may not be applicable to others.

INH achieves peak concentration around 2 hours post-drug administration with a therapeutic range of 3-6μg/ml [10]. Hence in our study, serum INH concentrations, post 2 hours drug administration were analyzed in all patients. A significant difference in serum INH concentrations was documented across the 3 acetylators status with slow acetylators showing maximum serum concentration in comparison to fast acetylators. Similar trends have been reported in other studies and our results are in concordance with the reported results. Higher INH concentrations in individuals with NAT2 SA or IA genotypes may lead to an increased risk of drug toxicity [15, 24-27]. Slow acetylators and intermediate acetylators metabolize INH at a slower rate, potentially resulting in the accumulation of the drug in the body when standard doses are administered. Differences in an individual's response to drugs can lead to adverse drug reactions, ineffective treatments, or increased susceptibility to other diseases. Similarly, genetic variations in the NAT2 gene can influence how different populations metabolize isoniazid.

The relationship between NAT2 genotypes, serum INH concentrations, and drug-induced hepatotoxicity has been extensively documented in numerous studies [4,24-26]. In our study, 26% of slow acetylators developed drug-induced hepatotoxicity, aligning with the reported incidence range of 2-39% across various countries [2]. The Indian sub-population however have shown a higher incidence of hepatotoxicity with antitubercular drugs as compared to

Western populations [28,29]. Our study has its limitations. In the present study we have only looked at some of the NAT2 alleles. Additionally, there are other genes too involved in INH metabolism. Also, serum levels of Isoniazid metabolites were not estimated in the study.

Conclusions

Knowledge of the genetic determinants that affect drug metabolism is essential to predict how individuals will respond to different medications which will help in guaranteeing effective therapy. Investigating the genetic mechanisms underlying anti-tubercular drug-induced hepatotoxicity and correlating these findings with serum drug levels is clinically important. This knowledge can provide the medical community with critical insights prior to initiating therapy, thus minimizing the risk of adverse reactions and hospitalizations.

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Online supplementary material:
Supplementary Table 1. Clinical data of tuberculosis patients as per their acetylator status.
Supplementary Table 2. Allelic and genotypic frequencies of *NAT2* polymorphism in tuberculosis patients (n=217).
Supplementary Table 3. Allelic, genotype frequency and serum INH concentrations of *NAT2* acetylators in Indian tuberculosis patients (n=217).
Supplementary Table 4. Relationship between *NAT2* acetylator and development of drug induced hepatoxicity and serum INH concentrations.

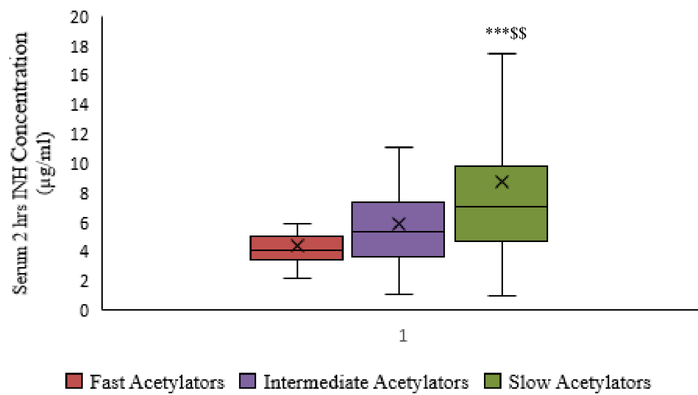

Figure 1. Serum INH concentrations amongst the NAT2 Acetylator genotype groups. The data is displayed using a box and whisker plot format depicting median and interquartile range (IQR). Significance levels are indicated as follows: *p<0.05; ***p<0.001 in comparison to Fast Acetylators and $^{\$\$}$p<0.01in comparison to Intermediate acetylators, utilizing the Kruskal-Wallis test followed by Dunn's multiple comparison test.

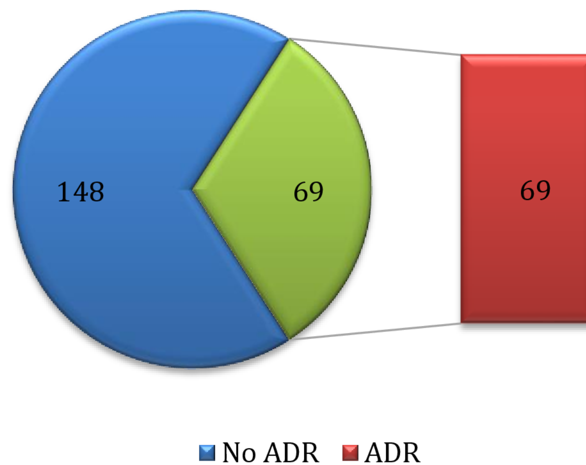

Figure. 2 Adverse drug reactions (ADRs) developed in TB patient receiving antitubercular drugs (n=69). The data is represented as number of patients.

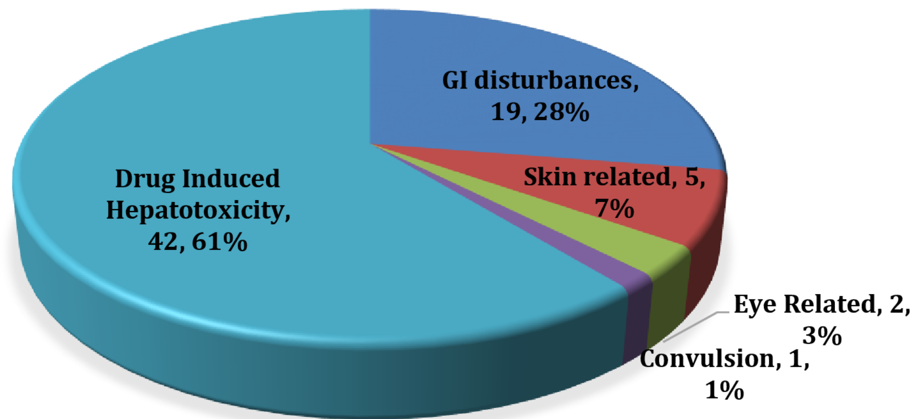

Figure. 3 Distributions of ADRs reported in a group of TB patients. The data is represented as number of patients and in percentage.