Pharmacogenetic study of 6-Mercaptopurine and L-Asparaginase based chemotherapy in pediatric Acute Lymphoblastic Leukemia patients in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia

Awol Mekonnen Ali

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A Thesis submitted to the School of Graduate Studies of Addis Ababa University, in Partial Fulfillment for the requirements of the Degree of Doctor of Philosophy in Pharmacology.

By: Awol Mekonnen Ali

Advisor: Prof. Tefera Abula

Co-Advisors: Dr. Raweligh Howe

Dr. Haileyesus Adam

Dr. Marieke Coenen

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DECLARATION

I, the undersigned, declare that this Ph.D. thesis is my original research work and has not been presented for a degree in any other university.

Name: Awol Mekonnen Ali

Signature: _____

This thesis has been submitted for examination with approval as Principal Supervisor.
Name: Professor Tefera Abula

Signature: _____

Place and date of submission Addis Ababa, Ethiopia, December, 2023

Approved by the examining Board:

Name	Date	Signature
Prof. Tefera Abula (Advisor)	_____	_____
Dr. Haileyesus Adam (Co-Advisors)	_____	_____
Dr. Rawleigh Howe (Co-Advisors)	_____	_____
Dr. Marieke Coenen (Co-Advisors)	_____	_____
Dr. Abiy Habtewold (External Examiner)	_____	_____
Dr. Teshome Nedi (Internal Examiner)	_____	_____
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Abstract

Background

Acute lymphoblastic leukemia is the most common type of childhood cancer, necessitating a tailored combination of chemotherapy. However, dose-limiting hematotoxicity is a significant challenge. Genetic variations, in the thiopurine metabolic pathway gene, can predict toxicities related to 6-mercaptopurine. While L-asparaginase is crucial in treating childhood ALL, it can cause hypersensitivity reactions and hepatotoxicity. Reports have suggested that genetic variants in the *CNOT3*, *GRIA1*, and *NFATC2* genes may contribute to hypersensitivity reactions, while *PNPLA3* has been linked to hepatotoxicity.

Objective

The objective of this thesis was to examine the frequency of genetic variants of drug metabolizing enzyme and transporter polymorphisms and their association with the occurrence of adverse events during 6-MP and L-ASP based chemotherapy in pediatric acute lymphoblastic leukemia patients at TASH, Addis Ababa, Ethiopia.

Methods

A Cohort study was used and the clinical profile of the patients was collected for the first 6 months of the maintenance phase treatment. Genotyping of *GRIA1* rs4958351, *PNPLA3* rs738409, *ITPA*, and *XDH* was performed using KASP genotyping assay, while that of *CNOT3* rs73062673, and *NFATC2* rs6021191, *TPMT*, *NUDT15*, and *ABCB1* with TaqMan® SNP genotyping assays. TPMT activity was measured in whole blood of the participant by HPLC.

Results

During the initial six months of the maintenance phase treatment, 52.8% of the patients experienced grade 4 neutropenia. The risk of developing neutropenia was found to be higher in children aged six years or less and those with low day 1 maintenance white blood cell counts. Furthermore, specific genetic variants in *ITPA* and *XDH*, were associated with 6-MP induced neutropenic fever and grade 4 neutropenia, respectively. Notably, patients with the CC genotype of *XDH* rs2281547 were found to have a nearly threefold increased risk of developing grade 4 neutropenia compared to individuals with the TT genotype (AHR 2.956, 95% CI=1.494-5.849, p = 0.002). Additionally, it was observed that 12.5% of the patients developed L-ASP hypersensitivity, but there were no significant differences between the frequency of hypersensitivity reactions and the risk alleles of the investigated genes.

Conclusion

In ALL patients, the genetic variant *XDH* rs2281547 was found to be a predictive factor for hematologic grade 4 toxicities caused by 6-MP. To detect hematotoxicity early, it is recommended to closely monitor the levels of white blood cells and neutrophils throughout the maintenance treatment. Furthermore, it is important to consider genetic variations apart from the *TPMT* gene that play a role in the metabolic pathway of 6-mercaptopurine in order to prevent hematological toxicity.

List of scientific papers

1. Awol Mekonnen Ali, Haileyesus Adam, Daniel Hailu, Marieke J.H. Coenen, Rawleigh Howe, Teferra Abula. **Incidence and determinants of hematotoxicity in Acute Lymphoblastic Leukemia children who received 6-Mercaptopurine based maintenance therapy in Addis Ababa, Ethiopia**. *PLoS ONE* **18**(6): e0286544. https://doi.org/10.1371/journal. pone.0286544

2. Awol Mekonnen Ali, Haileyesus Adam, Daniel Hailu, Ephrem Engidawork, Rawleigh Howe, Teferra Abula, and Marieke J. H. Coenen. **Genetic variants of genes involved in thiopurine metabolism pathway are associated with 6-mercaptopurine toxicity in pediatric acute lymphoblastic leukemia patients from Ethiopia**. *Front. Pharmacol*. **14**:1159307. doi: 10.3389/fphar.2023.1159307

3. Awol Mekonnen Ali, Haileyesus Adam, Daniel Hailu, Johanne Groothuismink, Marieke J.H. Coenen, Rawleigh Howe, Teferra Abula. **Relationship between thiopurine S-methyltransferase genotype and phenotype in pediatric acute lymphoblastic leukemia in Addis Ababa, Ethiopia**. BMC research note.

4. Awol Mekonnen Ali, Haileyesus Adam, Daniel Hailu, Rawleigh Howe, Teferra Abula, Marieke J.H. Coenen. **Evaluating the frequencies of *CNOT3, GRIA1, NFATC2,* and *PNPLA3* variant alleles and their association with L-asparaginase hypersensitivity in pediatric acute lymphoblastic leukemia in Addis Ababa, Ethiopia.** The Application of Clinical Genetics 2023:**16** 131–137. https://doi.org/10.2147/TACG.S404695

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List of acronyms and abbreviations

6-dTGDP	Deoxy-6-thioguanine diphosphate
6-dTGTP	Deoxy-6-thioguanine triphosphate
6-meMPR	6-Methylmercaptopurine riboside
6-MeMP	6-Methylmercaptopurine
6-MP	6-Mercaptopurine
6-MTG	6-Methyl thioguanine
6-TGN	6-Thioguanine
6-TIMP	6-Thioinosine monophosphate
ABC	ATP-binding cassette
ALL	Acute lymphoblastic leukemia
AO	Aldehyde oxidase
ANC*s*	Absolute neutrophil counts
BAZ	Body mass index for age Z score
BSA	Body surface area
CBC	Complete blood count
CNOT3	CCR4-NOT transcription complex subunit 3
CNS	Central nervous system
CTCAE	Common toxicity criteria for adverse events
DI	Dose intensity
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
FDA	US food and drug administration
GI	Gastrointestinal
GMPS	Guanosine monophosphate synthetase
GWAS	Genomewide association studies
HAZ	Height-for-age z-scores
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HPLC	High-performance liquid chromatography
HR	High Risk
IM	Intramuscular
IQR	Interquartile range
IR	Intermediate Risk
IV	Intravenous
IMPDH	Inosine monophosphate dehydrogenase type
IMPDH	Inosine 5´-monophosphate dehydrogenase
ITPA	Inosine triphosphatase pyrophosphatase
KOD	KASPar-On-Demand
L-ASP	L-asparaginase
LMICs	Low- and middle income countries
MAF	Minor allele frequency
MGL	Morisky, green, and levine medication adherence Qquestionnaire
MTX	Methotrexate
NFATC2	Nuclear factor of activated T cells 2
NUDT15	Nudix hydrolase 15

P-gp	P-glycoprotein
PGx	Pharmacogenetic
PNPLA3	Patatin-like phospholipase domain containing protein 3
RDI	Relative dose intensity
ROC	Receiver operating characteristics curve
SAM	S-adenosyl-L-methionine
SDs	Standard deviations
SLC28A3	Solute carrier family 28 member 3
SOPs	Standard operating procedures
SR	Standard risk
TASH	Tikur anbessa specialized hospital
TIMP	Thioinosine monophosphate
TPMT	Thiopurine methyltransferase
WAZ	Weight-for-age z-scores
WBC	White blood cell count
WHO	World health organization
WHZ	Weight-for-height z-scores
XO	Xanthine oxidase
XDH	Xanthine dehydrogenase
XTP	Xanthosine triphosphate

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1 Introduction

1.1 Cancer epidemiology

Cancer is a major hindrance to increasing global life expectancy and stands as the primary factor contributing to mortality on a global scale. According to Sung *et al*. (2021), the global incidence of cancer in 2020 was estimated to be 19.3 million new cases, resulting in 10 million cancer-related deaths. Cancer accounts for more than one in six deaths worldwide and is responsible for approximately 25% (5.1 million) of premature deaths before the age of 70 in 2019 (WHO, 2021). In the same year, there were 474,519 newly diagnosed cases and 311,594 deaths from leukemia worldwide. In Africa, the proportion of cancer incidence is 5.7%, accounting for about 1.1 million cancer cases by 2020. However, the share of cancer deaths in Africa is higher at 7.2% compared to the incidence rate. Among the most predominant cancers are female breast cancer (11.7%), lung cancer (11.4%), colorectal cancer (10.0%), prostate cancer (7.3%), and stomach cancer (5.6%) (Sung *et al*., 2021).

The occurrence of new cancer cases in Africa is projected to increase by 70% by 2030 because of aging and population growth. This highlights the need to effectively address cancer as an emerging health problem to control rising rates of incidence and mortality (Hamdi *et al*., 2021). Breast cancer, accounting for approximately 27.7% of total cancer cases, and cervical cancer, accounting for 19.6%, are the most common estimated cancer burden among African females. Among African males, prostate cancer (18.1%), liver cancer (9.7%), and colorectal cancer (6.9%) are the most prevalent cancers (Bahnassy *et al*., 2020). Cancer related deaths exceeded those of tuberculosis, malaria, and AIDS combined, with projections indicating that cancer-related deaths in Africa will surpass the global average by 30% within the next two decades (Hamdi *et al*., 2021). In Ethiopia, although a national figure of cancer prevalence is lacking, breast cancer (20.9%) is the primary type, followed by cervical cancer (9.6%), colorectal cancer (7.8%), and leukemia (5.6%), according to the Addis Ababa city cancer registry (GLOBOCAN 2020, 2021).

Pediatric cancers are a major contributor to the global disease burden, with an estimated 400,000 children aged 0-19 years affected each year worldwide. Surprisingly, about 90% of these cases happen in low- and middle-income countries (LMICs), which not only

affect the child but also their families (WHO, 2021). Acute lymphoblastic leukemia (ALL) is the primary childhood cancer globally, constituting 19% of total childhood cancer incidence (Johnston *et al*., 2021; WHO, 2021). Childhood cancer survival rate in African children is lower, reaching only 20%, in comparison to high-income countries, where it stands at 80% (Bahnassy *et al*., 2020).

The estimated incidence of cancer in children annually in Ethiopia ranges between 3,707 and 6,000, with leukemia being the most common (29%). Non-Hodgkin's lymphoma, Wilms tumor, and retinoblastoma follow (Memirie *et al*., 2018; Shad *et al*., 2013). Among all leukemia cases in children, acute leukemia accounted for 89%, with acute lymphocytic leukemia accounting for 91% and acute myeloid leukemia accounting for the remaining 9% (Memirie *et al*., 2018).

1.2 Acute lymphoblastic leukemia

ALL is a blood cancer in which lymphoid precursor cells undergo uncontrolled proliferation, resulting in an accumulation of abnormal cells that are blocked at an early stage of differentiation. This malignant transformation is believed to arise from somatic mutations in a single B- or T-lymphocyte progenitor cell (Amiwero *et al*., 2014). It is lymphoid neoplasms that show similarities in morphology and immunophenotype to B-lineage and T-lineage precursor cells. The classification of ALL involves three subtypes, L1, L2, and L3, which are determined by the visual characteristics of the cancerous cells (Onciu, 2009).

1.3 ALL risk factors

Despite significant progress in treating ALL with high cure rates, the cause of the disease remains unclear. Proposed factors contributing to the development of ALL include various genetic and environmental factors, but none have been definitively proven (Tebbi, 2021). While several genetic factors have been linked to the risk of ALL, a large number of patients do not possess any identified inherited factors. Genome-wide association studies (GWAS) have revealed that CCAAT enhancer binding protein epsilon, GATA Binding Protein 3, AT-rich interaction domain 5B, and DNA-binding protein ikaros genetic variants have been linked to an increased susceptibility to developing ALL. Rare germline mutations in ETS family transcription factor 6 and

Paired Box 5 have also been linked to familial ALL (Hunger and Mullighan, 2015). Additionally, certain congenital syndromes like Bloom syndrome, Fanconi anemia, Down's syndrome, Ataxia telangiectasia, and Nijmegen breakage syndrome carry a higher risk of developing ALL (Malard and Mohamad, 2020).

Environmental risk factors, including exposure to traffic density, paternal smoking during preconception and childhood, solvents and petroleum, nuclear facilities, low doses of ionizing radiation, and extremely low-frequency electromagnetic fields during childhood are linked to ALL. Other factors such as general pesticide exposure, domestic painting, consumption of coffee and cola, diabetes during pregnancy, and maternal fertility treatment have also been related to a higher risk of childhood ALL (Onyije *et al*., 2022).

Gender, race, and ethnicity can also impact the incidence of ALL. The disease is more common among white individuals than black individuals, and it is more frequently diagnosed in boys than in girls (Hunger and Mullighan, 2015; Lim *et al*., 2014).

1.4 Risk classification and risk-adapted therapy

A good systemic risk-directed chemotherapy has greatly improved the cure rates for ALL, with most centers in developed countries achieving a cure rate of over 90%. However, resource-limited nations still experience significantly lower cure rates, which can vary from 20% to 70%, depending on the standard of treatment facilities (Bahnassy *et al*., 2020; Tandon, 2020). Several factors contribute to the differences in outcomes for ALL in low- and middle-income countries (LMICs) compared to developed countries, including limited resources for patients and healthcare professionals. This can lead to a delayed diagnosis and unfavorable medical consequences. Addressing these resource gaps is essential to improving outcomes for ALL patients in LMICs (Jabeen *et al*., 2016; Tandon, 2020).

The classification of disease risk and treatment intensity for ALL is primarily based on disease stage, which can be determined by several factors. In resource-limited nations, easily accessible factors such as age, physical examination findings, initial white blood cell count (WBC), central nervous system (CNS) status, and early response are used to categorize patients into three groups (Hunger *et al*., 2009). Accordingly, patients are

grouped into Standard Risk (SR), Intermediate Risk (IR), and High Risk (HR) groups (Navarrete *et al*., 2014).

The treatment for ALL is divided into different phases, each including different chemotherapy combinations adapted to the patient's risk classification. The chemotherapies used during each phase of treatment are outlined in Table 1. Following a steroid pre-phase, SR patients receive a three-drug induction treatment (vincristine, prednisone, and L-asparaginase) for four weeks, while HR patients receive additional doxorubicin. The objective of the induction treatment is to attain complete remission (CR), which is a normocellular bone marrow and shows no indications of the primary disease (Navarrete *et al*., 2014). After achieving CR, therapy is modified to eliminate any remaining submicroscopic cancer and prevent relapse. The dosage and drug regimen during the consolidation phase vary based on the patient's risk factors. HR patients receive more intensive consolidation treatment compared to SR patients, resulting in significantly improved outcomes for this group (Rudin et al., 2017).

The maintenance phase is the last and longest stage of ALL treatment (Hunger and Mullighan, 2015). This phase involves daily oral administration of 6-mercaptopurine (6-MP), Methotrexate (MTX) orally once a week, and monthly pulses of vincristine and steroids. Maintenance treatment lasts for duration of 2.5 years starting from the date of diagnosis and is critical to achieving long-term remission (Hunger *et al*., 2009). The duration of the maintenance phase is a crucial factor in determining treatment outcomes. Shortening the maintenance phase by even six months can significantly reduce patient response and increase the risk of relapse (Rudin *et al*., 2017).

Table 1. Acute lymphoblastic leukemia chemotherapy phases (Hunger *et al.,* 2009; Rudin *et al.,* 2017).

Phase	Length of Treatment	Purpose
Induction	4 weeks	Intense chemotherapy is administered to put the patient into clinical remission.
Consolidation	4-9 weeks	This is designed to eradicate any remaining leukemic lymphoblasts that may be present after clinical remission has been attained.

Interim Maintenance	8 weeks	Administered in order to maintain remission while allowing the bone marrow to recover.
Delayed Intensification	8 weeks	Chemotherapy is administered to sustain remission while allowing the patient's bone marrow time to recover from the intense therapy.
Maintenance	Until 30 months from the start of therapy	Less intensive continuation of the chemotherapy regimen, aimed to keep patients in remission.

1.5 6-mercaptopurine

6-MP is a thiopurine drug that contains a sulfur-containing group substituted in place of carbon 6 of the purine molecule (Rashidi *et al.* 2007). It received approval for its medical use in the United States in 1953 (Hayhoe, 1955) and was considered the most effective and safe medication (WHO, 2021). This drug has been used for several medical conditions, including cancer and autoimmune diseases (Hayhoe, 1955). Specifically, it has been used to treat chronic myeloid leukemia, ALL, ulcerative colitis and Crohn's disease (Nadhum *et al.,* 2020).

6-MP is the pillar of ALL maintenance treatment; in addition, it also plays a role in other phases (Lee *et al*., 2017). In most of the United States, the United Kingdom, and the Nordic countries, the oral 6MP starting dose is 75 mg/m^2/d, whereas in most of continental Europe it is 50 mg/m^2/d (Schmiegelow *et al*., 2014). Dosing of 6-MP and MTX varies based on body surface area (BSA) and clinical side effects, with the goal of maintaining a target absolute neutrophil count (ANC) (500/µL-1500/µL) and platelet count (>50,000/µL) to prevent myelosuppression. Dose adjustments are often needed to maintain remission and prevent disease relapse while avoiding severe side effects such as bleeding and infection (Lee *et al*., 2017). In childhood ALL, the intensity of 6-MP dosing can impact treatment outcomes, with higher dose intensity potentially leading to better outcomes (Karol *et al*., 2022; Relling *et al*., 1999). Dose intensity refers to the amount of medication given per unit of BSA per unit of time (Longo *et al*., 1991).

The mechanism of action of 6-MP/thiopurine involves three pathways (Lim and Chua, 2018). Firstly, deoxy-6-thioguanosine metabolites induce apoptosis by inhibiting DNA-processing enzymes. Secondly, thioguanosine triphosphate (TGTP) exerts inhibitory

effects on Rac1, a protein that plays a role in regulating T-lymphocyte proliferation and repressing immunity. Thirdly, phosphoribosyl pyrophosphate amidotransferase, which catalyzes the initial step of purine *de novo* synthesis, is inhibited by methyl-thioinosine monophosphate (meTIMP) (Lim and Chua, 2018; Nadhum *et al*., 2020).

The oral absorption of 6-MP is rapid, with maximum concentrations reached in about 1.5 hours and a short plasma half-life of 0.5-1.5 hours. 6-MP has incomplete and variable bioavailability (about 16–50%) majorly due to extensive intestinal and hepatic metabolism. It has moderate plasma protein binding (19–30%) and the steady state volume of distribution is around 0.56 L/kg (Kumar *et al*., 2015; Ogungbenro and Aarons, 2015). Following intravenous (IV) administration of 6-MP, it showed systemic clearance of 23.02 L/h (Jacqz-Aigrain *et al*., 1997). Unlike the parent drug, both 6-MMPR and 6-TGN metabolites have a long half-life of 5 days and steady-state concentrations achieved after 4 weeks (Derijks *et al*., 2004).

6-MP can cause several life-threatening side effects, including myelotoxicity, which is characterized by leukopenia, anemia, and thrombocytopenia and can occur at any time during treatment. The complete blood count (CBC) is closely monitored during treatment, and dosages are adjusted or interrupted when there are indications of a decline (Burchenal *et al*., 1953). Due to general immunosuppression, 6-MP can also cause viral and bacterial infections (Nielsen *et al*., 2001). Rarely, 6-MP can cause oral lesions (Burchenal *et al*., 1953). Although most patients do not encounter serious gastrointestinal (GI)-related adverse effects, individuals who do experience GI-related toxicities pose a distinct therapeutic challenge, as these toxicities result from a skewed metabolism of 6-MP leading to an accumulation of the 6-MMP metabolite (Conneely *et al*., 2020).

Thiopurine/6-MP is metabolized through a complex metabolic pathway involving three competing routes: hypoxanthine-guanine phosphoribosyltransferase (HGPRT), xanthine oxidase (XO), and thiopurine methyltransferase (TPMT) (Ogungbenro and Aarons, 2015).

1.5.1 Thiopurine metabolic pathway

6-MP is a pro-drug converted to active metabolites via extensive metabolism (Figure 2). Its transport into the cell involves solute carrier family 28 member 3 (SLC28A3),

SLC29A1, SLC28A2, and SLC29A2 (Zaza *et al*., 2010), and it can enter either catabolic or anabolic metabolic pathways (Rashidi *et al*., 2007). Catabolic metabolic pathways degrade 6-MP to inactive forms, which explains its short half-life. Xanthine oxidase (XO), aldehyde oxidase (AO), and xanthine dehydrogenase (XDH) convert 6-MP to 8-OH-6-MP or thioxanthine (2-OH-6-MP), which then form the inactive product thiouric acid via XO and XDH. Thiouric acid is excreted in the urine (Choughule *et al*., 2014; Rashidi *et al*., 2007). XO is expressed both in intestinal epithelial cells and the liver (Rashidi *et al*., 2007), while XDH is mainly expressed in the liver (Choughule *et al*., 2014). TPMT methylates 6-MP to produce 6-methyl MP, which is an inactive metabolite and not a substrate of HGPRT. TPMT competes with inosine monophosphate dehydrogenase (IMPDH) for their common substrate, 6-thioinosine monophosphate (6-TIMP), to form another major metabolite, methyl-thioinosine 5´-monophosphate (MeTIMP). MeTIMP effectively suppresses the process of *de novo* purine synthesis and is believed to substantially contribute to the cytotoxic effects of 6-MP (Krynetski *et al*. 1995).

The anabolic metabolic pathway of 6-MP leads to the production of its active metabolites (Choughule *et al*., 2014). HGPRT converts 6-MP to 6-thioinosine monophosphate (6-TIMP), which is further metabolized via inosine 5´-monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS) to produce the 6-thioguanine nucleotides (6-TGNs) (Coulthard *et al*., 2002; Munshi *et al*., 2014). The 6-TGNs include thioguanosine 5´-mono, -di, and -tri-phosphates, as well as deoxy-6-TGNs. Additionally, phosphorylation by kinases can lead to the formation of 6-thioinosine dip- and tri-phosphates from 6-TIMP. ITPA can dephosphorylate 6-thioinosine triphosphate (6-TITP) back to 6-TIMP (Al Hadithy *et al*., 2005). Nudix hydrolase 15 (NUDT15) is an enzyme that efficiently converts the thiopurine active metabolite 6-TGTP to 6-TGMP (Moriyama *et al*., 2016) and deoxy-TGTP to deoxy-TGMP (Valerie *et al*., 2016).

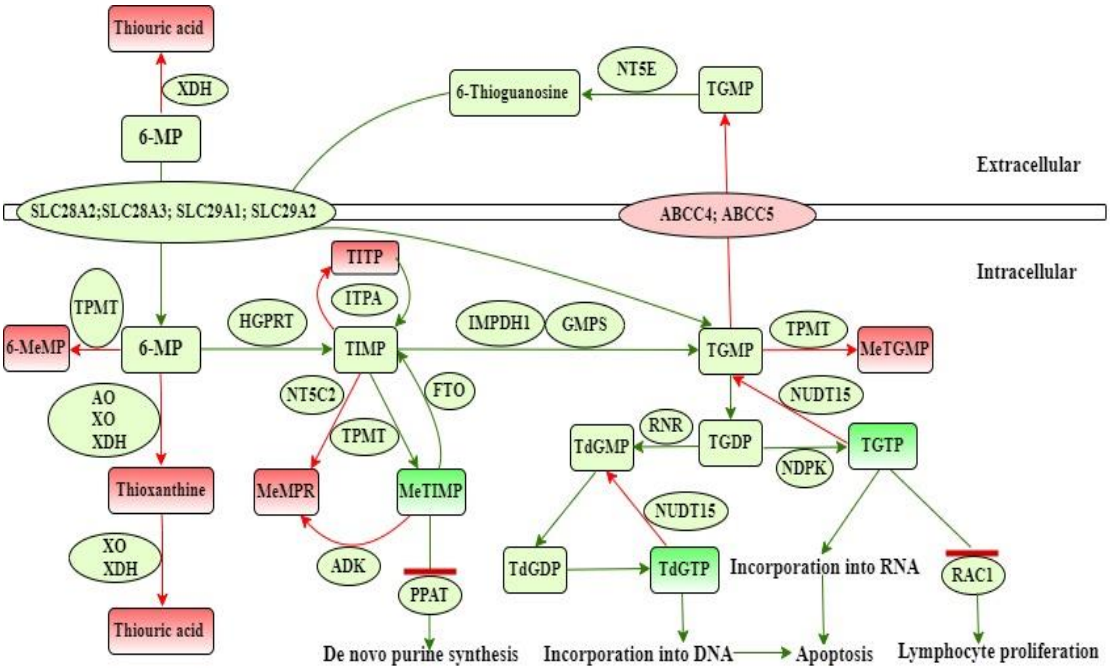

Figure 1: Thiopurine metabolism pathway and action (adopted from Lim and Chua, 2018). ecto-5´-nucleotidase (NT5E); fat mass and obesity-associated protein (FTO); nudix hydrolase 15 (NUDT15); hypoxanthine guanine phosphoribosyltransferase (HGPRT); ribonucleotide reductase (RNR); nucleotide diphosphate kinase (NDPK); inosine triphosphate pyrophosphatase (ITPA); inosine monophosphate dehydrogenase type 1 (IMPDH1); guanosine monophosphate synthetase (GMPS); thioinosine monophosphate (TIMP); thiopurine methyltransferase (TPMT); aldehyde oxidase type 1 (AOX1); phosphoribosyl pyrophosphate amidotransferase (PPAT); xanthine dehydrogenase (XDH); thioinosine triphosphoate (TITP); methyl-thioinosine monophosphate (meTIMP); thioguanine monophosphate (TGMP); methyl-mercaptopurine (meMP); methyl-thioguanine monophosphate (meTGMP); thioguanine diphosphate (TGDP); thioguanine triphosphate (TGTP); Adenosine kinase (ADK); thioxanthine (TX); methyl-mercaptopurine ribonucleotides (meMPR); deoxy-6-thioguanine diphosphate (6-dTGDP); Cytosolic 5´-nucleotidase II (NT5C2); deoxy-6-thioguanine triphosphate (6-dTGTP).

1.6 L-asparaginase

L-asparaginase (L-ASP) is an enzyme that has been used as a therapeutic agent with antineoplastic properties for nearly 40 years (Batool *et al*., 2016; Fu & Sakamoto, 2007). It is an established component in the treatment of acute leukemia and considered a cornerstone of ALL treatment protocols for both pediatric and adult patients. L-ASP is employed in pediatric regimens for remission induction and intensification treatment, as well as in the majority of adult treatment protocols (Pieters *et al*., 2011).

L-ASP is an amidohydrolase enzyme that has both L-asparaginase and L-glutaminase activities (Brumano *et al*., 2019). It hydrolyzes the amino acids L-asparagine and L-glutamine into aspartic acid and glutamic acid, respectively. L-ASP is present in mammals, rodents, birds, plants, yeast, and a wide range of bacteria but not found in humans (Cachumba *et al*., 2016; Holmquist, 1963). L-ASP is subdivided into type I and type II isozymes, both of which catalyze the deamidation of L-asparagine and L-glutamine. However, type II L-ASP exhibits a higher specific action against L-asparagine and shows the antitumor activity required for chemotherapeutic use in ALL (Campbell *et al*., 1967; Wriston, 1985).

Despite the presence of L-ASP in animals and plants, for medical use, only *E. coli* and *Erwinia chrysanthemi* are currently used as sources for its synthesis (Cachumba *et al*., 2016). In medical use, three L-ASP preparations are available: native L-ASP derived from *E. coli*, a pegylated form of native *E. coli* L-ASP (polyethylene glycol (PEG)-asparaginase), and a product isolated from *Erwinia chrysanthemi* (Brumano *et al*., 2019). *E. coli* possesses two types of L-ASP, EcA I and EcA II, with different characteristics. EcA I is constitutively expressed and found in the cytoplasm, whereas EcA II is an inducible enzyme located in the periplasm of bacteria and has a higher affinity for L-asparagine (Yun *et al*., 2007). PEG-asparaginase is created by the covalent linking of 5000-dalton units of monomethoxypolyethylene glycol to *E. coli* L-ASP, which has been developed to decrease immunogenicity of the L-ASP and prolong its plasma half-life (Avramis *et al*., 2002). PEG-asparaginase reduces the number of painful injections as it can replace up to 6-9 doses of native *E. coli* L-ASP (Fu & Sakamoto, 2007). *Erwinia chrysanthemi* L-ASP is less effective in hydrolyzing L-asparagine and should be used as a second line treatment if immunogenicity still arises in patients treated with PEG-asparaginase (Nguyen *et al*., 2016).

L-ASP is not administered orally due to presystemic degradation. *E. coli* L-ASP can be given intravenously or intramuscularly, while PEG-asparaginase and *Erwinia* L-ASP are preferably given by IM injection due to the potential for higher immunogenicity when administered intravenously (Cecconello *et al*., 2020). The half-life of *Erwinia* asparaginase is approximately ten hours, whereas *E. coli* L-ASP has a half-life of around 24 hours. PEG-asparaginase has a much longer half-life of approximately 15 days due to

its PEG group. The volume of distribution for all three preparations is slightly larger than the plasma volume (Keating *et al*., 1993). Neither the total enzyme nor fragments were detectable in the urine of patients collected within the first eight hours after L-ASP infusion. L-ASP is mostly eliminated by the reticuloendothelial system, with minimal enzyme activity (Brueck *et al*., 1989).

To achieve therapeutically sufficient asparagine depletion, serum asparaginase activity levels need to be maintained at or above 0.10 IU/mL (Vrooman *et al*., 2013). L-asparagine is an amino acid required for the synthesis of cellular proteins and cell survival. It can be synthesized by the body itself from aspartic acid and glutamine via L-asparagine synthetase within the cell or can be absorbed from the exogenous administration (El-Nagga *et al*., 2014). Unlike normal cells, tumor cells lack L-asparagine synthetase and can only synthesize L-asparagine slowly. Thus, they are dependent on an exogenous supply of L-asparagine (Chiu *et al*., 2020; Lee *et al*., 1989). Depletion of circulating L-asparagine starves the malignant cells, which are unable to complete protein synthesis, leading to the destruction of these malignant cells (El-Nagga *et al*., 2014).

Patients treated with L-ASP develop two types of adverse effects: the first is because of an immune response against the bacterial enzymes (Nguyen *et al*., 2016). All L-ASP, being large molecules of bacterial origin, often elicit an immune response with the production of anti-L-ASP antibodies (Kishore *et al*., 2015). The binding of these antibodies to the L-ASP molecule potentially decreases its enzymatic activity and initiates a number of downstream effects. Following L-ASP administration, the production of IgG and IgE antibodies has been related to the development of clinical allergy (Burke, 2014).

Immune responses to L-ASP can be classified into two types: clinical and subclinical hypersensitivity (silent inactivation) (Asselin and Fisher, 2014). Reduced L-ASP activity due to neutralizing antibodies has been seen in both types of hypersensitivity reactions. (Cecconello *et al*., 2020). In the literature, the rates of clinical hypersensitivity reactions vary in a range from 10–30% (Hijiya *et al*., 2016). Clinical hypersensitivity is described by symptoms that align with an immune response to a known antigen, commonly

manifested with a combination of symptoms that can range in severity from mild to severe (Burke, 2014). Mild-to-moderate allergic reactions [Common Toxicity Criteria for Adverse Events (CTCAE) Grades 1 and 2] are described by chills, flushing, fever, and dyspnea. Severe reactions (CTCAE Grades 3 and 4) can include angioedema, bronchospasm, and anaphylaxis (Asselin and Fisher, 2014; Burke, 2014).

The occurrence of hypersensitivity reactions to L-ASP can be influenced by a range of factors, such as the L-ASP preparation/formulation used, frequency and route of administration, concurrent chemotherapy, and timing of therapy (Burke, 2014). The IM administration has been associated with a higher incidence of skin reactions due to local tissue irritation caused by the injection (Pieters *et al*., 2011). Clinical hypersensitivity reactions to L-ASP predominantly manifest during the post-induction stages of treatment, suggesting that these reactions are more prevalent after a prolonged interruption in L-ASP therapy (Heo *et al*., 2014). The rarity of allergic reactions during induction chemotherapy can be attributed to various factors, including a delay in the immune response caused by the time required for antibody production and complement activation, the masking of allergic reaction symptoms by intensive corticosteroid treatment (Burke, 2014; Pieters *et al*., 2011), and the desensitizing effect of more intensive L-ASP dosing schedules (Pieters *et al*., 2011).

Subclinical hypersensitivity is characterized by decreasing L-ASP activity due to the production of anti-asparaginase antibodies (Asselin and Fisher, 2014). It has been reported that 8–29% of patients treated with *E. coli*-derived L-ASP experience subclinical hypersensitivity (Hijiya *et al*., 2016).

The second type of L-ASP treatment-related adverse effect is due to the enzyme's ability to hydrolyze the amino acid L-glutamine. Glutaminase activity of L-ASP has a potential role in the immunosuppressive effect of L-ASP prepared from *E. coli* and *Erwinia*, with glutaminase activity of 2 and 15%, respectively (Wriston, 1985). Nevertheless, L-ASP glutaminase activity has been linked with many of the side effects of this treatment, which include hepatotoxicity, pancreatitis, coagulation dysfunction, and immunosuppression (Nguyen *et al*., 2016).

1.7 Pharmacogenetics

Substantial variation between individuals in a certain population with regard to response and toxicity of different drugs have been observed (Kulkarni, 2016). Medications response differences have long been recognized by clinicians, but it was not until 1957 that Arno Motulsky published studies on drug response variations (Motulsky, 1957).

Pharmacogenetics (PGx) is concerned with the impact of genetic variation in drug metabolism, transport or molecular targets/pathways on variability of drug response (Kulkarni, 2016). Genetic variation refers to differences in nucleotide sequences in genes (Wright, 2005), which can affect protein activity and consequently influence drug exposure, treatment efficacy and toxicity (Hertz and Rae, 2015). Commonly studied genetic variants in PGx include SNPs, deletions, copy number variations, nucleotide insertions, tandem repeats, chromosomal translocations and gene expression of which SNP is the most common one (Weng *et al*., 2013). Mechanisms responsible for inactivation of drug-metabolizing enzymes include splice site mutations leading to exon skipping (e.g. *CYP2C19*), gene duplication (e.g. *CYP2D6*), microsatellite nucleotide repeats (e.g. *CYP2D6*), point mutations resulting in early stop codons (e.g. *CYP2D6*), altered promoter functions (e.g. *CYP2A5*), enhanced proteolysis (e.g. *TPMT*), critical amino acid substitutions (e.g. *CYP2D6*) or large gene deletions (e.g. *GSTM1*) (Evans and Johnson, 2001).

Modern therapeutics aims to use drugs with maximum benefit and minimum acceptable toxicity (Lee *et al*., 2005). To this end, many monogenic PGx variations have been identified, and the US Food and Drug Administration (FDA) has recommended pharmacogenomic consideration or package insert labeling for over 264 drugs in relation to genetic variations in more than 60 genes (Varnai *et al*., 2020). Oncology is the therapeutic area with the most drugs approved for PGx labeling (Kim *et al*., 2021). The efficacy and safety of various chemotherapeutic drugs in oncology show significant individual and population variability (Quinones & Lee, 2015), which can be attributed, in large part, to genetic variation that encodes drug targets, transporters and metabolizing enzymes, influencing pharmacokinetics and pharmacodynamics (Kulkarni, 2016). PGx approaches have been applied to many existing chemotherapy agents that are used for the

treatment of cancer in an effort to identify relevant inherited variations that may better predict patient response to chemotherapy (Lee *et al*., 2005).

PGx can be a valuable tool for dosage calibration in ALL treatment for several reasons (Rudin *et al*., 2017). ALL treatment drugs, including 6-MP and MTX, exhibit a limited therapeutic window (Lennard, 1999), which poses challenges in anticipating and mitigating potential toxic effects. Dose reduction poses a risk as it is commonly linked to decreased survival due to underdosage. Metabolizing enzymes genes of the drugs used in ALL treatments are highly variable (Lopez-Lopez *et al*., 2014). Several genetic variants have been identified that result in variations in expressed protein levels or activity within pathways, both between individuals and populations. The treatment response can be influenced by genetic variations that modify drug exposure or activity (Bárcenas-López *et al*., 2021).

1.7.1 Pharmacogenetics of 6-MP in ALL

In some patients, long-term use of 6-MP can lead to myelosuppression and hepatic toxicity, causing hospital admissions and treatment discontinuation (Lopez-Lopez *et al*., 2014). Therapeutic response or toxicity differences between individuals and populations can be partly attributed to the formation of variable quantities of the metabolites produced, which is influenced by genetic polymorphisms of genes encoding enzymes in thiopurine metabolism (Sousa *et al*., 2020). The clearance of both the pharmacologically active 6-Thioguanine (6-TGN) and toxic 6-Methylmercaptopurine (6-MeMP) metabolites of 6-MP in pediatric ALL patients exhibited significant interpatient variability, as indicated by a population pharmacokinetic model. This model also demonstrated that the *TPMT* genotype can impact the metabolic transformation rate of 6-MP into 6-TGN (Hawwa *et al*., 2008).

1.7.1.1 *TPMT*

TPMT is a cytosolic transmethylase enzyme that plays a significant role in catalyzing the S-methylation of heterocyclic sulphhydryl compounds, including thiopurine drugs (Larovere *et al*., 2003). The *TPMT* gene is located on chromosome 6p22.3 and comprises ten exons and nine introns over a span of 34 kb (Szumlanski *et al*., 1996). The gene's polymorphisms have been extensively studied (Abaji and Krajinovic, 2017), with

Weinshilboum and Sladek being the first to describe the *TPMT* genetic polymorphism (Weinshilboum and Sladek, 1980). *TPMT* gene has an autosomal codominant inheritance, and genetic polymorphism can affect TPMT enzyme activity, leading to a trimodal distribution. In most population, around 4% to 11% of individuals are heterozygous for *TPMT* gene and have an intermediate TPMT enzyme activity, while only 0.3% (1 in 300) of individuals are compound heterozygous or homozygous for mutated *TPMT* gene and have very low or absent TPMT activity, with the remaining percentage of individuals having a wild-type homozygous genotype and normal to high TPMT activity (Liu *et al*., 2015; Zalizko *et al*., 2020).

Several *TPMT* variant alleles have been identified, with the three most common SNPs being *TPMT*3A*, *TPMT*3C*, and *TPMT*2*, found in approximately 95% of individuals with low or absent TPMT activity (Rosdiana *et al.*, 2021). The tertiary structure of the TPMT protein is changed by these variants, resulting in instability and a decrease in catalytic activity (Chen *et al*., 2021). Patients who are heterozygous or homozygous for the 'low-activity ' mutation gene may have a higher risk of myelotoxicity with thiopurine therapy (Coulthard and Hogarth, 2005; Gazouli *et al*., 2012). Studies have shown that individuals who possess a heterozygous *TPMT* genotype exhibit 6-TGN concentrations that are 2.25 times greater than wild-type patients due to reduced TPMT catalytic activity (Adam De Beaumais *et al*., 2011). The first variant allele identified, *TPMT*2*, harbors a 238G→C transversion, which leads to the replacement of a flexible alanine residue (Ala80Pro) by a rigid proline residue (Krynetski *et al*., 1995). The second variant allele identified, *TPMT*3A*, harbors 2 transition SNPs, one in exon 7(460G→A) and the other in exon 10 (719A→G), each of which causes an amino acid change Ala154Thr and Tyr240Cys, respectively (Tai *et al*., 1996), whereas *TPMT*3C* harbors only one transition SNP in exon 10 (719A→G) (Loennechen *et al*., 1998). The *TPMT*3A* allele is the predominant mutant allele in whites, whereas the *TPMT*3C* allele is the prevailing *TPMT* mutant allele in populations of African, African American, and Asian descent (Rosdiana *et al*., 2021).

There are two methods for assessing the TPMT enzymatic activity status of a patient: genotyping for variant alleles and phenotyping (enzyme activity testing) (Booth *et al*.,

2011). The correlation between *TPMT* genotype and phenotype is high, ranging from 76 to 99% in different studies, although it is lower for intermediate metabolizers (Derijks and Wong, 2010; Kahlin *et al*., 2021). Non-genetic factors and genetic variations in the regulatory region of the *TPMT* gene can cause genotype-phenotype discrepancies (Derijks and Wong, 2010). While genotyping is a definitive technique for identifying defective *TPMT* alleles, phenotyping may be more appropriate in some specific cases since a variation in TPMT enzyme activity exists between individuals with the same allele (Adehin and Bolaji, 2018; Lennard, 2013; Zur *et al*., 2016). However, the phenotype is also influenced by blood transfusion and medication (Zur *et al*., 2016). However, blood transfusion and medication can also influence the phenotype (Adehin and Bolaji, 2018; Zur *et al*., 2016). The use of determining TPMT activity status, the timing of genotyping or phenotyping, and the clinical implications are still being discussed (Booth *et al*., 2011).

Patients with reduced TPMT activity (or an inherited variant allele) who are administered standard doses of thiopurines have been documented to exhibit elevated levels of 6-TGN active metabolites and an increased risk of adverse effects (Gazouli *et al*., 2012). Homozygous *TPMT* wild allele carriers can receive a conventional starting dose safely (Sanderson *et al*., 2004). TPMT heterozygous patients require 50-70% of the standard thiopurine dose while a substantial ten-fold dose reduction or switching to alternative agents are suggested for homozygous patients (Bertholee *et al*., 2017; Lennard, 2013).

1.7.1.2 *NUDT15*

Nudix hydrolases are a family of proteins with a highly conserved amino acid signature sequence called the Nudix motif $GX_5Ex_7REUXEEXGU$ (where U is one of the bulky, hydrophobic amino acids Ile, Leu or Val) (Sheikh *et al*., 1998). Besides the Nudix motif, specific amino acid residues that are distinctive for particular substrates are identified, and Nudix enzymes can be subdivided into subfamilies based on their preferred substrates (Dunn *et al*., 1999). *NUDT15*, a member of this family, catalyzes the hydrolysis of various nucleoside diphosphate derivatives, including dNTPs, NTPs, nucleotide sugars, dinucleoside polyphosphates, coenzyme A, NAD+, NADH+, and FAD+ (Olejnik et al., 2007). *NUDT15* converts TGTP to TGMP (also TdGTP to

TdGMP) and inhibits the incorporation of these thiopurine metabolites into DNA (DNA-TG), negatively regulating thiopurine activation and cytotoxicity (Moriyama *et al*., 2016).

There are several known *NUDT15* variant alleles. *NUDT15* variant has been found to have significantly different frequencies among different racial and ethnic groups. Alleles containing p.Arg139Cys (NUDT15*2 and NUDT15*3), are most commonly found in Asians, with a frequency of around 9.8%. It has also been detected in US Hispanics with a frequency of 3.9%, but it has not been observed in Africans (Yang *et al*., 2015). Mutation of *NUDT15* c.415C>T has been associated with decreased protein stability of the enzyme (Valerie *et al*., 2016). Exon 3 missense variant of the *NUDT15* gene (c.415C>T or the p.Arg139Cys variant) is linked with thiopurine induced bone marrow suppression in inflammatory bowel disease patients (Chao *et al*., 2017) and in children with ALL (Yang *et al*., 2015).

NUDT15 c.415C>T homozygous individuals for the variant allele were sensitive to 6-MP and were only able to tolerate 8% of the standard dose. This variant alone justifies 22% of the disparity in 6-MP tolerance (Moriyama *et al*., 2016). *NUDT15* p.Gly17_Val18dup or p.Gly17_Val18del are also linked with thiopurine-induced toxicity. According to Walker *et al*. (2019), the p.Gly17_Val18dup variant lessens NUDT15 activity to 15% of normal enzymatic function, while p.Gly17_Val18del and p.Arg139Cys have little to no enzyme activity.

In addition to *TPMT* genotypes, several studies have demonstrated that *NUDT15* genotypes play a crucial role in ensuring the safety of patients treated with thiopurines. The US FDA recommends genotyping of *NUDT15* in patients who are experiencing severe myelosuppression and suggests considering alternative treatment (Azathioprine) or drastic 6-MP dosage reduction in homozygous patients either for *TPMT* or *NUDT15* (Tanaka & Saito, 2021).

1.7.1.3 *ITPA*

The *ITPA* gene encodes for the ITPA enzyme that cleanses the nucleotide pool by hydrolyzing non-canonical purines like inosine and xanthosine triphosphate (ITP/XTP) and their deoxy forms (dITP/dXTP) (Lin *et al*., 2001; Simone *et al*., 2013). Deficiencies in ITPA have been linked to disease and as a risk factor for adverse drug reactions in

patients receiving certain drugs (Simone *et al.,* 2013). About five SNPs have been identified on the *ITPA* gene, two of which (C94A and IVS2 + A21C) are associated with 6-MP toxicity (Simone *et al*., 2013; Sumi *et al.,* 2002). The c.94C>A variant encodes for Pro32Thr substitution and has the most significant effect on ITPA enzyme activity. Heterozygous individuals for this variant have a 22.5% decrease in enzyme activity, while homozygotes for the mutated gene have none at all (Maeda *et al*., 2005). The heterozygotes individuals for the IVS + 21A>C variant showed 61% of the median wild-type ITPA activity and those homozygotes had only 27% enzyme activity (Atanasova *et al.,* 2007). For compound heterozygotes with both variant alleles (94C→A/IVS2+21A→C), enzyme activity was only 10% (Simone *et al.,* 2013).

The frequency of the ITPA c.94C>A SNP varies across different populations globally, with a lower occurrence observed in Central and South American populations (1-2%), while Asian populations exhibit the highest frequency (11-19%). In African populations, Caucasian, and African-American the allele frequency ranges from 5 to 7% (Marsh *et al*., 2004). ITPA g.IVS2+21 A>C allele frequency is 13% in Caucasian populations (Maeda *et al*., 2005).

ITPA deficiency has been associated with thiopurine toxicity as a result of 6-thio-ITP accumulation (Marinaki *et al*., 2004). Studies revealed that *ITPA* polymorphism emerges as a predictor of myelotoxicity when 6-MP doses are adjusted for an individual's TPMT activity. This myelotoxicity could be caused by a higher level of methylated metabolites (methyl-thioITP) (Stocco *et al*., 2009). *ITPA* deficiency is significantly associated with 6-MP toxicity in populations with low *TPMT* variant distribution (Hareedy *et al*., 2015; Tanaka *et al*., 2012). A meta-analysis showed that variations in the *ITPA* gene could serve as indicator for thiopurine toxicity (Barba *et al*., 2022).

1.7.1.4 *XDH*

Xanthine oxidoreductase is a molybdo-flavoenzyme that exists in the forms of XO and XDH (Bortolotti *et al*., 2021). Xanthine oxidoreductase contributes to the formation of the 6-TX intermediate as well as the final product 6-TUA in human liver cytosol and competes with TPMT to inactivate 6-MP (Choughule *et al*., 2014). Genetic polymorphism can either cause increased or decreased enzyme activity, leading to inter-

individual variability (Smith *et al*., 2009). Evidence suggests that *XDH* polymorphisms can affect thiopurine metabolite levels in a pattern attributable to decreased XDH activity, resulting in decreased levels of 6-thiouric acid (6-TU) and increased levels of 6-TGNs with dose-related toxicity in individuals with *XO/XDH* variant alleles (Choi *et al*., 2019; Hawwa *et al*., 2008; Smith *et al*., 2012).

1.7.1.5 *ABCB1*

The proteins encoded by ATP-binding cassette (ABC) genes possess several domains that come together to form a pore with intracellular nucleotide-binding domains that enable ATP-dependent transport of substrates across the plasma membrane (Linton and Higgins, 2007). Human *ABCB1* gene encodes a 170-kDa P-glycoprotein (P-gp), which belongs to ABC transporters (Kimchi-Sarfaty *et al*., 2007).

P-gp works as both a functional barrier and an efflux transporter for drugs in several tissues (Gregers *et al*., 2015), including the intestinal wall and proximal tubule of kidney cells (Schinkel, 1997). Many genetic polymorphisms have been detected in the *ABCB1* gene, with the most common variants being 1236C>T, 2677G>T/A/C, and 3435C>T among various population groups (Kimchi-Sarfaty *et al*., 2007). Polymorphism in *ABCB1* transporters has been linked to differences in drug efficacy, adverse side effects, and altered drug-drug interactions (Wolf *et al*., 2011). P-gp overexpression in malignant cells leads to multidrug resistance, which has been observed in several chemoresistant tumors such as renal cell, colon, and hepatocellular, among others, suggesting it as a marker of poor prognosis (Gregers *et al.,* 2015; Zhai *et al*., 2012). P-gp also serves as an efflux pump for several antileukemics used in the treatment of childhood ALL, such as 6-MP, doxorubicin, etoposide, and vincristine. The increased expression of the *ABCB1* gene may contribute to the failure of therapy or the recurrence of a medical condition, while the reduced functionality of P-gp can lead to increased toxicity from 6-MP due to decreased excretion (Milosevic *et al*., 2018).

1.7.2 Pharmacogenetics of L-ASP in ALL

L-ASP preparations have been reported to elicit an immune response (Lopez-Santilla *et al*., 2017), and the pharmacogenetics of L-ASP have been investigated to discover the genetic basis of interpatient variability in response (Abaji and Krajinovic, 2019). Several

genes, including *GRIA1*, *CNOT3*, *NFATC2*, and *PNPLA3*, have been linked with L-ASP toxicity.

1.7.2.1 *GRIA1*

The GluA1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, which are tetrameric ligand-gated ion channels responsible for transmitting glutamatergic signals in the brain, is encoded by the *GRIA1* gene (Ismail *et al*., 2022). Glutamate serves not only as a neurotransmitter but also as a crucial immunomodulator (Pacheco *et al*., 2007). Ganor *et al*. (2003) discovered the presence of AMPA-activated ionotropic GluRs in human lymphocytes. Furthermore, it has been observed that glutamate alone has the capability to activate and regulate the activity of T-cells. Additionally, the *GRIA1* gene is situated in chromosome 5q31-33, which has been identified as a susceptibility locus for various autoimmune or inflammatory diseases, such as asthma. Various findings indicate that there is a shared set of candidate genes between drug allergy and asthma (Lopez-Santilla *et al*., 2017).

There is an increasing body of evidence suggesting that intronic SNPs in the *GRIA1* gene are linked to hypersensitivity to *E. coli*-ASP (Chen *et al*., 2010; Kutszegi *et al*., 2015; Rajić *et al*., 2015). Several studies have focused on five specific SNPs located in the intronic region of the *GRIA1* gene, namely rs4958351, rs10070447, rs6890057, rs4958676, and rs6889909. However, these association studies have yielded inconsistent and controversial results (Lopez-Santilla *et al*., 2017).

1.7.2.2 *CNOT3*

CCR4–NOT complex consists of at least nine conserved “canonical” subunits that regulate gene expression at the post-transcriptional level in human (Vicente *et al*., 2018). CCR4-NOT Transcription Complex Subunit 3 (CNOT3) is a constituent of the CCR4-not complex, which exerts regulatory influence on the expression of genes and cellular signals (Collart *et al*., 2013). As part of the CCR4-NOT complex, CNOT3 plays a role in mRNA deadenylation and degradation, contributing to the regulation of human leucocyte antigen (HLA) transcription (Rodrıguez-Gil *et al*., 2017). Multiple studies have established a connection between L-ASP hypersensitivity in childhood ALL patients and specific HLA alleles (Gagne *et al*., 2020; Kutszegi *et al*., 2017). It is sensible to assume

that the regulation of *CNOT3* genes may play a role in L-ASP hypersensitivity. GWAS has demonstrated an association between the intronic variant rs73062673 (T > C) in the *CNOT3* gene and L-ASP hypersensitivity (Højfeldt *et al*., 2019).

1.7.2.3 *NFATC2*

Nuclear Factor of Activated T Cells 2 (*NFATC2*) is a member of the NFAT family that has been well studied as having roles in the immune system (Kitamura and Kaminuma, 2021; Lang *et al*., 2018). The *NFATC2* gene is responsible for encoding a cytoplasmic element of the NFAT transcription factor family. Upon stimulation by T-cell receptors, it undergoes dephosphorylation and becomes activated. Subsequently, it is translocated from the cytoplasm into the nucleus. Once in the nucleus, *NFATC2* plays a crucial role in regulating gene transcripts (Okamura *et al*., 2000; Rao *et al*., 1997). The effect of *NFATC2* on the susceptibility to drug-induced allergies remains uncertain; however, research has demonstrated that *NFATC2* can affect the formation and operation of regulatory T cells (Fernandez *et al*., 2015). However, numerous studies have provided evidence that NFAT pathway inhibition through the use of *NFAT* inhibitors or *NFATC2* knockout mice in various immune-related disease models can effectively reduce the intensity of an immune response (Choi *et al*., 2012; Rathod *et al*., 2020). Additionally, GWAS revealed a strong association between the rs6021191 variant in *NFATC2* and L-ASP hypersensitivity (Fernandez *et al*., 2015).

1.7.2.4 *PNPLA3*

Patatin-like Phospholipase Domain Containing Protein 3 (*PNPLA3* or adiponutrin) is a member of patatin-like lipolytic enzymes family which is involved in lipid metabolism and signaling (Liu *et al*., 2017). Unlike other family members, which exhibit nonspecific lipid acyl hydrolase activity and are soluble proteins, PNPLA3 is closely associated with lipid droplets and membranes (Bruschi *et al*., 2017). *PNPLA3* rs738409 variant results in an isoleucine to methionine substitution at position 148 which markedly reduces the catalytic velocity of *PNPLA3* (Huang *et al*., 2011). Several studies have shown that *PNPLA3* rs738409 is linked to constitutive liver dysfunction (Dai *et al*., 2019; Grimaudo *et al*., 2020; Hotta *et al*., 2010; Walker *et al*., 2020) and drug-induced liver toxicity following ALL therapy (Gutierrez-Camino *et a*l., 2017; Liu *et al*., 2017).

1.8 Rationale for the study

According to estimates, a significant number of individuals sustain injuries or lose their lives annually in healthcare facilities due to adverse drug events, resulting in substantial financial expenses each year. Patients exhibit varying responses when administered the same medication, and these variations in drug response cannot be solely attributed to non-genetic factors. The variation in drug response is frequently found to be more significant among a group of patients than within an individual or between identical twins. This suggests that a portion of the drug response differences between individuals are inherited. In fact, genetics explain anywhere from 20% to 95% of the variation in how drugs are processed by the body and exert their effects (Evans, 2004).

Most anticancer and immunosuppressant medications have narrow therapeutic indices, severe systemic toxicity, unpredictable efficacy, and low overall response rates. Hence, the field of pharmacogenomics research is particularly crucial in the context of cancer treatment as it plays a vital role in assisting clinicians in accurately anticipating variances in drug efficacy, response, toxicity, and resistance among patients. Moreover, it serves as a valuable tool in guiding the selection of optimal drugs and doses, thereby facilitating safer, more effective, and cost-effective treatment options (Feng *et al*., 2014).

Patients treated with 6-MP experience myelosuppression characterized by leukopenia, neutropenia, thrombocytopenia, anemia, alopecia, and GI adverse effects. 6-MP intolerance presents wide interindividual variability, partly arising from genetic polymorphisms. Genetic risk factors predictive of thiopurine-induced adverse effects should be studied in childhood ALL patients treated with 6-MP. Children are prone to drug-related adverse effects. The impact of adverse effects on children can be significantly more severe in comparison to adults. Studies have shown that adverse effects experienced by children not only lead to hospitalization but can also result in permanent disability or even death. ALL is the predominant form of childhood cancer observed at Tikur Anbessa Specialized Hospital (TASH). A number of children treated with 6-MP experienced severe hematotoxicity such as leukopenia, neutropenia and infection. Hematotoxicity necessitates dose-reductions of the chemotherapy, medication discontinuations and dose-delays, may affect short- and long-term outcomes. Poor

treatment outcome and relapse are related to frequent treatment interruption. It is very difficult to treat relapsed ALL in Ethiopia with the current treatment facility.

The *TPMT* genetic polymorphism is a prime illustration in the field of pharmacogenetics, showcasing its advancement and significance in tailoring drug therapy to individual patients. The 6-MP metabolism is catalyzed by several polymorphic enzymes, including *TPMT*, *NUDT15*, *ITPA*, and *XDH*. Genetic variations in these genes may explain the inter-individual differences in response to 6-MP. Nevertheless, there has been no research conducted on the clinical significance of 6-MP pharmacogenetics in Ethiopia. Insight into genetic polymorphism holds potential benefits for patients, health planners, and health care practitioners in various ways.

Although L-ASP is an effective antineoplastic agent, it frequently leads to hypersensitivity reactions. In such instances, it is often necessary to discontinue the treatment, and the production of anti-asparaginase antibodies may also reduce the activity of asparaginase, thereby compromising its effectiveness in fighting leukemia. Treatment discontinuation of L-ASP is linked to disease relapse. Recent studies implied that genetic polymorphisms partly explain interindividual difference to L-ASP induced hypersensitivity reaction.

The present study explored the predictors and prevalence of toxicities in individuals undergoing treatment with 6-MP and L-ASP based anticancer regimen. The study also determined the concordance between genotype and phenotype of *TPMT*. In addition, the risk factor and incidence of L-ASP induced hypersensitivity has been evaluated. The results of this study provide important baseline data that can be used for possible further dose optimization studies. The outcome of this study could also help drug regulatory authorities and health care practitioners better understand the risk factors for hematotoxicity induced by 6-MP in Ethiopian pediatric ALL patients. This could be used to design and implement methods to tailor therapy for a specific individual patient. As a result, the study findings have the potential to improve the quality of patient care.

2 Objectives

2.1 General objective:

- ✓ To determine the frequency of genetic variants of drug metabolizing enzyme and transporter polymorphism and their association with the occurrence of adverse events during 6-MP and L-ASP based chemotherapy in childhood acute lymphoblastic leukemia patients in TASH, Addis Ababa, Ethiopia.

2.2 Specific objectives:

- ✓ To determine the frequency and non-genetic determinants of hematologic toxicities induced by 6-MP based chemotherapy.
- ✓ To explore the association between *NUDT15*, *ITPA*, *TPMT*, *XDH*, and *ABCB1* polymorphisms and the occurrence of hematologic toxicities.
- ✓ To investigate the concordance between *TPMT* genotype and phenotype.
- ✓ To evaluate *CNOT3*, *GRIA1*, and *NFATC2* polymorphisms with their association to L-ASP hypersensitivity.

3 Methodology

3.1 Study location and setup

This study was done in the Department of Pediatric Oncology of TASH, which provides organized cancer care services. It is the largest teaching and tertiary level referral hospital in Ethiopia, established in 1973 and administered by the Addis Ababa University since 1998. TASH is a treatment center in Ethiopia that provides comprehensive services for both children and adults. Additionally, it serves as the referral center for childhood cancer diagnosis and treatment in the country, catering to children from all regions. The hospital's Department of Pediatric Oncology is situated on the 7^{th} floor (7D) of the main building, while its outpatient care can be found on the first floor of the hospital's cancer clinic. The Department has inpatient capacity of 31 beds. The Department operates a specialized ward with a dedicated Pediatric Oncology-hematology specialist. This ward provides comprehensive services for children who have been diagnosed with cancer, both on an inpatient basis (when they are admitted to the ward for diagnostic or treatment purposes) and on an outpatient basis (when they receive treatment while staying at a shelter or home).

Among all cancer cases, ALL is the leading type of pediatric cancer. ALL is diagnosed using a combination of different methods. Diagnoses begin by taking medical history and physical examination. Blood tests are performed for CBC and peripheral blood smear to check their morphology. Bone marrow tests are used to diagnose leukemia. Blood chemistry test is used to detect liver or kidney problems. Imaging tests might also be done in children with ALL to help determine the extent of the disease.

Patients were treated using a protocol for low- and middle-income countries (Hunger *et al*., 2009) with combination of chemotherapy for period of 2.5 years falls into separate phases. Moreover, they also get preventive treatment for the spinal cord. IV native *E. coli* L-ASP is part of the induction, consolidation, and delayed intensification treatment. A dose of 6000 U/m^2 is administered nine times, both in induction and delayed intensification, irrespective of the risk group. While patients in HR group receive additional 10000 U/m^2 dose of L-ASP eight times during the consolidation phase based on the protocol. The oral maintenance treatment includes daily administration of 6-MP

and weekly administration of MTX. Additionally, the patients are administered vincristine monthly at a dose of 1.5mg/m^2, along with dexamethasone (6mg/m^2 for 5 day/month) and intrathecal MTX using age-adjusted dosing. To provide prophylaxis against Pneumocystis jirovecii, trimethoprim/sulfamethoxazole is given as a co-medication at a dose of TMP 5 mg/kg three times per week.

3.2 Study population and design

A cohort of 160 pediatric patients that attended a pediatric hematology clinic in TASH between January 2019 and December 2021, were clinically confirmed to have ALL, received 6-MP and L-ASP based chemotherapy, and met the specified inclusion criteria, were recruited in this study.

3.2.1 Inclusion criteria

- ✓ Children under the age of 12 years
- ✓ Children treated with maintenance phase of ALL treatment protocol
- ✓ Children with normal kidney and liver function
- ✓ Conventional dosage of 6-MP of 75 mg/m^2
- ✓ Agree to sign informed consent/assent form.

3.2.2 Exclusion criteria

- ✓ Patients with terminal illness.
- ✓ Patients with impaired renal and hepatic functions.

In this cohort of patients, the frequency of genetic variations in drug metabolizing enzymes and transporter proteins polymorphisms and their association with hematologic toxicity were assessed. To monitor toxicity, all patients were observed for at least 6 months. The association between genetic polymorphism with the occurrence of adverse events during 6-MP therapy was investigated by comparing the children with wild type genes as reference or controls to those children who possess polymorphic genes as test groups.

3.3 Study outcomes

- ✓ Neutropenia of grade 4

- ✓ Grade 4 early-onset leukopenia/neutropenia. The occurrence of low level of WBC/neutrophil during the first 60 days of the maintenance therapy was defined as early-onset leukopenia/neutropenia (Zhou *et al*., 2018).
- ✓ Neutropenic fever. Neutronic fever was defined as a grade 4 neutropenia with temperature of 38 °C or greater.
- ✓ Drug discontinuation
- ✓ LASP hypersensitivity

3.4 Assessed explanatory variables

- ✓ Socio-demographics (gender, age, nutritional status)
- ✓ SNP
- ✓ Risk group
- ✓ Day 1 maintenance therapy WBC counts
- ✓ Day 1 maintenance therapy ANC

3.5 Chemicals and reagents

QIAamp Blood Midi Kit (Qiagen GmbH, Hilden, Germany), KASPar-On-Demand (KOD) assay (LGC Genomics, Hoddesdon, UK), KASP 5000 V4.0 Low ROX (2x; LGC Genomics, Hoddesdon, UK) TaqMan® SNP Genotyping Assays (40x; Applied Biosystems by Thermo Fisher Scientific, Warrington, UK), TaqMan® Universal PCR Master Mix (2x; Applied Biosystems by Thermo Fisher scientific, Warrington, UK)**,** S-adenosyl-L-methionine (SAM) (Sigma Aldrich, USA), 6- Thioguanine (Sigma Aldrich, USA), 6-methyl thioguanine (Sigma Aldrich, USA), NaH2PO4 (Merck KGaA, Germany), NaOH (Merck KGaA, Germany), Tetrahydrofuran (Merck KGaA, Germany), Acetonitrile (Merck KGaA, Germany), Absolute Ethanol (Merck KGaA, Germany), MilliQ grade water, Agarose (Merck KGaA, Germany), Ethidium bromide (Merck KGaA, Germany), Potassium cyanide, 2% dimethyllaurylamine oxide

3.6 Methods

3.6.1 Assessment of hematologic toxicity

The medical records were used to collect data on patients' demographic information, clinical characteristics such as clinical presentation, CBC, peripheral morphology,

peripheral and bone marrow blast, as well as their risk group. Additionally, during the initial 6 months of the maintenance treatment, various clinical profiles were gathered, including but not limited to CBC results, the presence of fever, admissions to emergency, dose reduction, and drug discontinuation. CBC tests were conducted every four weeks, unless there were specific clinical reasons to indicate otherwise. The grading of myelotoxicity was based on the CTCAE scale version 4.0 (CTCAE, 2010) as outlined in Table 2. Allergic reactions to L-ASP were assessed using the CTCAE version 3.0 (CTCAE, 2006).

Table 2. CTCAE, version 4.0

Myelotoxicity	Grade 4 (life-threatening)
Leukopenia	<1000/mm3
Neutropenia	<500/mm3
Thrombocytopenia	<25,000/mm^3
Anemia	<6.5 g/dl

The collection of data on family demography, economic status, drug adherence, and reported adverse effects was conducted through the use of a questionnaire. The four-item Morisky, Green, and Levine Medication Adherence Questionnaire (MGL) was utilized to evaluate the adherence of the patients. The scores for adherence were computed, and individuals were classified into three categories based on their level of adherence. These categories include low adherence, which is defined as having answered "Yes" to 3 or 4 items; moderate adherence, which is characterized by answering "Yes" to 1 or 2 items; and high adherence, which is indicated by answering "Yes" to 0 items (Morisky *et al*., 1986).

The World Health Organization Anthro Version 3.2.2 was utilized to calculate height-for-age z-scores (HAZ), weight-for-height z-scores (WHZ), weight-for-age z-scores (WAZ), and Body mass index (BMI) for age Z score (BAZ) for children aged up to 5 years. The calculation of the HAZ, WAZ, and BAZ was performed utilizing WHO AnthroPlus Version 1.0.4 specifically for children aged above 5 years. However, the WAZ was exclusively produced for children under the age of ten. Children who have z-scores below

-2 standard deviations for WHZ, HAZ, WAZ, and BAZ were categorized as wasted, stunted, underweight, and thin, respectively (WHO, 2006).

3.6.2 Blood collection

Whole blood for DNA and TPMT enzyme activity measurement was collected by EDTA vacutainer tubes from each eligible study participant at the same time of their routine schedule. Blood samples were collected before the administration of vincristine when children had an indwelling cannula for vincristine therapy. On a single occasion, a 3 mL blood sample was collected from each patient in an EDTA tube for the purpose of conducting a genotype-phenotype study. An extra blood was obtained from patients who finished their treatment in order to measure the TPMT activity of the enzyme.

3.6.3 DNA isolation and assessment of the quality and quantity of genomic DNA

The QIAamp Blood Midi Kit was utilized to extract genomic DNA from peripheral leukocytes in 1mL of whole blood. The NanoDrop™ ND-2000c Spectrophotometer (Thermo Scientific, Isogen, the Netherlands) was used to evaluate the amount and quality of the DNA that was extracted. A gel made of 1.5% agarose and 0.5% ethidium bromide was prepared, and the DNA bands were captured using the syngene G:BOX, Qdoc imaging system.

3.6.4 Genotyping

The genotyping was performed at the Division of Human Genetics at Radboud University Medical Center, located in Nijmegen, the Netherlands. The KOD assay was utilized to conduct genotyping of SNP rs738409 in the *PNPLA3*, rs1127354 and rs7270101 in the *ITPA*, rs2281547 in the *XDH*, and rs4958351 in the *GRIA1* gene following a previously described method with minor modifications (Vos *et al*., 2016). The total volume was 5 µL, comprising 1 µL of DNA (10 ng/µL), 0.0625 µL of the KASPar assay (40x), 2.5 µL of KASP 5000 V4.0 Low ROX (2x; LGC Genomics), and 1.44 µL of MilliQ grade water. The PCR protocol involved an initial denaturation step at 94°C for duration of 15 minutes. Afterward, there were 10 rounds, with each cycle comprising denaturation at 94°C for a period of 20 seconds and annealing/extension at 61°C for 60 seconds including a drop of 0.6°C. Then, there were 26 rounds of denaturation occurring at 94°C

for a period of 10 seconds, followed by annealing/extension at 55°C for a period of 60 seconds. This was then followed by an additional 12 cycles of denaturation at 94°C for a period of 20 seconds, and annealing/extension at 57°C for a period of 60 seconds.

The genotyping of *TPMT*3B*, *TPMT*2*, *TPMT*3C*, *ABCB1* rs1045642, *NFATC2* rs6021191, *CNOT3* rs73062673, *NUDT15* rs746071566 and rs116855232 variants was conducted using TaqMan® SNP Genotyping Assays (Assay ID numbers used were C__30634116_20 for TPMT*3B, C__12091552_30 for TPMT*2, C_____19567_20 for TPMT*3C, C___7586657_20 for rs1045642, C__29689803_10 for rs6021191, C__98092291_20 for rs73062673 and C_154823200_10 for rs116855232). This genotyping procedure was described in detail somewhere else in Kumagai *et al*., (2019). The final reaction volume of 5 µL was prepared by mixing 1 µL of DNA (10 ng/µL), 2.5 µL of TaqMan® Universal PCR Master Mix (2x; Applied Biosystems by Thermo Fisher scientific, Warrington, UK), 0.0625 µL of TaqMan® SNP Genotyping Assay (40x; Applied Biosystems by Thermo Fisher Scientific), and 1.44 µL of MilliQ grade water. The PCR conditions included an initial stage at 95°C for 12 min and followed by a stage for 50 cycles step 1 with 92°C for 15 s and step 2 with 60°C for 90 s. For *NUDT15* rs746071566 custom designed assay (Id: ANDKGXA) was used. The final reaction volume of 10 µL was prepared by mixing 1 µL of DNA, 5 µL of TaqMan® Universal PCR Master Mix, 0.25 µL of TaqMan® SNP Genotyping Assay, and 3,75 µL of MilliQ grade water. The PCR conditions included an initial stage at 95°C for 10 min and followed by a stage for 50 cycles; step 1 with 95°C for 15 s and step 2 with 60°C for 60 s and post-read stage with 60°C.

MilliQ grade water and positive controls were included for quality control in both Taqman® and KASP assay. The DNA samples were amplified in Veriti™ 96-well Fast Thermal Cycler PCR (Applied Biosystems, Singapore). The results were analyzed with the software v1.5.0 for QuantStudio™ 3 Real-Time PCR (Applied Biosystems by Thermo Fisher Scientific, Singapore).

3.6.5 TPMT phenotyping (TPMT enzymatic assay)

TPMT enzyme activity was measured at the Department of gastroenterology at Radboud University Medical Center. The activity of TPMT enzyme was analyzed in whole blood

by high-performance liquid chromatography (HPLC) method as outlined by Ford *et al*. (2006). Briefly, the process involved in lysing 200 µL of whole blood by freezing it at a temperature of -80ºC. The lysed cells were defrosted in a 600 µL suspension buffer (0.1 mol/L phosphate buffer, pH 7.4). A reaction mixture of 500 µL, consisting of enzyme substrates (0.1 mol/L phosphate buffer, pH 7.4, SAM, and 6-thioguanine), was combined with 200 µL of whole-blood lysate. This mixture, along with 200 µL of whole-blood lysate as a blank, was incubated for a duration of 1 hour at 37°C with shaking in a water bath. Following that, the blank was mixed with 500 µL of the reaction mixture by vortexing. Immediately after, the reaction was halted, and proteins were precipitated by subjecting the incubate to rapid heating at a temperature of 90ºC. After centrifugation, the supernatant was subjected to HPLC analysis in order to determine the reaction product, 6-methyl thioguanine (6-MTG).

The HPLC analysis was conducted using an isocratic pump and an autosampler Chromsystems equipped with a 20 µL loop. The stationary phase consisted of a Kingsorb reversed phase column (C18, 75 mmX64.6 mm, 3µm particle size) maintained at ambient temperature. To protect the column, a 4 mm X 3 mm Kingsorb guard column (Security Guard, Phenomenex) was utilized. The mobile phase was a mixture of phosphate buffer (50 mM)-acetonitrile-tetrahydrofuran (ratio of 89:7:4 (v/v). After the addition of organic solvents, the final pH of the mobile phase was adjusted to 6.2. 0.80 ng of 6-MTG (per 20 µl loop) calibrant was used to standardize the assay. The samples were analyzed for a period of 10 minutes, with a flow rate of 1.5 mL/min.

The lysate hemoglobin concentration was determined by colorimetric technique. Bayer ADVIA Hemoglobin reagent which is 20mM potassium cyanide in a 2% dimethyllaurylamine oxide solution was used. Hem iron in the hemoglobin was oxidized from the ferrous to the ferric state. Subsequently, the oxidized hem iron combined with cyanide to generate a reaction product, which was measured at 546 nm.

3.6.6 Data management

The clinical nurses and laboratory technologists working in the cancer center at TASH collected the blood samples. All specimens were collected and stored in accordance with standard operating procedures (SOPs). The SOPs were also adhered for the collection of

data from patients' charts, pretreatment of blood samples, genotyping, and HPLC assays. Data were collected data using questioner, and case report form and coded and entered. The collected data were checked for consistency and completeness by the investigators. Furthermore, before data entry, the data were checked for completeness. To ensure data quality, the data were double-entered.

3.6.7 Statistical analysis

The entry and analysis of the data were carried out using SPSS version 26. The chi-square test was employed to evaluate the Hardy-Weinberg equilibrium. The descriptive statistics, the median with interquartile range (IQR) or frequencies and percentages, were utilized to present the demographic characteristics, anthropometric measurements, and clinical profiles of the participants in the study.

Hazard ratios and the analysis of risk factors for outcome variables were determined by Cox regression. The assessment of hematotoxicity determinants was carried out using bivariable and multivariable analyses. In multivariable analyses, variables with a p-value of ≤ 0.20 in the bivariable analysis were included using the enter method for variable selection. To prevent the potential impact of the interaction between WBC and ANC on the regression analysis, two distinct multivariable regression models were employed. Model 1, all variables from the bivariable analysis, excluding ANC, were incorporated into the multivariable. Model 2, all variables were modeled except for WBC. The variables employed in the association study encompass the age and gender of the child, marital status of parents/guardians, their place of residence, the educational level of their caregivers, adherence, BAZ, HAZ, food items, risk group classification, ANC, WBC, and genotype.

Column proportions tests were used to determine the concordance between *TPMT* genotype and phenotype. The receiver operating characteristics curve (ROC) was utilized to estimate the TPMT activity cutoff value that distinguishes intermediate- and high-activity groups. A comparison of groups was carried out by either the chi-square test for categorical data or the Mann-Whitney U test for continuous data, depending on appropriateness. A p-value below 0.05 is deemed statistically significant.

3.6.8 Ethical consideration

The institutional review board (IRB) of the College of Health Sciences at Addis Ababa University, the AHRI/ALERT ethics review committee, and the national research ethics committee (NREC) under the Federal Ministry of Science and Technology in Ethiopia granted ethical clearance. Prior to their involvement in the study, participants parents/guardians were asked to give written informed consent. During the process of obtaining consent, the participants were given an information sheet that outlined the purpose of the study, the reasons for their selection to participate, and what was expected of them. It was also emphasized that they had the right to terminate their involvement in the study at any moment. The participants were reassured about the confidentiality of their information by ensuring that no personal identifiers were used and that the data would be analyzed in aggregated form. Furthermore, the investigators treated each participant with respect and equality.

4 Results

4.1 Chemotherapy induced grade 4 hematotoxicity (Paper I)

The study included a total of 160 children; however, due to insufficient data (death, relapse, lost to follow up, and transferred to other treatment site) eighteen children were excluded from statistical analysis. Of the remaining participants, 92 (64.8%) were boys and 50 (35.2%) were girls. Most of the children in the study resided in urban places. Most of the caregivers were older than 26 years of age (90.1%), and half of the caregivers were male. A large proportion of the individuals providing care were in a marital relationship (89.4%) and 22.5% had not received any formal education (Paper I, Table 1).

4.1.1 Patients characteristics

During the maintenance treatment, all participants were in remission. A little over half 76 (53.5%) consumed food that contained protein, while 131 (92.3%) consumed three or more meals on a daily basis. According to the WHO Z-score, 31 (21.8%) were thin, 8 (14.3%) were wasted, 35 (24.6%) were stunted, and 25 (22.1%) were underweight. The scores measuring medication adherence indicated that 111 (78.2%) of the children had a high level of adherence, while 31 (21.8%) had a medium level of adherence (Paper I, Table 2). Among those with medium adherence, 7 (22.6%) showed a lack of attentiveness about administering medication, 9 (29%) discontinued medication when they experienced worsening symptoms, and 15 (48.4%) forgot to administer medication.

The initial clinical profiles of the patients revealed that splenomegaly 76 (54.7%) and hepatomegaly 84 (60.4%) were present either individually or in combination. Examination of peripheral morphology indicated that 39 (33.1%) patients had L1, 78 (66.1%) had L2, and only 1 (0.7%) had L3. Approximately 73 (51.4%) of the study participants were identified as high risk, with the remaining individuals being classified as standard risk. Upon diagnosis, the median WBC count was 12,340/mm^3, with 31 (23.4%) of the patients exhibiting WBC counts exceeding 50,000/mm^3. In the maintenance treatment initiation, the median WBC count and ANC were recorded as 3,500/mm^3 and 1,600/mm^3, respectively.

4.1.2 Perceptions of caregivers about chemotherapy induced side effects

Of the 142 respondents, fever/flu-like symptoms were reported most frequently, which was acknowledged by 95 (66.9%) of caregivers. Following closely, itching or skin rash was the second most frequent side effect, reported by 82 (57.8%) of respondents. Decreased appetite ranked third, with 71 (50%) of caregivers reporting it as a side effect. Behavior alterations were noted as the fourth most frequent side effect, with 61 (43%) of caregivers mentioning it (Paper I Figure 1).

4.1.3 Incidence of grade 4 hematotoxicity, and treatment discontinuation

During the initial 60-days of the maintenance treatment, 28 (19.7%) of the study participants exhibited early-onset leukopenia, while 46 (32.4%) of study participants displayed early-onset neutropenia. Within the initial six months of the maintenance treatment, 45 (31.7%) of the patients encountered leukopenia, while neutropenia affected 75 (52.8%) of them. 83 (58.5%) required interruption of their medication. 19 (13.4%) of the individuals reported having anemia, while only 8 (5.6%) of the participants had encountered thrombocytopenia (Paper I, Table 3).

4.1.4 Predictors of grade 4 neutropenia

The age of the child and the WBC on the first day of maintenance were found to have a significant association with grade 4 neutropenia, according to bivariable Cox regression analysis. The multivariable analysis further indicated that children under the age of 6 had approximately twice the rate of developing grade 4 neutropenia compared to those above the age of 6, as indicated by both model 1 and 2. The incidence of grade 4 neutropenia in children who had a WBC count lower than 4,500 on the first day of maintenance treatment was found to be 2.477 times higher compared to those who had normal WBC counts (model 1). Likewise, children who had an ANC value less than 2,500 on the first day of maintenance treatment were observed to have a 2.11 times higher risk of developing grade 4 neutropenia than those with normal ANC counts (model 2) (Paper I, Table 4).

4.1.5 Predictors of treatment interruption, neutropenic fever, and early-onset grade 4 leukopenia/neutropenia

Child's age, and day 1 maintenance WBC counts showed association with a treatment interruption during bivariable regression analysis. Child's age (≤ 6 year) (AHR 2.1, 95% CI= 1.33-3.317, p = 0.001), and low day 1 maintenance WBC counts (AHR 2.024, 95% CI=1.249-3.282, p = 0.004) were found to be independently associated with a treatment interruption after multivariable regression analysis (model 1). In model 2, low ANC count (AHR 1.938, 95% CI=1.065–3.527, p= 0.03), and child's age (≤ 6 year) (AHR 2.018, 95% CI=1.282-3.179, p = 0.002) were independent risk factors of treatment interruption (Paper I, Table 5).

Child's age, and day 1 maintenance WBC counts were significantly associated with early-onset grade 4 leukopenia/neutropenia following bivariable regression analysis. Multivariable analysis showed that child's age (≤ 6 year), and low day 1 maintenance WBC counts were independent predictors of early-onset leukopenia (Model 1). Similarly, child's age (≤ 6 year), and low day 1 maintenance WBC counts were found to be independent risk factor for early-onset neutropenia (Model 1).

When considering the incidence of neutropenic fever in the bivariable analysis, only the age of the child was found to have a significant contribution.

4.2 Genotyping and its association with grade 4 hematotoxicity (Paper II)

The frequencies of genotypes were observed to align with Hardy-Weinberg Equilibrium (HWE) (p > 0.05). The frequencies of the variants *TPMT*3C*, *ITPA* rs1127354, *ITPA* rs7270101, *XDH* rs2281547, and *ABCB1* rs1045642 were 0.35, 4.6, 11.6, 31, and 77.1%, respectively. However, the *TPMT*3B*, *TPMT*2*, *NUDT15* rs116855232, and rs746071566 did not show any variant alleles identified. The analysis of genotype frequencies in patients who experienced grade 4 neutropenia and those who tolerated the treatment revealed that individuals with the *XDH* rs2281547 CC/TC had a notably elevated incidence of grade 4 neutropenia (p = 0.01) in contrast to those with the TT genotype (Paper II, Table 2). The same trend was observed in the allele analysis. However, there were no statistically significant variations in the frequencies of other alleles between patients with grade 4 neutropenia and those who tolerated the treatment.

The analysis using multivariable Cox regression for grade 4 neutropenia indicated that patients with the CC genotype of *XDH* rs2281547 had a significantly increased risk (AHR 2.956, 95% CI=1.494-5.849, p = 0.002) of developing grade 4 neutropenia compared to individuals with the TT genotype (Paper II, Table 3). Additionally, the Kaplan-Meier hazard curves demonstrated that the cumulative risk of developing grade 4 neutropenia was observed to be notably elevated in individuals with the TC and CC genotype in comparison to those with the TT genotype (Paper II, Figure 1).

The multivariable analysis demonstrated that individuals carrying the *ITPA* rs1127354 AC genotype exhibited a risk of developing neutropenic fever that was slightly more than two times higher than those without this genotype. Moreover, patients carrying the *XDH* rs2281547 CC variant (AHR 2.704, 95% CI=1.382-5.289, p = 0.004) were found to be more prone to treatment discontinuation compared to those with the TT genotype. The Kaplan-Meier hazard curves also demonstrated that patients with the *XDH* rs2281547 CC and TC variant had a higher hazard of treatment interruption compared to those with the TT variant (p = 0.005). Likewise, patients with the *ITPA* rs1127354 AC genotype had a higher hazard of developing neutropenic fever compared to those with the CC genotype (p = 0.041) (Paper II, Table 4). Following multivariable analysis, no significant association was found between the tested genetic variants and grade 4 early-onset leukopenia. Nonetheless, patients who have the *ITPA* rs7270101 AC genotype demonstrated a twofold higher likelihood of developing grade 4 early-onset neutropenia (p = 0.035).

4.3 Genotype-phenotype relationship (Paper III)

A total of 98 ALL patients were included, with the majority being male. Only two patients (1.9%) were found to be heterozygous for *TPMT*1/*3C* variant, while no other tested variants (*TPMT*2* and *3B) were identified, and homozygous *TPMT* mutants were not detected. In this paper, the subsequent analysis of TPMT enzyme activity is divided into two groups: patients who are currently undergoing treatment and patients who have completed their treatment. The enzyme activity measurements revealed a significant number of patients with negative activity, and therefore those samples were excluded from the analysis. The study found no statistically significant differences in TPMT

activity based on gender and age within any of the groups. The median TPMT enzyme activity (mU/L) did not differ significantly between patients on maintenance therapy and those who completed therapy. However, it was noted that the TPMT enzyme activity (measured in nmol6MTG/gHb/h) in the treatment group was higher significantly than in the treatment completed group.

The correlation analysis demonstrated a strong positive correlation between TPMT activity in mU/L and TPMT activity in nmol6MTG/gHb/h in both on the treatment and treatment completed groups ($r > 0.9$, $p < 0.001$) (Paper III, Table 2). In both groups, there was a minor (insignificant) correlation between the activity of TPMT and the levels of Hb. The enzyme activity measured in nmol6MTG/gHb/h showed a negative correlation, whereas the TPMT activity measured in mU/L exhibited a positive correlation with the Hb levels, although statistical significance was not achieved. Furthermore, in the treatment group, the activity of TPMT (nmol6MTG/gHb/h) was found to be higher significantly in individuals with a hemoglobin (Hb) level below 12 g/dL compared to individuals with a Hb level above 12 g/dL.

The TPMT enzyme activity exhibited a range of values, spanning from 15 to 77 nmol6MTG/gHb/h, with a median value of 32. Similarly, the enzyme activity ranged from 27 to 144 mU/L, with a median value of 60.3. Within the treatment group, 8.2% displayed TPMT activity below 18 nmol6MTG/gHb/h in whole blood. The majority of the treatment group (91.8%) demonstrated enzyme activity falling within the range of 18-77 nmol6MTG/gHb/h. The threshold value distinguishing between normal and intermediate metabolizers was established as 18 nmol6MTG/gHb/h or 37 mU/L, based on the analysis of ROC and previously published data by Adehin and Bolaji, (2018). However, due to the insufficient presence of heterozygous genotypes within the study cohort, a well-defined cut-off value could not be determined.

The relationship between genotype and phenotype according to the SNPs in the treatment group showed that most patients who did not carry a variant in *TPMT* had normal enzyme activity (93%) (Paper III, Table 3). Only one of the two patients with the *TPMT*1/*3C* genotype had decreased enzyme activity, while the other patient had an activity of 32 nmol6MTG/gHb/h, which is higher than the cut-off value.

4.4 Asparaginase hypersensitivity (paper IV)

In this sub-study, of the total of 160 patients, 144 (90%) were included in the present study regarding L-ASP hypersensitivity association. The remaining 16 patients were excluded from the analysis due to absence of data on hypersensitivity. Among the participants, 50 (34.7%) were females. More than half of the patients population presented with hepatomegaly 83 (59.7%), splenomegaly 76 (54.7%), either in isolation or combination. Furthermore, a majority of the study participants, accounting for 122 (84.7%), were aged 10 years or younger. Among the entire study participants, 77 (53.5%) were classified as being part of the high-risk group, whereas the remaining 67 (46.5%) were designated as belonging to the standard risk group. Eighteen individuals, which accounts for 18 (12.5%) of the total study participants, experienced hypersensitivity reactions ((Paper IV, Table 1).

4.4.1 Allele frequencies and determinants of L-asparaginase hypersensitivity

The genotypes of all patients involved in the study (n=160) were evaluated (Paper IV, Table 2). The minor allele frequency (MAF) of GRIA1 rs4958351 is (17.8%) and It was discovered that 52 individuals (32.5%) carried the rs4958351 variant allele. The MAF of CNOT3 rs73062673 is 6.9% and only 2 individuals (1.3%) had a homozygous mutation for rs73062673, while 18 individuals (11.3%) had the heterozygous variant. MAF of NFATC2 rs6021191 was 5.6% and the rs6021191 polymorphisms were found to be heterozygous mutations, accounting for 18 cases (11.3%). No homozygous mutations for rs6021191were detected in this study. The MAF of PNPLA3 rs738409 was observed to be 16.9%. Genotyping of PNPLA3 rs738409 revealed that among the participants, 44 individuals (27.5%) had heterozygous variants, while 5 individuals (3.1%) had homozygous variants.

Association analysis was performed to examine the correlation between L-ASP hypersensitivity and different factors in a group of 144 patients. The results of the bivariable logistic regression analysis confirmed that there was no significant association between the patients' profile (including age, gender, and risk group) and genetic factors with L-ASP hypersensitivity. It is important to note that no association analysis was conducted for PNPLA3 rs738409 due to a lack of clinical data on hepatotoxicity.

5 Discussion

Chemotherapy induced hematologic toxicity incidence, drug interruption, and emergency admission and associated non-genetic risk factors were investigated (**Paper I**). This study also investigated the impact of pharmacogenetic variations in drug transporter gene and drug mobilizing enzymes genes relevant for metabolism of chemotherapeutic agents (**Paper II**). Further, this study examined the relationship between genotype-phenotype of the most important gene in disposition of 6-MP (**Paper III**). Besides, the present study investigated the L-ASP hypersensitivity reaction and associated non-genetic and genetic risk factors (**Paper IV**).

A prolonged maintenance phase therapy with low intensity is necessary to eliminate any remaining leukemic clones and reduce relapse in ALL patients (Schmiegelow *et al*., 2014). However, the main challenge during this phase is the occurrence of dose-limiting hematotoxicity. In certain cases, chemotherapy is discontinued due to hematotoxicity, which subsequently increases the risk of relapse (Teachey *et al*., 2021). In the present study, we conducted an investigation into the occurrence of grade 4 hematotoxicity and the factors that influence the maintenance treatment of ALL patients. The findings of this research reveal a notably higher prevalence of grade 4 neutropenia, reaching 52%, in comparison to previous investigations conducted by Choi *et al*. (2019) (29%) and Rosdiana *et al*. (2021) (2.8%). Study conducted in China (Fan *et al*., 2022) and Thailand (Puangpetch *et al*., 2020) reported a comparable incidence of grade 4 neutropenia, which was found to be 47%. On the other hand, a study conducted in Korea (Lee *et al*., 2021) and Sweden (Wahlund *et al*., 2020) revealed a greater occurrence of grade 4 neutropenia.

Additionally, this study revealed that the rate of 6-MP discontinuation and neutropenic fever was higher compared to previous reports (Linga *et al*., 2014; Tanaka *et al*., 2012). However, the current study reported lower 6-MP discontinuation rates compared to previous studies (Choi *et al*., 2019; El-Rashedy *et al.,* 2015). The global distribution of 6-MP related toxicity exhibits variations, which can be ascribed to multiple factors such as patient characteristics, genetic variation, follow-up period, and disparities in 6-MP dosage and dose adjustment protocol. The disparity between this study and previous studies is attributed to the difference in 6-MP dosing and follow-up period.

This is among a few studies that present the reported side effects of maintenance therapy by caregivers. In this study, the most commonly observed side effects were fever or flu-like symptoms; following this, itching or a skin rash, decreased appetite, and alterations in behavior. However, a study conducted in Indonesia (Sitaresmi *et al*., 2009) found that the most commonly observed side effect was changes in behavior, from there on an increase in appetite and susceptibility to infection. The potential cause of the increased occurrence of fever/flu-like symptoms observed in this study could be attributed to neutropenia.

The risk factors associated with hematotoxicity induced by chemotherapy can be classified into three main categories: disease factors, patient characteristics, and therapeutic factors (Lyman *et al*., 2005; Rosdiana *et al*., 2019). The risk factors associated with neutropenia, which is the primary dose-limiting toxicity (Crawford *et al*., 2004), have not been extensively studied in pediatric patients with ALL, especially in Ethiopia.

This study found that the age of a child and the WBC/ANC levels on the initial day of maintenance treatment were independent determinants of the occurrence of grade 4 neutropenia. Individuals who did not develop grade 4 neutropenia had higher age and day 1 maintenance WBC/ANC levels compared to patients who experienced grade 4 neutropenia. These findings align with previous studies, which reported that younger age (Ouyang *et al*., 2013) and low baseline WBC and ANC counts (Ahmed *et al*., 2019) were significant risk factors for chemotherapy-induced neutropenia. Additionally, a previous study conducted on pediatric patients with ALL demonstrated that being younger was linked to a higher likelihood of developing neutropenia (Wahlund *et al*., 2020).

This study highlighted that younger age is a risk factor for febrile neutropenia. This observation is in line with pervious study which indicates that younger age is a risk factor for febrile neutropenia (Wahlund *et al*., 2020). But, another study presented conflicting findings, indicating that age might not pose a risk for febrile neutropenia (Sulviani *et al*., 2007). Neutropenia is the primary cause for discontinuing treatment and reducing dosage in this particular group of patients. Younger patients, aged six years or below, have been found to be linked with treatment interruption, possibly due to the common occurrence of

neutropenia in younger children. Besides, patients with low WBC/ANC counts on the first day of maintenance therapy are more likely to experience interruptions in their 6-MP treatment compared to patients who have normal WBC/ANC counts. The relationship between treatment outcome and treatment intensity is widely acknowledged in various drug-sensitive cancers, such as pediatric ALL (Relling *et al*., 1999). Therefore, it is crucial to minimize treatment interruptions. Our findings suggest that focusing on younger children and monitoring the WBC and ANC level could be beneficial in this regard. In addition to non-genetic factors, studies have also implicated genetic factors in the development of side effects associated with 6-MP treatment.

The investigation presented in **paper II** focused on exploring the genetic risk factors related to drug mobilizing enzymes and transporters that play a role in the disposition of 6-MP in the Ethiopian pediatric ALL patients. In 2019, the Clinical Pharmacogenetics Implementation Consortium made updates to the dosing recommendation of 6-MP in a clinical setting based on the *NUDT15* and *TPMT* genotype (Relling *et al*., 2019). However, the predictive value of *NUDT15* and *TPMT* alleles is affected by variations in genotype frequency and distribution across different ethnic populations.

In this cohort, *NUDT15* rs116855232 and rs746071566 were not detected, likely due to their low frequency in African populations. *TPMT*3C* is the commonest variant among black populations, but in this cohort, its frequency was much lower than in previous studies in black populations (Ameyaw, 1999; McLeod *et al*., 1999; Adehin *et al*., 2017). The frequency of *TPMT*3C* was only 0.35%, which is approximately seven times lower than the report by Ronen *et al*. (2010) in Ethiopian Jews. In this study, it was not feasible to predict toxicity by using *TPMT* genotyping due to the limited frequency of known variants associated with reduced TPMT activity. Therefore, it is crucial in the future to screen a larger group from Ethiopia in order to ascertain the precise frequency.

TPMT polymorphisms alone cannot fully explain the variability in 6-MP toxicity, which makes thiopurine side effects frequently observed in patients without a *TPMT* variant (Pai *et al*., 2021). Compared with the observed incidence of thiopurine toxicity, *TPMT* variant alleles are relatively rare in the population (Kurzawski *et al*., 2012). Studies have shown that *TPMT* variants can only account for up to 25% of thiopurine side effects (Broekman

et al., 2017). Other genetic polymorphisms, including *ITPA* and *NUDT15*, have been linked to thiopurine toxicity in individuals who do not possess *TPMT* polymorphisms (Chiengthong *et al*., 2016; Mao *et al*., 2021). In a recent study, a variant in the *XDH* gene was found to be linked to 6-MP induced toxicity. XDH plays a role in both the production of the 6-thioxanthine intermediate from 6-MP and the subsequent conversion of this intermediate into the final product (Choughule *et al.*, 2014).

The results of this study revealed that individuals who had two copies of the minor allele of XDH rs2281547 were at a greater risk of grade 4 neutropenia. It is important to note that rs2281547 is an intronic variant, which has the potential to affect gene expression, although the specific functionality of this particular SNP is currently unknown. Therefore, studies in the future are necessary to confirm the association of rs2281547 with neutropenia. The risk of hematological toxicity related to 6-MP is increased in individuals with poor XDH enzyme activity, while those who are rapid metabolizers have an elevated risk of thiopurine failure as a result of reduced production of 6-TGTP (Dewit *et al*., 2010). The concurrent use of 6-MP with allopurinol or febuxostat, which are inhibitors of XDH and XO enzymes, has been found to increase the production of both active and toxic compounds (Chocair *et al*., 1993; Dewit *et al*., 2010). Although XDH plays a role in the disposition of 6-MP, limited studies have been conducted to investigate the role of *XDH* polymorphisms. Variants of molybdenum cofactor sulfurase c.362C > T and c.2107C > A have been identified to reduce the metabolic ability of XDH (Kurzawski *et al*., 2012; Stiburkova *et al*., 2018). These variants result in a slower thiopurine metabolism, leading to increased production of the hematotoxic nucleotide 6-thioguanine. A recent study investigating the association between pathway genes has highlighted the role of *XDH* in thiopurine toxicities, including hepatotoxicity, neutropenia, and treatment discontinuation (Choi *et al*., 2019). Additionally, a case report study conducted by Serre-Debeauvais *et al*. (1995) has identified that xanthine oxidase deficiency exacerbates hematotoxicity caused by azathioprine.

This study additionally found connections between *ITPA* polymorphisms and hematological toxicity of 6-MP. ITPA serves as an enzyme responsible for house-cleaning functions by hydrolyzing 6-TITP back to 6-TIMP, thereby hindering the buildup of toxic 6-TITP (Zamzami *et al*., 2013; Barba *et al*., 2022). Meta-analysis has indicated

that populations characterized by low *TPMT* and high *ITPA* variants are more likely to be affected by *ITPA* variants (Barba *et al*., 2022). In this study, it was observed that the frequency of *TPMT* variants was low, and notably, the ITPA rs1127354 variant showed a significant association with the occurrence of neutropenic fever. This finding aligns with a prior investigation by Stocco *et al*. (2009), which revealed a considerably greater probability of experiencing severe neutropenic fever in patients who possessed a variation in *ITPA*. According to a study conducted in Egypt and reported by Hareedy *et al*. (2015), it was found that the *ITPA* rs7270101 gene variant was linked to a high risk of neutropenia and leukopenia. The study's findings were further supported by our own finding, suggesting a connection between the presence of the *ITPA* rs7270101 variant and the development of neutropenia within the first 60 days of maintenance therapy. This study is the first of its kind conducted on the Ethiopian ALL patients, highlighting the association between *ITPA* alleles and hematological toxicity related to 6-MP during maintenance therapy. Despite extensive research, there remains a lack of agreement regarding the influence of *ITPA* gene variants on the development of hematologic toxicity induced by thiopurine medications. Furthermore, the implementation of these findings in a clinical setting has not been standardized, particularly when considering different ethnic populations (Zhou *et al*., 2018; Mao *et al*., 2021).

In **paper III** of the present study, both the TPMT phenotype and genotype were examined in pediatric ALL patients who were undergoing maintenance therapy with 6-MP, as well as in children who had completed their ALL therapy. TPMT plays a crucial role in the control of 6-thioguanine nucleotides, impacting both the effectiveness and safety of thiopurine treatment (Gisbert *et al*., 2006). It is suggested by multiple studies that it is important to assess the TPMT status of patients before starting thiopurine therapy. This assessment can be done using either TPMT phenotype or genotyping of known TPMT variants that are linked to enzyme deficiency (Lennard, 2013; Zalizko *et al*., 2020).

Due to enzyme induction effects of drug, the TPMT activity in ALL children who were undergoing maintenance therapy with 6-MP was found to be significantly higher compared to those who had completed the entire ALL therapy. As previously reported by Lennard *et al*. (1990), the TPMT activity in children undergoing chemotherapy has been

found to be significantly higher compared to long-term survivors of ALL and normal control children. Several other studies (Brouwer *et al*., 2005; Chrzanowska *et al*., 2012; Lennard *et al*., 2013), have also indicated that TPMT activity significantly rises during chemotherapy, surpassing the levels observed in both healthy children and children diagnosed with ALL.

The activity of the TPMT enzyme is influenced by gender, with males exhibiting higher TPMT enzyme activity compared to females (Schaeffeler *et al*., 2004; Zimdahl Kahlin *et al*., 2019). However, the current study found no significant effect of gender on TPMT activity. This finding is consistent with other studies conducted by Brouwer *et al*. (2005) and Lennard *et al*. (2013), which also failed to detect any influence of gender on TPMT enzyme activity. The absence of a gender effect in this study may be attributed to the young age of the study participants. In younger children, the impact of gender on TPMT enzyme activity is reduced as testosterone has less influence on TPMT activity (Schaeffeler *et al*., 2004).

The observation made in this study contradicts the well-established understanding that TPMT activity typically follows a trimodal distribution (Weinshilboum & Sladek, 1980). Similarly, other studies also failed to observe the expected trimodal frequency distribution of TPMT activity (Hindorf & Appell, 2012; Milek *et al*., 2006; Mozafari *et al*., 2018). This discrepancy can likely be attributed to the absence of individuals with genetic variants linked with reduced enzyme activity.

Schaeffeler *et al*. (2004) presented a comprehensive overview of various research studies that investigated the correlation between genotypes and phenotypes, revealing an overall concordance rate ranging from 76% to 100%. However, in the intermediate metabolizer group, most studies reported a lower concordance rate ranging from 50% to 95.2%. Similarly, Kahlin *et al*. (2021) also reported a lower rate of concordance (64.4%) specifically in the intermediate metabolizer genotype-phenotype relationship. In the present study also a lower concordance rate of 50% was observed between heterozygous genotypes and phenotypes. This may be explained by the fact that only two participant are heterozygous.

Patients with anemia may exhibit elevated Hb-corrected TPMT activity, when the activity expressed in nmol6MTG/gHb/h in individuals with low Hb concentration This resolved by measuring TPMT activity in mU/L. The presence of this misleadingly high TPMT activity could potentially result in inappropriate treatment decisions (Bahrehmand *et al*., 2017; Barlow *et al*., 2010). This study's findings align with previous research, as individuals with low Hb levels demonstrated higher TPMT activities compared to those with normal Hb levels when TPMT activity was expressed in nmol6MTG/gHb/h.

The identification of individuals with a decreased TPMT activity cannot be guaranteed solely by either their phenotype or genotype. However, in most populations, genotyping *TPMT*2* and the *TPMT*3* family variants can cover approximately 95% of inactivating *TPMT* variant alleles. To ensure that no variants have been missed, phenotyping can be used as a double-check. It is estimated that this may occur in 1 in 7,416 *TPMT* heterozygote individuals due to the presence of rare or novel variant alleles (Ford et al., 2009). The utilization of phenotyping as the initial test poses a higher risk of misclassifying an individual with TPMT deficiency as having intermediate activity, in comparison to genotyping (Hindorf & Appell, 2012). A study conducted on pediatric ALL patients with intermediate TPMT activity demonstrated that genotyping is more effective than phenotyping in determining the activity, as evidenced by the measurement of 6-MP metabolite concentration (Lennard *et al*., 2013). In this study, Heterozygote patients are at risk of misclassification when only phenotyping is used for TPMT status evaluation. Therefore, genotyping is recommended for the pre-treatment classification of the patients before initiation of thiopurine therapy.

In **paper IV** describes risk factors associated with L-ASP hypersensitivity reactions. L-ASP-antibodies complexes cleared more rapidly from the circulation by the reticuloendothelial system. Patients who experience hypersensitivity reactions to L-ASP exhibit increased clearance of the drug, which results in suboptimal concentrations of the drug in their serum (Burke & Zalewska-Szewczyk, 2022; Zalewska-Szewczyk *et al*., 2007). It has been observed that failure to complete the full course of L-ASP treatment due to toxicities is associated with unfavorable outcomes in ALL (Højfeldt *et al*., 2021; Silverman, 2001). In our investigation, we examined genetic variations in genes that have been previously linked to hypersensitivity to L-ASP. However, in our study, none of the

three genetic variants in GRIA1, CNOT3, and NFATC2 showed any association with hypersensitivity.

The incidence of hypersensitivity to L-ASP in this particular group is very lower (12.5%) when compared to the reported incidences from Spain (55%) (Ovalle *et al*., 2021), the United States (41%) (Chen *et al*., 2010; Panosyan *et al*., 2004), and Slovenia (49.3%) (Rajić *et al*., 2015). There is no definitive explanation for this inconsistency; however, the occurrence of hypersensitivity to L-ASP has been linked to various factors such as the specific L-ASP preparation used, the route of administration, the time point in therapy, concurrent chemotherapy, the intensity and consistency of dosing, racial ancestry, and patient genetics (Burke, 2014; Chen *et al*., 2010; Fernandez *et al*., 2014). In this study, racial ancestry appears to play a significant role in the lower incidence of L-ASP hypersensitivity, as the rate of hypersensitivity is lower among individuals of black descent.

In this study, genetic variants that had been previously reported in *GRIA1*, *CNOT3*, *NFATC2*, and *PNPLA3* were examined. The variant in *GRIA1* and *NFATC2* exhibited lower allele frequencies when compared to the overall African population. Furthermore, MAF of *GRIA1* rs4958351 in this study is lower (17.8%) compared to the reported frequencies in America (27%) (Chen *et al*., 2010), Hungary (34%) (Kutszegi *et al*., 2015), and Caucasians (36.9%) (Rajić *et al*., 2015). On the other hand, the MAF of *NFATC2* rs6021191 (5.6%) was higher than that observed in a study conducted in the USA (2.6%), but lower than the MAF of *NFATC2* rs6021191 among African Americans (14.2%) (Fernandez *et al*., 2014). In comparison to the overall African population, higher frequencies of the *CNOT3* and *PNPLA3* alleles were observed. Additionally, the MAF of the *CNOT3* rs73062673 variant was found to be lower than previous findings, with a percentage of 6.9% as opposed to 10% reported by Højfeldt *et al*. in (2019). The MAF of *PNPLA3* rs738409 (16.9%) was found to be lower in comparison to other studies conducted in North India (29.7%) (Dutta, 2013), Germany (31.3%) (Nischalke *et al*., 2011), and Brazil (43.3%) (Manchiero *et al*., 2017). However, it was observed to be higher than the MAF of African Americans (13.9%) (Romeo *et al*., 2008).

The association analysis for *PNPLA3* was not conducted due to a lack of complete clinical data. Nevertheless, previous studies by Chen *et al.* (2015) and Romeo *et al.* (2008) have demonstrated a significant association between this SNP and nonalcoholic fatty liver disease. Additionally, Liu *et al.* (2017) found a correlation between the *PNPLA3* SNP and liver toxicity induced by L-ASP. The associations between *GRIA1* rs4958351, *CNOT3* rs73062673, and *NFATC2* rs602119 with L-ASP hypersensitivity was not observed in this cohort. However, previous studies (Chen *et al*., 2010; Fernandez *et al*., 2014; Rajić *et al*., 2015), have identified a significantly higher incidence of L-ASP hypersensitivity in patients carrying the risk allele of *GRIA1* rs4958351. Our study aligns with the findings of Kutszegi *et al*. (2015), who also observed no association between *GRIA1* and rs4958351 with *E. coli*-ASP hypersensitivity. The first identification of an association between *CNOT3* and PEG-asparaginase hypersensitivity was made in a genome-wide association study (Højfeldt *et al*., 2019). To our understanding, we are the first to conduct a validation study on this topic; however, our findings did not demonstrate any association between the *CNOT3* SNP rs73062673 and L-ASP hypersensitivity. In a prior investigation, it was discovered that the rs6021191 variant in the *NFATC2* gene exhibited an association with hypersensitivity to L-ASP (Fernandez *et al*., 2014). However, the current study does not show the same results. Similarly, Liu *et al*. (2021) conducted a study and found no association between the *NFATC2* rs6021191 variant and hypersensitivity to PEG-asparaginase.

It is worth mentioning that the incidence of L-ASP hypersensitivity tends to be higher in individuals who carry variant alleles compared to those who carry non-risk alleles for all three genotyped SNPs. The number of participants in this study is small compared to most of the previous studies conducted for the L-ASP association study. It is possible that there could still be an association, but further evidence from a larger population is required to establish this.

The risk of L-ASP hypersensitivity has previously been linked to non-genetic factors such as gender, age, racial ancestry, and risk arm (Chen et al., 2010; Fernandez et al., 2014). Other report suggesting that there is no association between age and gender with L-ASP hypersensitivity (Rajić et al., 2015). Similarly, in the present study no association was observed between non-genetic factors and L-ASP hypersensitivity. In the present

investigation, a potential explanation for the absence of correlation between the predictive factors and L-ASP hypersensitivity might be attributed to a lower occurrence of hypersensitivity reactions, which is further compounded by the smaller size of the sample.

The study encountered various limitations. Despite the limited number of participants and the fact that it was conducted in a single institution, this research offers crucial insights into the factors associated with hematologic toxicities. The findings of the present study are constrained due to the examination of only five genes in the thiopurine pathway. Additionally, a relatively high drop-out rate was observed during the measurement of enzyme activity, which could largely be attributed to one sample batch. We excluded all negative enzyme activity results. We are also unable to sequence the *TPMT* gene to identify possible variants that may impact enzyme activity, aside from the common variants. Furthermore, it was not possible to establish a well-defined cut-off value. The availability of data on liver function was significantly limited, resulting in our inability to address all of the research inquiries. Additionally, it would be valuable to ascertain the activity of the asparaginase enzyme, as this would facilitate the identification of subclinical L-ASP hypersensitivity. The strength of our study lies in the investigation of a cohort of patients from a large African country, where information regarding allele frequencies and the translatability of previously reported associations to this population is often lacking.

Conclusions

In conclusion, this study revealed a high frequency of hematotoxicity, specifically grade 4 neutropenia, among ALL patients who received 6-MP treatment in the maintenance therapy. Grade 4 hematotoxicity incidence was significantly influenced by the age of the child and the blood counts on the first day of maintenance. Patients aged 6 years and younger, as well as those with low WBC or ANC on day 1 of maintenance, require support prior to starting chemotherapy. The study found that the presence of *XDH* and *ITPA* variants was linked to grade 4 hematologic toxicities in pediatric patients undergoing maintenance therapy. This research contributes valuable insights into the identification of high-risk individuals within the Ethiopian population who are

susceptible to chemotherapy-induced toxicity in the context of pediatric ALL. This study revealed that the frequently observed genetic variations in *TPMT* are uncommon among pediatric ALL patients from Ethiopia. Patients who have one copy of the variant gene are at risk of being misclassified when only phenotyping is used to determine their TPMT status. Hence, it is advisable to conduct genotyping prior to commencing thiopurine treatment for pre-treatment classification of the patients. Besides, the incidence of hypersensitivity to L-ASP in pediatric patients with ALL at TASH, Addis Ababa, Ethiopia, is relatively low. In the present study, no significant association was found between hypersensitivity and three previously examined candidate genes. However, it is worth noting that the frequency of hypersensitivity is higher in patients who carry the risk allele.

Recommendations

We suggest conducting future studies involving large cohorts from diverse ethnic backgrounds to ascertain the specific genes that play a significant role in predicting grade 4 hematological toxicities across different ethnic populations. Additionally, it is suggested to sequence the *TPMT* pathway in the future to potentially identify genetic risk factors for hematotoxicity induced by 6-MP. Furthermore, sequencing of the *TPMT* gene is recommended to identify other variants that may impact enzyme activity, apart from the common ones. Measuring 6-MP metabolite and investigation of the association between *TPMT* pathway gene variants with variation in metabolite concentration is recommended. Besides, we suggest future investigations in larger sample size to ascertain association between genetic predictors with L-ASP induced toxicity and measuring asparaginase enzyme activity. Exploring the role of PNPLA3 rs738409 in liver toxicity induced by L-ASP is also recommended as considerable proportion (30.6%) of the participants in this study possess the risk allele.

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Annex: Publications

PLOS ONE

RESEARCH ARTICLE

Incidence and determinants of hematotoxicity in acute lymphoblastic leukemia children who received 6-mercaptopurine based maintenance therapy in Addis Ababa, Ethiopia

Awol Mekonnen Ali[1]*, Haileyesus Adam[2], Daniel Hailu[2], Marieke J. H. Coenen[3¤], Rawleigh Howe[4], Teferra Abula[1]*

1 Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia, **2** Department of Pediatrics and Child Health, School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia, **3** Department of Human Genetics, Radboud Institute for Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands, **4** Armauer Hansen Research Institute, Addis Ababa, Ethiopia

¤ Current address: Erasmus Medical Center, Erasmus University, Rotterdam, Netherlands
* awolalim@gmail.com (AMA); abula_teferra@yahoo.com (TA)

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Abstract

Introduction

The maintenance phase of acute lymphoblastic leukemia treatment is the final and longest stage of treatment, mainly focused on antimetabolite therapy. This phase is essential to eliminate residual leukemic clones and prevent relapse. However, dose-limiting hematotoxicity is a major problem during this phase resulting in dose reduction or treatment discontinuation.

Objective

In this cohort study, the clinical features and risk factors of hematological toxicity during the maintenance phase of treatment were analyzed in pediatric patients from Ethiopia.

Methods

A total of 160 patients from Tikur Anbessa specialized hospital were included in the study of which 142 had sufficient data available for analysis. Patient characteristics as well as information about the care-givers, sides-effects as reported by the care-givers and clinical factors were collected. Bivariable followed by multivariable analysis was performed to investigate which factors were associated with hematological toxicity during the maintenance phase.

Results

During the first six months of maintenance phase treatment grade 4 neutropenia was detected in 52.8% of the patients. The risk of developing grade 4 neutropenia was increased by about two fold in children with the age of 6 years and less compared to those with the age

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of more than 6 years. Similarly, the rate of developing grade 4 neutropenia among children with less than 4,500 maintenance day 1 white blood cell counts was significantly higher than that of children with normal maintenance day 1 white blood cell counts (AHR 2.477, 95% CI = 1.461–4.200, $p = 0.001$).

Conclusion

In conclusion, child's age and day 1 maintenance white blood cell/absolute neutrophil counts significantly affected the occurrence of grade 4 hematotoxicity. Close monitoring for white blood cell and absolute neutrophil counts during maintenance phase treatment is recommended for early diagnosis of hematotoxicity.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer. It is estimated to account for 19% of total childhood cancer incidences globally [1]. In Ethiopia, the annual incidence of childhood cancers has been estimated between 3,707 and 6000 cases, with leukemia being the common (29%), followed by non-Hodgkin's lymphoma, Wilms tumor, and retinoblastoma [2, 3]. Acute leukemia is observed in 89% of all leukemia cases in children in Ethiopia, ALL accounts for 91% of these cases and 9% can be attributed to acute myeloid leukemia [2]. The outcomes for acute leukemia are lower in low- and middle-income countries (LMICs) compared to developed ones [4]. Lack of resources for both the patients and healthcare professionals is the key factor, leading to delayed diagnosis and/or adverse clinical outcomes [5]. Socioeconomic factors like malnutrition, high infection rate, comorbidities, and disease biology also contribute to the inferior survival observed in these settings [4, 5]. The cure rates range from 20% to 70% in LMICs compared to >80% in high income countries [5, 6].

The disease risk group, and thus treatment intensity, is mainly determined by disease stage in LMICs. Hence, patients are stratified into three disease risk groups based on physical examination, age, initial white blood cell count (WBC), central nervous system (CNS) status, and early response [7]. To optimize treatment chemotherapies are combined differently in each phase of treatment based on the disease risk groups [8]. To maintain long-term remission, ALL patients require maintenance phase therapy for up to 2.5 years from the start of treatment with daily oral 6-mercaptopurine (6-MP), weekly oral methotrexate (MTX), and monthly vincristine/steroid pulses [7]. Maintenance phase therapy is directed to keep patients in remission [9], however, it can lead to hematotoxicity (including anemia, leukopenia, neutropenia, and thrombocytopenia) [10, 11]. Hematotoxicity is the main dose-limiting toxicity of chemotherapy [12]. ALL patients with severe neutropenia or thrombocytopenia may require 6-MP dose reduction or even discontinuation [10]. In addition, severe neutropenia can lead to mortality as a result of vulnerability to infections and sepsis [13]. During maintenance phase therapy toxicity is a vital issue to consider; it is the main reason for drug discontinuation, leading to relapse. Moreover, hematotoxicity can be life-threatening, and hence knowledge on factors that influence hematotoxicity can be of added value for the treatment of the patients.

In contrast to low and middle income countries the incidence and factors associated with chemotherapy induced hematotoxicity in ALL are well investigated in developed countries. Therefore, this study aimed to determine the incidence and predictors of hematotoxicity during 6-mercaptopurine based maintenance therapy among pediatric ALL patients from Ethiopia.

Methods

Study setting and patient recruitment

This cohort study was carried out at Tikur Anbessa specialized hospital (TASH), a tertiary care center affiliated with College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. A total of 160 pediatric ALL patients were enrolled from 2019 to 2021 at the pediatric oncology department of TASH. TASH is the only compressive cancer care and treatment center for both children and adults in Ethiopia. The pediatric oncology department of the hospital is located on the 7D, seventh floor of the main building. The outpatient pediatric oncology department is located in the first floor of cancer clinic of the hospital. Patients with renal disease, liver disease, and heart failure were excluded from the study. Patients were stratified into standard risk (SR), intermediate risk (IR), and high risk group (HR) based on a physical examination, age, initial white blood cell count, central nervous system status, and early prednisolone response. Written informed consent was obtained from all participants' caregivers before study enrolment. Ethical approval to conduct this study was obtained from the Institutional Review Board of the College of Health Sciences, Addis Ababa University, the Armauer Hansen Research Institute Ethical Review Committee, and the Ethiopian National Research Ethics Review Committee. The identities of the study participants were kept confidential. Patients were treated using a protocol for low- and middle-income countries [7]. The maintenance phase is always initiated with 75 mg/m^2 of 6-MP based on the protocol for North America. The dose of 6-MP is either discontinued or reduced primarily due to severe neutropenia. Furthermore, trimethoprim/sulfamethoxazole (TMP/SMX) at a dose of TMP 5 mg/kg/d 3 times per week was given for *Pneumocystis jirovecii* prophylaxis as a co-medication.

Data collection

Patients' demographic, clinical characteristics including clinical presentation, complete blood count (CBC), peripheral morphology, peripheral and bone marrow blast, and risk group were collected from medical records. Clinical profiles such as CBC, fever, emergency admission, dose reduction, and drug discontinuation were collected for the first 6-months after start of the maintenance phase. A CBC was performed at a 4-week interval unless it was indicated for any clinical reasons. Family demographics, economic status, child feeding, drug adherence, and reported adverse events were collected from caregivers using questionnaires.

Medication adherence was assessed using the four-item Morisky, Green, and Levine Medication Adherence Questionnaire (MGL). The adherence scores were calculated, and participants were categorized as low adherence (3 or 4 items answered Yes), moderate adherence (1 or 2 item/s answered Yes), and high adherence (0 item answered Yes) [14].

Weight-for-height z-scores (WHZ), height-for-age z-scores (HAZ), weight-for-age z-scores (WAZ), and Body mass index (BMI) for age Z score (BAZ) were computed using WHO Anthro Version 3.2.2 for children up to five years. The WAZ, HAZ, and BAZ were computed using WHO AnthroPlus Version 1.0.4 for children older than five years. The WAZ was generated for children younger than 10 years. Children with z-scores of less than −2 standard deviations (SDs) for HAZ, WHZ, WAZ, and BAZ were classified as stunted, wasted, underweight, and thin, respectively [15].

Study outcomes

Grading of hematologic toxicity was based on the Common Terminology Criteria for Adverse Events (CTCAE), version 4.0 [16]. Accordingly, toxicity classified for grade 4 when WBC $<1000/mm^3$; ANC $<500/mm^3$; anemia <6.5 g/dl and thrombocytopenia $<25{,}000/mm^3$. The primary outcome measure was the occurrence of grade 4 neutropenia during maintenance treatment. The

secondary outcomes were the drug discontinuation, neutropenic fever and early-onset grade 4 leukopenia/neutropenia. Early-onset leukopenia/neutropenia was defined as the occurrence of leukopenia/neutropenia during the first 60 days of the maintenance therapy [17].

Statistical analysis

Data were analyzed using SPSS version 26. Study participants' demographic characteristics, anthropometric, and clinical profiles were presented using descriptive statistics as median (IQR) or as frequency and percentages.

Risk factor analysis and hazard ratios for the primary and secondary outcomes were calculated using Cox regression. Bivariable followed by multivariable analysis were performed to identify predictive factors associated with hematotoxicity. In all multivariable analysis, variables with $p \leq 0.20$ in bivariable analysis were used with enter as variable selection method. To avoid the interaction between WBC and ANC in the regression, two multivariable regression models were used. First, all variables selected from bivariable analysis, except ANC, were entered into multivariable model. In model 2, all variables except WBC were modeled. Significance threshold was set at p-values <0.05. Variable used in the association study for the primary outcome are:—child's age, sex, place of residence, caregivers' educational level, marital status, adherence, HAZ, BAZ, food item, risk group, WBC and ANC.

Results

Socio-demographic characteristics

In total 160 children participated in the study, eighteen children were not included in statistical analysis due to incomplete data. Socio-demographic characteristics of participants included in the statistical analysis are presented in Table 1. 92 (64.8%) were boys and 50 (35.2%) were girls. The mean age was 6.2±3.1 years with 55.6% under the age of 6. Most study participants were from urban area. A majority of the caregivers were married (89.4%) and 22.5% of caregivers had no schooling. 45.1% of the children came from families with more than five members and the majority of the caregivers (90.2%) were biological parents of the children.

All participants were in remission during maintenance phase of treatment. A bit more than half (53.5%) of the children obtained food containing protein and 92.3% used three or more meals daily. Based on the WHO Z-score definition (<-2 ZS), 14.3% of the children were wasted, 22.1% were underweight, 24.6% were stunted, and 21.8% were thin. The medication adherence scores revealed that 78.2% of children have a high level of adherence, while 21.8% have a medium level of adherence as depicted in Table 2. Among medium adherents, 48.4% forgot to give medicines regularly, 22.6% were careless about giving medications, and 29% stopped medication on feeling worse.

The patient baseline clinical profiles are depicted in S1 Table in S1 File. Hepatomegaly (60.4%) and splenomegaly (54.7%) presented in isolation or in combination. A bit more than half of the patients (51.4%) were classified as high risk, all others fall in the standard risk group. BSA of the children ranged from 0.46 to 1.53 m^2 with a median value of 0.79. The median WBC counts at diagnosis was 12,340/mm^3. One-fourth of the patients (23.4%) had WBC counts over 50,000/mm^3. The median WBC and ANC were 3,500/mm^3 and 1,600/mm^3 respectively at the commencement of the maintenance phase of treatment.

Caregivers' perceptions of chemotherapy-related side effects

Fig 1 shows the frequency of caregivers' perceptions of chemotherapy-related side effects. Fever/flu-like symptoms were the most frequently noted side effect with 66.9% of the

Table 1. Socio-demographic characteristics of the study population in outpatient pediatric oncology department of TASH (n = 142).

		Number (percentage)
Child's sex	Male	92 (64.8%)
	Female	50 (35.2%)
Child's age (years)	≤ 6	79 (55.6%)
	> 6	63 (44.4%)
Caregivers' age (years)	18–25	14 (9.9%)
	≥ 26	128 (90.1%)
Caregivers' sex	Male	72 (50.7%)
	Female	70 (49.3%)
Caregivers' marital status	Single	7 (4.9%)
	Married	127 (89.4%)
	Divorced	3 (2.1%)
	Widowed	5 (3.5%)
Place of residence	Urban	82 (57.7%)
	Rural	60 (42.3%)
Caregivers' educational level	Can't read or write	32 (22.5%)
	Elementary	32 (22.5%)
	Secondary education	48 (33.8%)
	Vocational education	9 (6.3%)
	University education	21 (14.8%)
Caregivers' occupation	House wife	44 (31.0%)
	Farmer	34 (23.9%)
	Daily Laborer	4 (2.8%)
	Government Employee	19 (13.4%)
	Retired	1 (0.7%)
	Merchant/Trade	29 (20.4%)
	Self employed	11 (7.7%)
Caregivers' relation to child	Mother	62 (43.7%)
	Father	66 (46.5%)
	Sister	3 (2.1%)
	Brother	4 (2.8%)
	Aunt	2 (1.4%)
	Uncle	2 (1.4%)
	Grand mother	3 (2.1%)
Family size	≤ 5	78 (54.9%)
	> 5	64 (45.1%)
Average monthly income of the family (ETB)	Low (446–1200)	43 (30.3%)
	Average (1201–2500)	26 (18.3%)
	Above average (2501–3500)	29 (20.4%)
	High (>3501)	44 (31.0%)

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caregivers reporting this. Itching or skin rash (57.8%), decreased appetite (50%), and behavior alterations (43%) were consecutively the second, third, and fourth most frequent side effects.

Incidence of grade 4 hematotoxicity, treatment interruption, and emergency admission

A description of grade 4 hematotoxicity, and treatment interruption during the first 6 months of maintenance treatment is presented in Table 3. Early-onset leukopenia was observed in

Table 2. Frequency distribution of child general heath, nutritional status, and adherence in outpatient pediatric oncology department of TASH (n = 142).

		Number (percentage)
Child general health	Excellent	3 (2.1%)
	Very good	97 (68.3%)
	Good	42 (29.6%)
WHZ	Wasted	8 (14.3%)
	Normal	48 (85.7%)
WAZ	Underweight	25 (22.1%)
	Normal	88 (77.9%)
HAZ	Stunted	35 (24.6%)
	Normal	107 (75.4%)
BAZ	Thin	31 (21.8%)
	Normal	111 (78.2%)
Number of meals per day	2	11 (7.7%)
	3	68 (47.9%)
	4	62 (43.7%)
	5	1 (0.7%)
Food item	Meat, egg, Milk, Vegetable	76 (53.5%)
	Other	66 (46.5%)
Adherence	High	111 (78.2%)
	Medium	31 (21.8%)

WHZ = Weight-for-height z-scores, HAZ = Height-for-age z-scores, WAZ = Weight-for-age z-scores, BAZ = Body mass index (BMI) for age Z score

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19.7% of the study participants. About one-third (32.4%) of patients developed early-onset neutropenia. During the first six months of maintenance phase treatment, leukopenia and neutropenia were seen in 31.7% and 52.8% of the patients, respectively. A bit more than half (58.5%) of the patients required drug interruption. Sixty-five (45.8%) patients were admitted to the emergency unit mainly due to neutropenic fever (31%). Anemia was reported in 13.4% of the patients. Only 5.6% experienced thrombocytopenia.

Predictors of grade 4 neutropenia

Table 4 provides an overview of the risk factors linked to the development of chemotherapy-induced grade 4 neutropenia in the bivariable (only factors with a $p<0.02$ are shown) and multivariable cox proportional hazard regression analysis. Bivariable cox proportional hazard regression analysis showed that the child's age, and day 1 maintenance WBC were significantly associated with grade 4 neutropenia. Multivariable analysis revealed that the rate of developing grade 4 neutropenia was about 2 times higher in a child under the age of 6 compared to those above 6 (model 1 and 2). The rate of developing grade 4 neutropenia among children with a WBC less than 4,500 (at day 1 of the maintenance therapy) was 2.477 times (AHR 2.477, 95% CI = 1.461–4.200, $p = 0.001$) higher than that of children with normal WBC counts (model 1). Similarly, the risk of developing grade 4 neutropenia among children with ANC less than 2,500 (at day 1 of the maintenance therapy) was 2.11 times (AHR 2.11, 95% CI = 1.105–4.029, $p = 0.024$) higher than that of children with normal ANC counts (model 2).

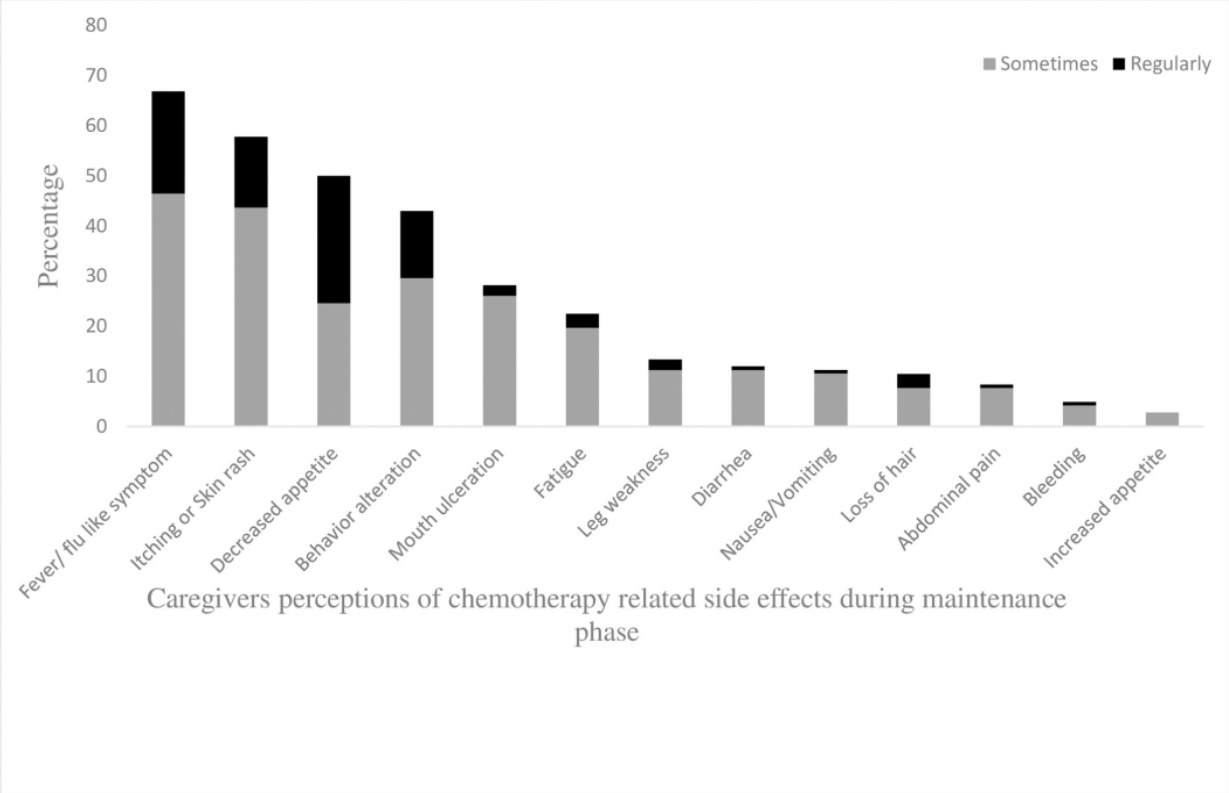

Fig 1. Perceptions of caregivers of children toward the frequency and the severity of chemotherapy related side effects during maintenance phase (n = 142).

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Predictors of grade 4 early-onset leukopenia/neutropenia, treatment interruption and neutropenic fever

Child's age, and day 1 maintenance WBC counts showed association with a treatment interruption during bivariable regression analysis (Table 5). Child's age (≤ 6 year) (AHR 2.1, 95%

Table 3. Incidence of grade 4 hematotoxicity, treatment interruption, and emergency admission, among the study participants (n = 142) during the first 6 months of maintenance therapy in outpatient pediatric oncology department of TASH.

	Number (percentage)
Early-onset leukopenia	28 (19.7%)
Leukopenia	45 (31.7%)
Early-onset neutropenia	46 (32.4%)
Neutropenia	75 (52.8%)
Treatment interruption	83 (58.5%)
Neutropenic fever	44 (31%)
Emergency admission	65 (45.8%)
Anemia	19 (13.4%)
Thrombocytopenia	8 (5.6%)

6-MP = 6-Mercaptourine

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Table 4. Cox proportional hazard regression results for incidence of grade 4 neutropenia in outpatient pediatric oncology department of TASH (n = 142).

Predictor Factor	Multivariable					
	Bivariable		Model 1		Model 2	
	CHR (95% CI)	*p*-value	AHR (95% CI)	*p*-value	AHR (95% CI)	*p*-value
CAY						
> 6	1		1		1	
≤ 6	1.891 (1.174–3.045)	0.009	2.189 (1.351–3.548)	0.001	2.09 (1.291–3.383)	0.003
MD1WBC						
≥4500	1		1			
<4500	2.155 (1.28–3.63)	0.004	2.477 (1.461–4.200)	0.001		
MD1ANC						
≥4500	1				1	
<4500	1.806 (0.952–3.426)	0.07			2.11 (1.105–4.029)	0.024

CAY = Child's age (Years), MD1WBC = Maintenance day 1 WBC, MD1ANC = Maintenance day 1 ANC, AHR = Adjusted hazard ratio, CHR = Crude hazard ratio

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CI = 1.33–3.317, $p = 0.001$), and day 1 maintenance WBC counts (AHR 2.024, 95% CI = 1.249–3.282, $p = 0.004$) were found to be independently associated with a treatment interruption after multivariable regression analysis (model 1). In model 2, low ANC count (AHR 1.938, 95% CI = 1.065–3.527, $p = 0.03$), and child's age (≤ 6 year) (AHR 2.018, 95% CI = 1.282–3.179, $p = 0.002$) were independent risk factors of treatment interruption. Only child's age contributed significantly to the occurrence of neutropenic fever in the bivariable analysis (Table 5). Child's age, and day 1 maintenance WBC counts were significantly associated with early-onset grade 4 leukopenia/neutropenia following bivariable regression analysis (S2 Table in S1 File). Multivariable analysis showed that child's age (≤ 6 year) (AHR 3.024, 95% CI = 1.282–7.136, $p = 0.012$), and day 1 maintenance WBC counts (≤ 4500) (AHR 4.498, 95% CI = 1.555–13.01, $p = 0.006$) were independent predictors of early-onset leukopenia.

Table 5. Cox regression results for neutropenic fever and treatment interruption among the study participants in outpatient pediatric oncology department of TASH (n = 142).

SNPs	Neutropenic fever Treatment interruption							
	Bivariable		Bivariable		Model 1 (Multivariable)		Model 2 (Multivariable)	
	CHR (95% CI)	*p*-value	CHR (95% CI)	*p*-value	AHR (95% CI)	*p*-value	AHR (95% CI)	*p*-value
CAY								
> 6	1		1		1		1	
≤ 6	2.448 (1.26–4.754)	0.008	1.848 (1.179–2.896)	0.007	2.1 (1.33–3.317)	0.001	2.018 (1.282–3.179)	0.002
MD1WBC								
≥4500	1		1		1			
<4500	1.241 (0.658–2.34)	0.505	1.753 (1.09–2.82)	0.021	2.024 (1.249–3.282)	0.004		
MD1ANC								
≥4500	1		1				1	
<4500	1.03 (0.495–2.142)	0.93	1.678 (0.928–3.034)	0.087			1.938 (1.065–3.527)	0.03

CAY = Child's age (Years), MD1WBC = Maintenance day 1 WBC, MD1ANC = Maintenance day 1 ANC, AHR = Adjusted hazard ratio

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Similarly, child's age (≤ 6 year) (AHR 2.919, 95% CI = 1.505–5.659, $p = 0.002$), and day 1 maintenance WBC counts (≤ 4500) (AHR 2.73, 95% CI = 1.349–5.524, $p = 0.005$) were found to be independent risk factor for early-onset neutropenia (S2 Table in S1 File, model 1).

Discussion

In this study we investigated incidence of grade 4 hematotoxicity and factors affecting maintenance treatment of patients with ALL. This study shows a higher incidence of grade 4 neutropenia (52%) as compared to previous reports performed in high income countries [18, 19]. A relatively close incidence of grade 4 neutropenia (47%) was reported in studies conducted in China [20] and Thailand [21]. Conversely, a higher incidence of grade 4 neutropenia was reported in a study performed in Korea [22] and Sweden [23]. This study also showed that the incidence of 6-MP interruption and neutropenic fever were higher than that of the previous reports [24, 25]. However, other studies reported higher treatment interruption than the current study [18, 26]. In general, there are differences in the distribution of 6-MP induced toxicity across the globe, and this discrepancy can be attributed to several factors, including patient characteristics, follow-up period, genetic variation, and differences in 6-MP dose and dose adjustment protocol.

This is one of the very few studies that shows data of side-effects of maintenance therapy as reported by the care-givers. This study revealed that fever/flu-like symptoms followed by itching or skin rash, decreased appetite, and behavior alterations were the most frequently noted side effect. Another study from Indonesia [27] however, reported that behavior alterations were the most frequently noted side effect followed by increased appetite and infection. Higher number of fever/flu-like symptoms in the current study might be due to neutropenia.

Child's age and day 1 maintenance blood counts significantly influence the occurrence of neutropenia. Risk factors for chemotherapy induced hematotoxicity can be categorized into three classes: disease factors; patient characteristics (age, comorbidity, abnormal laboratory results before therapy, and nutrition); and therapeutic factors (types and doses of chemotherapy) [10, 28]. This is among a few studies that assessed factors associated with 6-MP-based chemotherapy side effects in the maintenance phase of pediatric ALL treatment. Despite the importance of neutropenia as the primary dose-limiting toxicity of chemotherapy [29], its risk factors have not been well investigated in pediatric ALL patients, particularly in Ethiopia. Overall the result of this study revealed that child's age, and day 1 maintenance WBC/ANC counts were independent predictors of grade 4 neutropenia. Patients who developed grade 4 neutropenia had a significantly lower age and day 1 maintenance WBC/ANC counts than patients who did not develop grade 4 neutropenia. These findings are in agreement with previous reports on chemotherapy induced neutropenia in adult populations, who documented younger age [12] and low baseline WBC/ANC counts [30] as significant risk factors. Pervious study in pediatric ALL patients also showed that younger age was associated with an increased risk of neutropenia [23].

Failure of neutrophil production in the bone marrow or peripheral neutrophil destruction may cause neutropenia. There are multiple acquired causes of neutropenia such as infection, nutritional deficiencies, copper deficiency, protein malnutrition, immune reactions, and chemotherapy-induced neutropenia [10, 31]. However, the findings of this study depicted that patients' gender, nutritional status, and risk group were not related to neutropenia. These observations are consistent with the study by Rosdiana *et al*., [10], where patients' gender, BMI, nutritional status, and risk group were not associated with neutropenia in pediatric ALL patients. Another study also showed that patients' gender, BMI, and disease risk stratification and stage were not related to the neutropenia occurrence in pediatric cancer patients [32].

However, these findings contradict several reports in adult populations, where BMI, disease stage, and gender are significant predicting factors for the occurrence of neutropenia [29, 33, 34]. In addition to patients' characteristics, no significant association between any of the caregivers' characteristics (age, gender, place of residence, educational level, and marital status) and the risk of neutropenia were identified in this study.

This study highlighted that younger age is a risk factor for febrile neutropenia. This observation is in line with pervious study which indicates that younger age is a risk factor for febrile neutropenia [23]. However, another study showed that age was not a risk factor for febrile neutropenia [35]. Neutropenia is the main reason for treatment discontinuation and dose reduction in this cohort of patients. Age and day 1 maintenance WBC/ANC counts were shown to be associated with the treatment interruption. Patients with a younger age ($\leq$6 years) and low day 1 maintenance WBC/ANC counts had more often 6-MP interruption compared to a patient with older age and normal day 1 maintenance WBC/ANC counts. It is widely accepted that treatment outcome is related to treatment intensity in many drug sensitive cancers, including childhood ALL [36]. The delivered dose intensity is a major determinant of the treatment outcome [37]. Thus, every effort should be made to reduce the occurrence of treatment interruption and our study suggests that a focus on younger children and a low WBC/ANC count might be worthwhile.

Despite the small sample size and a single institutional study, this study provides essential information on factors associated with hematologic toxicities. Unfortunately, studies reporting similar data are limited. Multi-centered future studies with a large sample size are required to further validate these findings. Close monitoring for WBC and ANC counts during maintenance phase treatment is recommended for early diagnosis of hematotoxicity.

Conclusions

In conclusion, this study showed a high incidence of hematotoxicity particularly grades 4 neutropenia in ALL patients who underwent 6-MP treatment during the maintenance phase of treatment. Child's age and day 1 maintenance blood counts significantly affect the occurrence grade 4 hematotoxicity. Patients with the age of 6 year and younger and low day 1 maintenance WBC/ANC counts need prior support before initiation of chemotherapy. However additional studies are necessary to confirm the findings of this study.

Supporting information

S1 File.
(DOCX)

S1 Data.
(XLSX)

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Author Contributions

Conceptualization: Awol Mekonnen Ali, Haileyesus Adam, Daniel Hailu, Teferra Abula.

Data curation: Awol Mekonnen Ali.

Formal analysis: Awol Mekonnen Ali, Marieke J. H. Coenen, Teferra Abula.

Funding acquisition: Awol Mekonnen Ali, Rawleigh Howe, Teferra Abula.

Investigation: Awol Mekonnen Ali, Haileyesus Adam, Daniel Hailu.

Methodology: Awol Mekonnen Ali, Teferra Abula.

Resources: Rawleigh Howe.

Supervision: Haileyesus Adam, Daniel Hailu, Marieke J. H. Coenen, Rawleigh Howe, Teferra Abula.

Writing – original draft: Awol Mekonnen Ali, Marieke J. H. Coenen.

Writing – review & editing: Haileyesus Adam, Daniel Hailu, Marieke J. H. Coenen, Rawleigh Howe, Teferra Abula.

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Supplementary

Supplementary Table 1. Baseline clinical characteristics of study participants in outpatient pediatric oncology department of TASH (n=142).

		Number (percentage)
Hepatosplenomegaly	Hepatomegaly	84 (60.4%)
	Splenomegaly	76 (54.7%)
RBC peripheral	Normocytic & normochromic	84 (63.2%)
	Anisopoikilocytosis	12 (9%)
	Difficult to comment	37 (27.8%)
Peripheral morphology WBC	Normal	21 (16.7%)
	Increase	65 (51.6%)
	Decrease	40 (31.7%)
Risk group	Standard risk	69 (48.6%)
	High risk	73 (51.4%)
Continuous variable		Median (IQR)
Body surface area (m^2) (n=142)		0.79 (0.68-1.01)
WBC at diagnosis (cells/mm^3) (n=137)		12340 (4500-44850)
Lymphocyte at diagnosis (%) (n=119)		77.7 (59.9-87.5)
ANC at diagnosis (cells /mm^3) (n=126)		930 (351-3682)
Hemoglobin at diagnosis (gm/dL) (n=134)		7.55 (6.1-9.2)
Platelet at diagnosis (cells /mm^3) (n=133)		35000 (18500-79000)
Peripheral blast (%) (n=122)		25 (6-54.25)
Bone marrow blast (%) (n=127)		88 (60-100)
WBC at the beginning of maintenance therapy (cells /mm^3) (n=142)		3500 (2675-5450)
ANC at the beginning of maintenance therapy (cells /mm^3) n=142)		1600 (1075-2352)

ANC = Absolute neutrophil count, IQR = Interquartile range, RBC = Red blood cell WBC = White blood cell

Supplementary Table 2. Cox regression results for predictors of the early-onset grade 4 leukopenia and neutropenia in outpatient pediatric oncology department of TASH (n=142).

CAY= Child's age (Years), MD1WBC=Maintenance day 1 WBC, MD1ANC= Maintenance day 1 ANC, AHR = Adjusted hazard ratio

	Early-onset grade 4 leukopenia				Early-onset grade 4 neutropenia			
	Bivariable		Model 1 (Multivariable)		Bivariable		Model 1 (Multivariable)	
	CHR (95% CI)	*p*-value	AHR (95% CI)	*p*-value	CHR (95% CI)	*p*-value	AHR (95% CI)	*p*-value
CAY								
> 6	1		1		1		1	
≤ 6	2.603 (1.106-6.124)	0.028	3.024 (1.282-7.136)	0.012	2.594 (1.343-5.011)	0.005	2.919 (1.505-5.659)	0.002
MD1WBC								
≥4500	1		1		1		1	
<4500	3.934 (1.364-11.342)	0.011	4.498 (1.555-13.01)	0.006	2.38 (1.181-4.798)	0.015	2.73 (1.349-5.524)	0.005
MD1ANC								
≥2500	1				1			
<2500	1.377 (0.524-3.623)	0.517			1.198 (0.578-2.482)	0.628		

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EDITED BY
Funda Tuzun,
Dokuz Eylül University, Türkiye

REVIEWED BY
Eng Wee Chua,
National University of Malaysia, Malaysia
Nathalie K. Zgheib,
American University of Beirut, Lebanon

*CORRESPONDENCE
Awol Mekonnen Ali,
awolalim@gmail.com

†PRESENT ADDRESS
Marieke J. H. Coenen, Erasmus Medical Center, Erasmus University, Rotterdam, Netherlands

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Genetic variants of genes involved in thiopurine metabolism pathway are associated with 6-mercaptopurine toxicity in pediatric acute lymphoblastic leukemia patients from Ethiopia

Awol Mekonnen Ali[1]*, Haileyesus Adam[2], Daniel Hailu[2], Ephrem Engidawork[1], Rawleigh Howe[3], Teferra Abula[1] and Marieke J. H. Coenen[4†]

[1]Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia, [2]Department of Pediatrics and Child Health, School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia, [3]Armauer Hansen Research Institute, Addis Ababa, Ethiopia, [4]Department of Human Genetics, Radboud Institute for Health Sciences, Radboud University Medical Center, Nijmegen, Netherlands

Introduction: Genetic variation in the *thiopurine S-methyltransferase* (*TPMT*) gene by and large predicts variability in 6-mercaptopurine (6-MP) related toxicities. However, some individuals without genetic variants in *TPMT* still develop toxicity that necessitates 6-MP dose reduction or interruption. Genetic variants of other genes in the thiopurine pathway have been linked to 6-MP related toxicities previously.

Objective: The aim of this study was to evaluate the effect of genetic variants in *ITPA*, *TPMT*, *NUDT15*, *XDH*, and *ABCB1* on 6-MP related toxicities in patients with acute lymphoblastic leukemia (ALL) from Ethiopia.

Methods: Genotyping of *ITPA*, and *XDH* was performed using KASP genotyping assay, while that of *TPMT*, *NUDT15,* and *ABCB1* with TaqMan® SNP genotyping assays. Clinical profile of the patients was collected for the first 6 months of the maintenance phase treatment. The primary outcome was the incidence of grade 4 neutropenia. Bivariable followed by multivariable cox regression analysis was performed to identify genetic variants associated with the development of grade 4 neutropenia within the first 6 months of maintenance treatment.

Results: In this study, genetic variants in *XDH* and *ITPA* were associated with 6-MP related grade 4 neutropenia and neutropenic fever, respectively. Multivariable analysis revealed that patients who are homozygous (CC) for *XDH* rs2281547 were 2.956 times (AHR 2.956, 95% CI = 1.494–5.849, p = 0.002) more likely to develop grade 4 neutropenia than those with the TT genotype.

Conclusion: In conclusion, in this cohort, *XDH* rs2281547 was identified as a genetic risk factor for grade 4 hematologic toxicities in ALL patients treated with 6-MP. Genetic polymorphisms in enzymes other than *TPMT* involved in the 6-mercaptopurine pathway should be considered during its use to avoid hematological toxicity.

KEYWORDS

acute lymphoblastic leukemia, xanthine dehydrogenase, neutropenia, pharmacogenetics, 6-mercactopurine

1 Introduction

Globally, acute lymphoblastic leukemia (ALL) is the most common childhood cancer (Johnston et al., 2021). In Ethiopia, the annual incidence of childhood cancers has been estimated between 3,707 and 6,000 cases, with leukemia being the most prevalent cancer (29%) (Aziza et al., 2013; Memirie et al., 2018). Over 90% of patients with childhood ALL in developed countries can be cured as a result of risk-adapted and good systemic chemotherapy. However, treatment outcomes in resource-limited countries are lower, with cure rates ranging from 40% to 70% depending on where the patient is treated (Bahnassy et al., 2020; Tandon, 2020).

In addition to its role in other treatment phases, 6-mercaptopurine (6-MP) is the backbone of the maintenance phase of ALL treatment (Lee et al., 2017). Dose intensity of 6-MP is a key component determining treatment outcome of ALL patients (Relling et al., 1999). Despite good treatment outcomes the downside of 6-MP treatment is that it can induce severe life-threatening hematological and hepatotoxicity. Myelosuppression is particularly complicated when it is accompanied by a severe infection which leads to dose reductions, drug discontinuation, or even treatment interruption and death (Mao et al., 2021). To avoid myelosuppression, 6-MP is titrated to the desired absolute neutrophil count (ANC) value and therefore dose adjustments are routinely made (Karol et al., 2021).

Pharmacogenomics can play a role in increasing the efficacy and reducing toxicity of 6-MP in ALL patients by assisting optimal dose selection for the individual patient. 6-MP is a pro-drug, which requires extensive metabolism to the active metabolite 6-thioguanine. Genetic variants in drug metabolizing enzymes and transporters are known to alter 6-MP treatment outcomes (Jantararoungtong et al., 2021). For instance, it is well established that genetic variants in the thiopurine S-methyltransferase (TPMT) gene are associated with 6-MP treatment intolerance (Wang and Weinshilboum, 2006). To avoid toxicities, 6-MP dose adjustment can be made based on genetic variation in *TPMT*. *TPMT**2*, *TPMT**3B*, and *TPMT**3C* variants alter the tertiary structure of the *TPMT* protein, leading to instability and reduced catalytic activity (Chen et al., 2021). Patients that show an intermediate TPMT enzyme activity are advised to start with a reduced dose (30%–50%) and a 10 fold reduction or alternative treatment is recommended for patients with a complete TPMT deficiency (Bertholee et al., 2017). More recently, *NUDT15* rs116855232 and rs746071566 variants have been linked to 6-MP intolerance (Chiengthong et al., 2016; Walker et al., 2019) and treatment guidelines have also been developed for this gene (Relling et al., 2019). *NUDT15* (rs116855232) mutation adversely affects the protein stability of the enzyme, leading to rapid degradation (Valerie et al., 2016). Whereas, rs746071566 variant reduces NUDT15 activity (Walker et al., 2019). Besides *TPMT* and *NUDT15,* other genes in the 6-MP metabolism have also been investigated in relation to development of side effects. For example, genetic variants in *ITPA* (rs1127354 and rs7270101) reduce enzymatic activity which may increase toxicity risk due to an accumulation of potentially toxic metabolite thioITP (Marinaki et al., 2004; Maeda et al., 2005; Atanasova et al., 2007). A more recent study showed that genetic variants in *XDH* were associated with thiopurine metabolism and thiopurine related toxicities, including neutropenia, hepatotoxicity, and treatment interruption (Choi et al., 2019). The ATP-binding cassette sub-family B member 1 (*ABCB1*) gene encodes for P-glycoprotein. ABCB1 overexpression could be accountable for therapy failure or relapse while the reduced activity can lead to more severe 6-MP toxicity (Milosevic et al., 2018).

In the Caucasian population, genetic variants for *TPMT* have been identified in about 11% (Zeglam et al., 2015) of the population. On the other hand, variants in *NUDT15* are rare with frequencies ranging from 0.2% (Coenen, 2019) to 2.4% (Schaeffeler et al., 2019). There is, however, a scarcity of pharmacogenetic studies focusing on other genes in the 6-MP metabolic pathway, especially in non-Caucasian populations. This study therefore aimed to investigate the frequency of variants coding for drug metabolizing enzymes (*TPMT*, *ITPA*, *NUDT15*, and *XDH*), and transporter (*ABCB1*) in the thiopurine metabolic pathways in Ethiopian children with ALL and their association with 6-MP-related adverse events.

2 Materials and methods

2.1 Patient recruitment and 6-MP treatment

This study included 160 pediatric patients under the age of 12 years at the time of diagnosis, who were treated from 2019 to 2021 at the pediatric oncology wards of Tikur Anbessa specialized hospital (TASH), which provides organized cancer care services. Patients were treated using a protocol for low- and middle-income countries (Hunger et al., 2009). The maintenance phase of this protocol includes daily oral 6-MP and weekly oral methotrexate (MTX). The patients also received monthly vincristine 1.5 mg/m^2, 5 days of dexamethasone per month (6 mg/m^2/d for 5 days/mo) and intrathecal MTX (age-adjusted dosing) until 2.5 years from the date of diagnosis. Trimethoprim/sulfamethoxazole (TMP) at a dose of 5 mg/kg/d 3 times per week was given for *Pneumocystis jirovecii* prophylaxis as co-medication. Patients were stratified into three groups based on a physical examination, age, initial white blood cell count (WBC), central nervous system (CNS) status, and early prednisolone response. Informed consent was obtained from all participants parents or guardians. The study was approved by the Institutional Review Board (IRB) of the College of Health Sciences, Addis Ababa University (021/18), Armauer Hansen Research Institute Ethical Review Committee (P051/18), and National Research Ethics Review Committee of the Federal Democratic Republic of Ethiopia.

2.2 Sample and data collection

EDTA whole blood samples were collected from ALL patients. For all patients, the following information was collected from

medical records; demographic data, clinical presentation during diagnosis, complete blood count (CBC) at diagnosis, peripheral morphology, peripheral and bone marrow blast, liver, and kidney function test, and risk group. Clinical data such as CBC, fever, emergency admission, dose reduction, and drug discontinuation were collected for the first 6 months of the maintenance phase treatment. CBC was performed at a 4-week interval unless it was indicated for any clinical reasons.

2.3 DNA isolation and genotyping

Genomic DNA was extracted from 1 mL of whole blood using QIAamp Blood Midi Kit (Qiagen GmbH, Hilden, Germany). DNA quality was checked using gel electrophoresis and NanoDrop™ ND-2000c Spectrophotometer.

Genotyping of SNP rs1127354 and rs7270101 in *ITPA* and rs2281547 in *XDH* was performed using a KASPar-On-Demand (KOD) assay (LGC Genomics, Hoddesdon, United Kingdom) as described previously (Vos et al., 2016), with little modification. The final volume for each reaction was 5 µL containing 1 µL of DNA, 2.5 µL of KASP 5000 V4.0 Low ROX, 0.0625 µL of the KASPar assay (40x), and 1.44 µL of MilliQ grade water. The PCR conditions consisted of an initial denaturation at 94°C for 15 min, followed by 10 cycles at 94°C for 20 s and annealing/extension at 61°C for 60 s including a drop of 0.6°C for each cycle. This was followed by 26 cycles of denaturation at 94°C for 10 s and annealing/extension at 55°C for 60 s, followed by 12 cycles of denaturation at 94°C for 20 s and annealing/extension at 57°C for 60 s. *TPMT**2, *TPMT**3B, *TPMT**3C, *NUDT15* rs116855232, *NUDT15* rs746071566, and *ABCB1* rs1045642 variants were genotyped by TaqMan® SNP Genotyping Assays (Assay ID number C__12091552_30 for *TPMT**2, C__30634116_20 for *TPMT**3B, C_____19567_20 for *TPMT**3C, C_154823200_10 for rs116855232, and C___7586657_20 for rs1045642) as described elsewhere (Kumagai et al., 2019). The final reaction volume of 5 µL was prepared by mixing 1 µL of DNA, 2.5 µL of TaqMan® Universal PCR Master Mix (2x; Applied Biosystems by Thermo Fisher scientific, Warrington, United Kingdom), 0.0625 µL of TaqMan® SNP Genotyping Assay (40x; Applied Biosystems by Thermo Fisher Scientific), and 1.44 µL of MilliQ grade water. The PCR conditions included an initial stage at 95°C for 12 min and followed by a stage for 50 cycles step 1 at 92°C for 15 s and step 2 at 60°C for 90 s. For *NUDT15* rs746071566 custom designed assay (Id: ANDKGXA) was used. The final reaction volume of 10 µL was prepared by mixing 1 µL of DNA, 5 µL of TaqMan® Universal PCR Master Mix, 0.25 µL of TaqMan® SNP Genotyping Assay, and 3,75 µL of MilliQ grade water. The PCR conditions included an initial stage at 95°C for 10 min and followed by a stage for 50 cycles; step 1 at 95°C for 15 s and step 2 with 60°C for 60 s and post-read stage at 60°C.

2.4 Study outcomes

Myelotoxicity was graded according to Common Terminology Criteria for Adverse Events (CTCAE), version 4.0 (CTCAE, 2010). Accordingly, toxicity was classified as grade 4 when WBC <1,000/mm^3; ANC <500/mm^3; anemia <6.5 g/dL and thrombocytopenia <25,000/mm^3. The primary outcome measure was the occurrence of grade 4 neutropenia within the first 6 months of the initiation of maintenance treatment. The secondary outcomes were, drug discontinuation, neutropenic fever, and early-onset grade 4 leukopenia/neutropenia. Early-onset leukopenia/neutropenia was defined as the occurrence of leukopenia/neutropenia during the first 60 days of the maintenance therapy (Zhou et al., 2018). Neutronic fever was defined as a grade 4 neutropenia with temperature of 38°C or greater.

2.5 Statistical analysis

Data were entered and analyzed using SPSS statistical package for Windows SPSS version 26. The Chi-square test was used to assess Hardy–Weinberg equilibrium (HWE). Descriptive statistics were used to examine the demographic characteristics, clinical profiles, and genotype frequencies of participants. Risk factor analysis and hazard ratios for grade 4 neutropenia, early-onset grade 4 leukopenia/neutropenia, treatment interruption, and neutropenic fever were calculated using cox proportional hazard regression analysis. Bivariable followed by multivariable analysis was computed with enter as the variable selection method. Results were expressed as hazard ratios (HRs) and 95% confidence intervals. For all test a p-value of 0.05 was considered significant. All genetic variants except *TPMT* and *NUDT15* were included in multivariable analysis to see the influence of the variants on hematotoxicity.

3 Results

3.1 Demographic, clinical characteristics and incidence of grade 4 hematological toxicity

In this study, 142 patients were included in the final analysis. Due to incomplete data, 18 study participants were not included in the final analysis. The clinical characteristics of the patients are shown in Table 1. The study group consisted of 92 (64.8%) males, and 50 (35.2%) females, and the mean age at diagnosis was 6.2 years old. Sixty-nine (48.6%) participants were categorized as standard risk, while the remaining 73 (51.4%) were high risk. The overall incidence of chemotherapy-induced grade 4 neutropenia was 52.8%. Fifty-six (39.4%) patients received full doses of chemotherapy as scheduled, while therapy was interrupted in 83 (58.5%) patients because of adverse events in the first 6 months of the maintenance treatment. The most frequent adverse event causing discontinuation was myelosuppression.

3.2 Genotyping and its association with the primary outcome

All genotype frequencies were according to HWE ($p > 0.05$). The overall allele frequencies of *TPMT**3C, *ITPA* rs1127354, *ITPA* rs7270101, *XDH* rs2281547, and *ABCB1* rs1045642 variants were 0.35, 4.6, 11.6, 31, and 77.1%, respectively. No variant alleles were

TABLE 1 Characteristics of the study population in the outpatient pediatric oncology department of TASH (n = 142).

		Number (%)
Child's sex	Male	92 (64.8%)
	Female	50 (35.2%)
Child's age (years)	≤ 6	79 (55.6%)
	> 6	63 (44.4%)
Risk group	Standard risk	69 (48.6%)
	High risk	73 (51.4%)
WBC at the beginning of maintenance therapy (cells/mm^3)	< 4500	90 (63.4%)
	≥ 4500	52 (36.6%)
ANC at the beginning of maintenance therapy (cells/mm^3)	< 2500	111 (78.2%)
	≥ 2500	31 (21.8%)
Neutropenia		75 (52.8%)
Neutropenic fever		44 (31%)
Treatment interruption		83 (58.5%)

Data are presented as number and percentage.
ANC, absolute neutrophil counts; 6-MP, 6-Mercaptopurine; WBC, white blood cell.

TABLE 2 Genotype and allele frequencies of candidate drug metabolizing enzymes and transporter genes by grade 4 neutropenia.

	SNP	Genotype/variant allele	Grade 4 neutropenia		p-value
			No, n (%)	Yes, n (%)	
Genotype frequency	*TPMT**3C	TT TC	67 (100%)	74 (98.7%) 1 (1.33%)	0.343
	ITPA rs1127354	CC CA	62 (92.5%) 5 (7.5%)	67 (89.3%) 8 (10.7%)	0.509
	ITPA rs7270101	AA AC	54 (80.6%) 13 (19.4%)	55 (73.3%) 20 (26.7%)	0.306
	XDH rs2281547	TT TC CC	39 (58.2%) 26 (38.8%) 2 (3.0%)	29 (38.7%) 34 (45.3%) 12 (16%)	0.01
	ABCB1 rs1045642	AA AG GG	7 (10.4%) 22 (32.8%) 38 (56.7%)	2 (2.7%) 25 (33.3%) 48 (64.0%)	0.158
Allele frequency	*TPMT**3C *ITPA* rs1127354 *ITPA* rs7270101 *XDH* rs2281547 *ABCB1* rs1045642	C A C C G	0 (0%) 5 (3.7%) 13 (9.7%) 30 (22.3%) 98 (73.1%)	1 (0.67%) 8 (5.3%) 20 (13.3%) 58 (38.6%) 121 (80.6%)	0.343 0.519 0.34 0.003 0.131

ABCB1, ATP Binding Cassette Subfamily B Member 1; *ITPA*, inosine triphosphate pyrophosphatase; TPMT, thiopurine methyltransferase; XDH, Xanthine dehydrogenase.
The reference genotype is indicated first.

identified for *TPMT**2, *TPMT**3B, *NUDT15* rs116855232, and rs746071566. Table 2 shows a comparison of genotype and allele frequencies between patients who developed grade 4 neutropenia *versus* treatment tolerant patients. Patients carrying the *XDH* rs2281547 CC/TC genotype had a significantly higher incidence of grade 4 neutropenia (p = 0.01) as compared to TT genotype. Similar results were found for the allele analysis. The incidence of grade 4 neutropenia was higher in

TABLE 3 Cox proportional hazard regression results for incidence of grade 4 neutropenia.

SNPs	Bivariable		Multivariable	
	CHR (95% CI)	*p*-value	AHR (95% CI)	*p*-value
XDH rs2281547				
TT	1		1	
TC	1.432 (0.872–2.35)	0.156	1.416 (0.86–2.331)	0.171
CC	3.053 (1.550–6.012)	0.001	2.956 (1.494–5.849)	0.002
ITPA rs7270101				
AA	1			
AC	1.362 (0.816–2.274)	0.237		
ITPA rs1127354				
CC	1			
CA	1.224 (0.588–2.548)	0.589		
ABCB1 rs1045642				
AA	1			
AG	3.021 (0.715–12.758)	0.133		
GG	3.321 (0.807–13.67)	0.096		

AHR, adjusted hazard ratio; CHR, crude hazard ratio; SNPs, Single nucleotide polymorphisms; XDH, xanthine dehydrogenase; ABCB1, ATP Binding Cassette Subfamily B Member 1; ITPA, inosine triphosphate pyrophosphatase; TPMT, Thiopurine methyltransferase.
All of the variants in bivariable analysis were included in multivariable analysis to see the effects of all the variants for the development of grade 4 neutropenia.

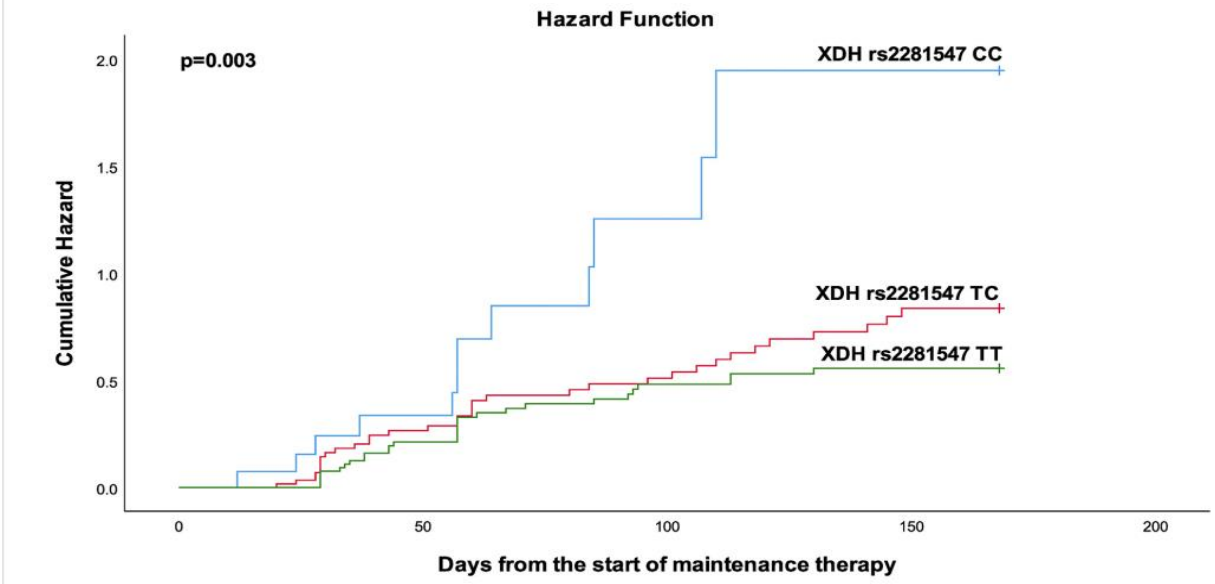

FIGURE 1
Kaplan-Meier curves to estimate cumulative hazard for the development of Chemotherapy-induced grade 4neutropenia stratified by XDH rs2281547.

patients carrying *XDH* rs2281547 C allele (38.6%% vs. 22.3%, p = 0.003). The other allele frequencies did not differ significantly between patients with grade 4 neutropenia compared to treatment tolerant patients.

Detailed data of cox proportional hazard regression analyses for grade 4 neutropenia are depicted in Table 3. Multivariable analysis showed that patients who carry the *XDH* rs2281547 CC genotype had a higher hazard (AHR 2.956, 95% CI = 1.494–5.849, p = 0.002) to

TABLE 4 Cox proportional hazard regression for incidence of neutropenic fever and treatment interruption.

SNPs	Neutropenic fever				Treatment interruption			
	Bivariable		Multivariable		Bivariable		Multivariable	
	CHR (95% CI)	p-value	AHR (95% CI)	p-value	CHR (95% CI)	p-value	AHR (95% CI)	p-value
XDH rs2281547								
TT	1				1		1	
TC	1.348 (0.718–2.53)	0.353			1.609 (1.008–2.569)	0.046	1.61 (1.007–2.573)	0.047
CC	1.541 (0.572–4.15)	0.392			2.785 (1.43–5.426)	0.003	2.704 (1.382–5.289)	0.004
ITPA rs7270101								
AA	1				1			
AC	1.524 (0.798–2.914)	0.202			1.36 (0.834–2.217)	0.217		
ITPA rs1127354								
CC	1		1		1			
CA	2.269 (1.01–5.094)	0.047	2.526 (1.102–5.79)	0.029	1.192 (0.575–2.474)	0.636		
ABCB1 rs1045642								
AA	1				1			
AG	2.437 (0.317–18.74)	0.392			2.184 (0.662–7.202)	0.199		
GG	3.712 (0.507–27.194)	0.197			2.426 (0.758–7.767)	0.135		

AHR, adjusted hazard ratio; CHR, crude hazard ratio; SNPs, Single nucleotide polymorphisms; XDH, xanthine dehydrogenase, ABCB1 = ATP, Binding Cassette Subfamily B Member 1, ITPA, inosine triphosphate pyrophosphatase; TPMT, Thiopurine methyltransferase.
All of the variants in bivariable analysis were included in multivariable analysis to see the effects of all the variants for the development of grade 4 neutropenia.

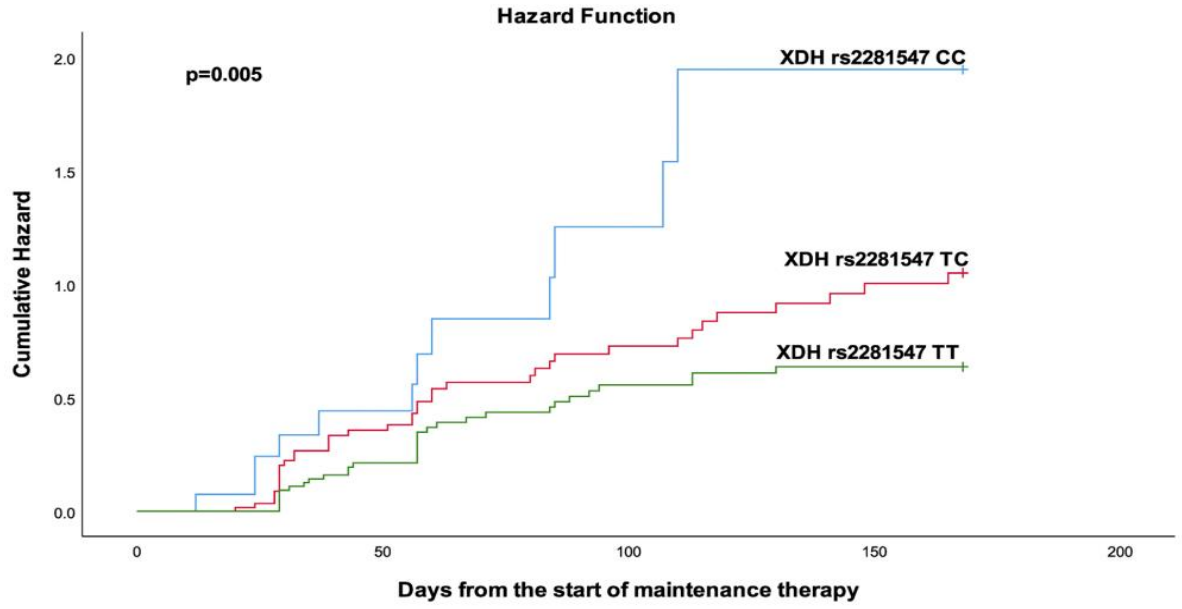

FIGURE 2
Kaplan-Meier curves to estimate cumulative hazard for treatment interruption stratified by XDH rs2281547.

develop grade 4 neutropenia than those with the *XDH* rs2281547 TT genotype. The result indicated that *XDH* rs2281547 was an independent genetic predictor of toxicity. Further, Kaplan–Meier hazard curves (Figure 1) show that the risk of developing grade 4 neutropenia increases over time during the first 6 months of maintenance treatment. The cumulative risk of

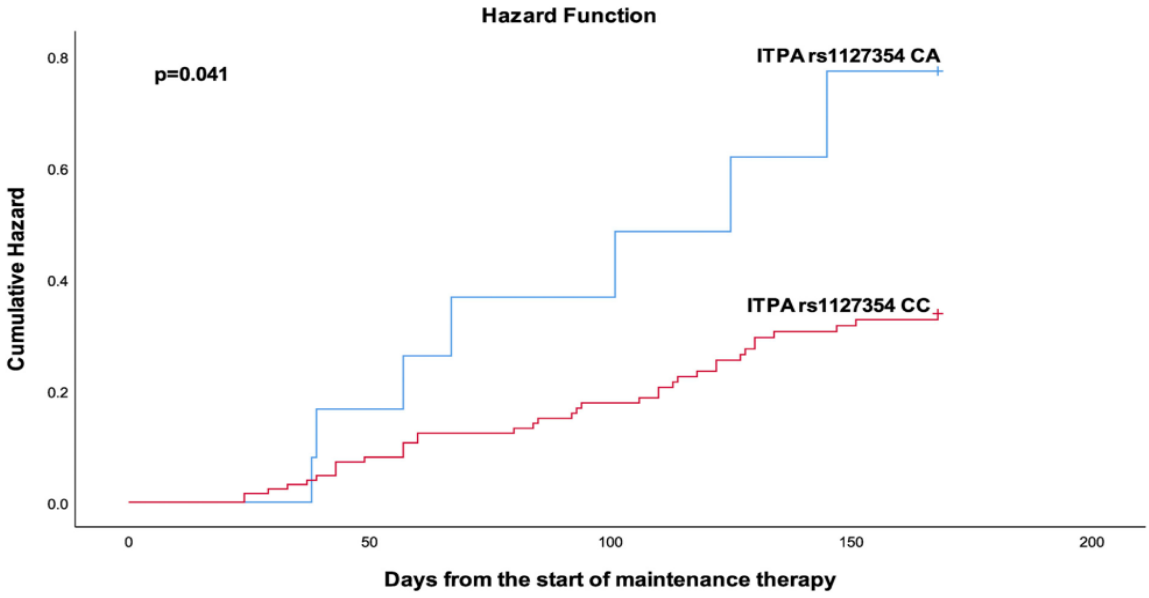

FIGURE 3
Kaplan-Meier curves to estimate cumulative hazard for neutropenic fever stratified by ITPA rs 1127354.

developing grade 4 neutropenia was higher in patients with the CC and TC genotype compared to patients with the TT genotype (p = 0.003).

Effects of both genetic and clinical factor on developing grade 4 neutropenia is shown in Supplementary Table S1. Patients with low day 1 maintenance WBC counts were 2.093 times more at risk to develop grade 4 neutropenia than those with normal day 1 maintenance WBC counts. Similarly, patients with age ≤6 years were 2.273 times more at risk to develop grade 4 neutropenia than those who were >6 years of age.

3.3 Genotyping and its association with the secondary outcomes

Table 4 shows the results from the bivariable and multivariable cox regression analyses for the secondary outcomes. Multivariable analysis demonstrated that patients with the *ITPA* rs1127354 AC had a bit more than two-fold (p = 0.029) increased risk of developing neutropenic fever. Furthermore, patients with *XDH* rs2281547 TC genotype (AHR = 1.61, 95% CI 1.007–2.573, p = 0.047), and CC genotype (AHR 2.704, 95% CI = 1.382–5.289, p = 0.004) were also had to experience more treatment interruption than those carrying the TT variant. Kaplan–Meier hazard curves (Figure 2) show that time to treatment interruption hazard was higher in patients with the *XDH* rs2281547 CC and TC genotype compared to persons with the TT genotype (p = 0.005). Similarly, the time to neutropenic fever hazard was higher in patients with the *ITPA* rs1127354 AC genotype compared to persons with the CC genotype (p = 0.041) (Figure 3).

Multivariable analysis showed that none of the genetic variants tested were associated with early-onset grade 4 leukopenia (Supplementary Table S2). On the other hand, patients with the *ITPA* rs7270101 AC genotype had about two-fold (p = 0.035) increased risk of developing early-onset grade 4 neutropenia.

4 Discussion

Inter-individual genetic variations in drug-metabolizing enzymes and transporters affect the toxicity and efficacy of 6-MP, which poses a challenge to the management of patients. Understanding risk factors linked to individual 6-MP intolerance in different ethnicities is vital to facilitate the development of individualized treatment protocol. This is vital for genetic factors as differences in genotype distribution among different ethnic populations limits the predictive value for toxicities. In this study, the incidence of chemotherapy-induced hematologic toxicity and associated risk factors including genetic variations in drug mobilizing enzymes and transporter relevant for the disposition of 6-MP were investigated in childhood ALL patients from Ethiopia.

NUDT15 rs116855232 and rs746071566, which are considerably lower in frequency in African population, were not detected in this cohort. *TPMT*3C* is the most prevalent allele among the black population (Ameyaw, 1999; McLeod et al., 1999; Adehin et al., 2017). However, in our cohort, the risk allele frequency of *TPMT**3C was 0.35%, about seven times less than described by Ronen *et al.* (Ofri et al., 2010) in Ethiopian

Jews. Besides this, other *TPMT* variants were not found at all (*2 and *2A). Thus, the value of predicting hematologic toxicity in this population by genotyping *TPMT* was hindered by the low frequency of well-known variants to be associated with decreased TMPT activity. It is essential to screen a larger group from Ethiopia to determine the exact frequency.

Literature shows that only up to 25% of thiopurine side effects can be explained by TPMT variants (Broekman et al., 2017). Genetic polymorphisms other than *TPMT* such as *ITPA* and *NUDT15* polymorphisms have been shown to be associated with thiopurine toxicity in patients without *TPMT* polymorphisms (Chiengthong et al., 2016; Mao et al., 2021). Recently study linked *XDH* genetic variant with 6-MP induced toxicity. *XDH* contributes to production of the 6-thioxanthine intermediate from 6-MP and it is also involved in the conversion of the intermediate to the final product (Choughule et al., 2014). This is the first study in Ethiopian ALL patients showing that persons homozygous for the minor allele of *XDH* rs2281547 have a higher risk on grade 4 neutropenia. rs2281547 is an intronic variant, which could alter gene expression and yet the functionality of this SNP is not known. Hence, confirmation of the association should be sought in future studies. Poor XDH enzyme activity increases the risk for 6-MP related hematological toxicity, whereas rapid XDH metabolizers have an increased risk of thiopurine failure due to low 6-TGTP formation (Dewit et al., 2010). Co-administration of 6-MP and allopurinol or febuxostat (XDH/XO inhibitors) increased production of active as well as toxic compounds that can lead to severe forms of thiopurine-induced toxicity (Chocair et al., 1993; Dewit et al., 2010). Despite the fact that *XDH* has a role in the metabolism of 6-MP, the potential clinical significance is not yet clear. There are few studies investigating the role of genetic variants in *XDH*. Molybdenum cofactor sulfurase c.362C > T and c.2107C > A variants alter and reduce the metabolic capacity of XDH, slowing thiopurine metabolism that increases formation of the nucleotide 6-thioguanine, which is hematotoxic (Kurzawski et al., 2012; Stiburkova et al., 2018). A recent pathway genes association study reported the role of *XDH* in thiopurine-related toxicities; neutropenia, hepatotoxicity, and treatment interruption (Choi et al., 2019). A case report study also identified that hematotoxicity caused by azathioprine is aggravated by xanthine oxidase deficiency (Serre-Debeauvais et al., 1995).

This study also identified associations between ITPA polymorphisms and 6-MP related hematological toxicity. The minor allele frequency of *ITPA* rs1127354 and *ITPA* rs7270101 was 4.6%, and 11.6%, respectively, which is similar to other studies (William et al., 2011; Smid et al., 2014; Hareedy et al., 2015). *ITPA* acts as a house-cleaning enzyme since it hydrolyzes 6-TITP back to 6-TIMP, thus preventing the accumulation of 6-TITP, which is toxic (Zamzami et al., 2013; Barba et al., 2022). A recent meta-analysis suggested that populations with low TPMT and high ITPA variant allele frequencies, such as Asians are more susceptible to the influence of ITPA variants (Barba et al., 2022). In the present study, the *TPMT* allele frequencies were also low and interestingly *ITPA* rs1127354 was significantly associated with the incidence of febrile neutropenia. This is in agreement with a previous study (Stocco et al., 2009), showing a significantly higher probability of severe febrile neutropenia in patients with a variant in *ITPA* among patients who received a *TPMT* genotype-guided dose of 6-MP. As reported in an Egyptian ALL study, *ITPA* rs7270101 was associated with the risk of neutropenia and leukopenia (Hareedy et al., 2015). In agreement with these results, we show that *ITPA* rs7270101 was associated with neutropenia that developed within the initial 60 days of the maintenance therapy. This is suggesting that *ITPA* rs7270101 could be an important genetic predictor for determining the 6-MP dose intensity with the risk allele carriers requiring a low 6-MP dose. This is the first study in Ethiopian population showing that *ITPA* alleles are associated with 6-MP related hematological toxicity during maintenance therapy. Nevertheless, there is no consensus on the impact of genetic polymorphisms in ITPA on thiopurine induced hematologic toxicity yet and its application in a clinic setting has not been unified, especially in different ethnic populations (Zhou et al., 2018; Mao et al., 2021).

This study showed that a higher incidence of grade 4 hematologic toxicities (mainly manifested as neutropenic toxicity) caused treatment interruption (Table 1). The finding of this study revealed that *XDH* rs2281547 are strong genetic predictor of grade 4 hematologic toxicities in this cohort of Ethiopian children. This study provides first information on genetic factors associated with hematologic toxicities in Ethiopian childhood ALL patients. However, the outcome of the current study is limited as only five genes in thiopurine pathway were tested. Other limitations include small sample size, a single institutional study, and lack of validation cohorts.

5 Conclusion

In the current study, the rate of hematological toxicity was high. XDH and ITPA variants were associated with grade 4 hematologic toxicities during maintenance therapy. *TPMT*3C* variant frequency was very low in this cohort. This study adds knowledge on how to identify pediatric ALL patients at high risk for chemotherapy induced toxicity in a population from Ethiopia. In the future, it is essential to study large groups of patients from different ethnic background to determine which genes are important to predict grade 4 hematological toxicities in different ethnic populations.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the study protocol was approved by the Institutional Review Board of the College of Health Sciences, Addis Ababa University (021/18/Pharma), the Armauer Hansen Research Institute Ethical Review Committee (PO51/18), and the Ethiopian National Research Ethics Review Committee. Written consent is obtained from guardians of all the study participants. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

AA, RH, TA, HA, and MC designed the study. AA collected the data. AA and MC did genotyping, analyzed the data and draft the manuscript. AA, HA, DH, EE, RH, TA, and MC involved in the investigation and/or in the discussion of results and critical review of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1159307/full#supplementary-material

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Supplementary

Supplementary Table 1. Cox proportional hazard regression results for incidence of grade 4 neutropenia

Predictor Factor	Bivariable		Multivariable	
	CHR (95% CI)	*p*-value	AHR (95% CI)	*p*-value
Child's age (Years)				
> 6	1		1	
≤ 6	1.891 (1.174-3.045)	0.009	2.273 (1.395-3.704)	0.001
Maintenance day 1 WBC				
≥4500	1		1	
<4500	2.155 (1.28-3.63)	0.004	2.093 (1.223-3.580)	0.007
XDH rs2281547				
TT	1		1	
TC	1.432 (0.872-2.35)	0.156	1.493 (0.906-2.460)	0.116
CC	3.053 (1.550-6.012)	0.001	2.481 (1.247-4.934)	0.01
ABCB1 rs1045642				
AA	1			
AG	3.021 (0.715-12.758)	0.133		
GG	3.321 (0.807-13.67)	0.096		

ANC = Absolute neutrophil count, AHR = Adjusted hazard ratio, CHR = Crude hazard ratio WBC = White blood cell count, *XDH* = Xanthine dehydrogenase. It was tested whether the independent variables (child's age, sex, risk group, WBC and genotype) could predict the outcome (grade 4 neutropenia). Factors with a p<0.2 in bivariable are depicted in the table and included in the multivariable analysis to see the influence of both clinical and genetic factor.

Supplementary Table 2. Cox proportional hazard regression for predictors of the early-onset grade 4 leukopenia and neutropenia

	Early-onset grade 4 leukopenia				**Early-onset grade 4 neutropenia**			
	Bivariable		**Multivariable**		**Bivariable**		**Multivariable**	
	COR (95% CI)	***p*-value**	**AOR (95% CI)**	***p*-value**	**COR (95% CI)**	***p*-value**	**AOR (95% CI)**	***p*-value**
***XDH* rs2281547**								
TT	**1**				**1**			
TC	**1.838 (0.795-4.246)**	**0.154**			**1.267 (0.676-2.375)**	**0.46**		
CC	**3.019 (1.012-9.012)**	**0.048**			**2.170 (0.912-5.164)**	**0.08**		
***ITPA* rs7270101**								
AA	**1**				**1**		**1**	
AC	**0.856 (0.347-2.111)**	**0.736**			**1.825 (0.995-3.349)**	**0.052**	**1.926 (1.046-3.547)**	**0.035**
***ITPA* rs1127354**								
CC	**1**				**1**			
CA	**1.184 (0.358-3.923)**	**0.782**			**0.921 (0.33-2.567)**	**0.874**		
***ABCB1* rs1045642**								
AA	**1**				**1**			
AG	**1.447 (0.178-11.762)**	**0.73**			**3.088 (0.406-23.48)**	**0.276**		
GG	**2.343 (0.314-17.465)**	**0.406**			**3.972 (0.542-29.10)**	**0.175**		

AHR = Adjusted odds ratio, CHR = Crude odds ratio, SNPs =Single nucleotide polymorphisms, *XDH* = Xanthine dehydrogenase, *ABCB1* = ATP Binding Cassette Subfamily B Member 1, *ITPA* = Inosine triphosphate pyrophosphatase, *TPMT* = Thiopurine methyltransferase. All of the variants in bivariable analysis were included in multivariable analysis to see the effects of all the variants for the development of early-onset grade 4 leukopenia/neutropenia.

Relationship between thiopurine S-methyltransferase genotype and phenotype in pediatric acute lymphoblastic leukemia in Addis Ababa, Ethiopia.

Awol Mekonnen Ali[1*], Haileyesus Adam[2], Daniel Hailu[2], Johanne Groothuismink[3], Marieke J.H. Coenen[3,4], Rawleigh Howe[5], Teferra Abula[1]

* awolalim@gmail.com

1 Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

2 Department of Pediatrics and Child Health, School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

3 Department of Human Genetics, Radboud Institute for Health Sciences, Radboud university medical center, Nijmegen, The Netherlands

4 Current address: Erasmus medical center, Erasmus University, Rotterdam, Netherlands

5 Armauer Hansen Research Institute, Addis Ababa, Ethiopia

Abstract

Thiopurine S-methyltransferase (TPMT) is a cytosolic transmethylase enzyme that catalyzes the S-methylation of thiopurine drugs, the mainstay of acute lymphoblastic leukemia (ALL) treatment. TPMT enzyme activity shows interindividual variability which can partly be explained by genetic variants in the *TPMT* gene. In this study we aimed to investigate the concordance between genotype and phenotype of *TPMT* in a cohort of patients with ALL from Ethiopia.

In the present study 73 on treatment and 32 treatment completed patients were included. TPMT activity was measured in whole blood of the participant by HPLC. The enzyme activity was expressed both in nmol6MTG/gHb/h and mU/L. The three most common variants in the *TPMT* gene (238G>C, 460G>A and 719A>G) were genotyped.

Only two patients carried the *3C variant (719A>C) (both heterozygous), no other variants were detected. In the treatment group TPMT activity (nmol6MTG/gHb/h) was significantly higher than the treatment completed group (32 vs. 25.55, p = 0.018). In the treatment group TPMT activity

(nmol6MTG/gHb/h) was significantly higher in individuals with Hb < 12 g/dL compared to individuals with Hb ≥12 g/dL (34.05 vs 27, p=0.037).

This study showed that the commonly genotyped variants in *TPMT* are rare in pediatric ALL patients from Ethiopia.

Key words: Acute lymphoblastic leukemia, Thiopurine S-methyltransferase, Genotype, Phenotype

1. Introduction

Thiopurine S-methyltransferase (*TPMT*) is a cytosolic transmethylase enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulphhydryl compounds, including thiopurine drugs [1]. The gene coding for the TPMT enzyme is located on chromosome 6p22.3, it is 34 kb consisting of 10 exons [2]. Genetic variation in the *TPMT* gene can have an effect on the activity of the enzyme. Persons carrying genetic variants in the *TPMT* gene linked to a decreased enzyme activity might have an increased susceptibility to myelotoxicity with thiopurine treatment [3].

Population studies showed that the three most common alleles *TPMT*3A* (460G>A and 719A>G), *TPMT*3C* (only 719A>G), and *TPMT*2* (238G>C) predict low or absent TPMT enzyme activity in approximately 95% of the population [4]. *TPMT*3A* is the most prevalent allele in Caucasians while *TPMT*3C* is the predominant allele reported in Southeast Asian and African populations [5]. In most populations, about 4%-11% of the individuals are heterozygous for a genetic variant in *TPMT* and have an intermediate *TPMT* enzyme activity; whereas nearly 0.3% (1 in 300) individuals are compound heterozygous or homozygous for a variant and have very low or absent TPMT enzyme activity [6, 7]. TPMT enzyme activity can be assessed directly, by phenotyping (enzyme activity measurement), or indirectly, by genotyping for variant alleles [8]. The correlation between *TPMT* genotype and phenotype is high. The overall correlation ranges from 76 to 99% depending on the population studied, though the concordance is lower in the case of intermediate metabolizers [9, 10]. These genotype- phenotype discrepancies might be explained by non-genetic factors or genetic variants in other regions of the gene [9]. Case studies have shown that other variants located in the *TPMT* gene region are known to alter the enzyme activity [11, 12].

Genotyping is an unambiguous method to detect non-functional *TPMT* alleles [13]. As most laboratories do not test all known genetic variants in the *TPMT* gene and as variation in TPMT enzyme activity exists between individuals with the same allele it might for specific cases be more relevant to phenotype [13, 14]. However, the TPMT phenotype can be affected by many factors e.g. blood transfusion and medication [14]. The exact moment when to use genotyping or phenotyping for determining *TPMT* activity status and the clinical implications are subject for discussion [8].

Genetic polymorphisms that influence the TPMT enzyme activity can contribute to interindividual variation in susceptibility to thiopurine therapy [15, 16]. *TPMT* is one of the most important metabolic factors for the regulation of 6-thioguanine nucleotides and affects not only the efficacy but also the safety of treatment with thiopurine [17]. Thus, patients with TPMT deficiency are prone to develop severe hematological toxicity and most cases require a five- to tenfold dose reduction of thiopurine [18, 19]. TPMT activity also seems to contribute to survival and prognosis of childhood ALL, reflecting the therapeutic efficacy of 6-MP treatment [20, 21]. Previous reports indicated that TPMT activity in the children on ALL chemotherapy is higher than children at diagnosis [22, 23]and children who completed treatment [15].

In the present study, TPMT enzyme activity was measured and phenotype-genotype concordance was determined in children receiving 6-MP during ALL maintenance therapy and children who completed maintenance therapy.

2. Materials and Methods

2.1. Study setting and Patient recruitment

The study was conducted at the pediatric oncology department Tikur Anbessa Specialized Hospital (TASH), College of Health Sciences, Addis Ababa University, Ethiopia. *TPMT* enzyme activity was assessed in 73 children with ALL during maintenance therapy and in 32 children that completed ALL treatment. Patients were treated using a protocol for low- and middle-income countries [24]. Patients with renal disease, liver disease, and heart failure were excluded from the study. Informed consent was obtained from caregivers for each child before enrolment in the study. Ethical approval was obtained from the Institutional Review Board (IRB) of the College of Health Sciences, Addis Ababa University (AAU), the Armauer Hansen Research Institute Ethical Review Committee (AAERC), and the Ethiopian National Research Ethics Review Committee.

2.2.TPMT enzyme activity measurement

TPMT enzyme activity was measured in whole blood using high-performance liquid chromatography (HPLC) method described previously [25]. Briefly, 200 µL of whole blood was lysed by freezing at -80ºC. The lysed cells were thawed in 600 μL suspension buffer (0.1 mol/L phosphate buffer, pH 7.4). 500 μL reaction mixture containing enzyme substrates (0.1 mol/L phosphate buffer, pH 7.4, S-adenosyl methionine, and 6-thioguanine) was added to 200 μL of whole-blood lysate and the mixture was incubated at 37ºC for one hour. The reaction was stopped and proteins precipitated by rapid heating of the incubate at 90ºC. Following centrifugation, the enzyme reaction product, 6-methyl thioguanine (6-MTG), was analyzed from the supernatant by HPLC. The enzyme activity was expressed both in nmol6MTG/gHb/h and mU/L.

2.3. Genotyping

Genomic DNA was isolated from 1ml of whole blood using QIAamp Blood Midi Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The DNA quality was assessed using NanoDrop™ ND-2000c Spectrophotometer and gel electrophoresis. *TPMT**2, *TPMT**3B, and *TPMT**3C variants were genotyped by TaqMan® SNP Genotyping Assays (Assay ID number C__12091552_30 for *TPMT**2, C__30634116_20 for *TPMT**3B, and C_____19567_20 for *TPMT**3C) as describer before [26].

2.4. Statistical analysis

Data was analyzed using SPSS version 26. The Hardy-Weinberg equilibrium was assessed using the chi-square test. Column proportion test was used to determine concordance between *TPMT* genotype and phenotype. The TPMT activity cutoff value between intermediate- and high-activity groups was estimated by the receiver operating characteristics curve (ROC). A comparison of groups was carried out with the chi-square test or Mann-Whitney U test as appropriate. $p<0.05$ was considered to be statistically significant.

3. Results

Patient demographics, TPMT enzyme activity, and allelic frequency of common *TPMT* polymorphisms of the patients included in this study (n=98) are presented in Table 1. The majority of the participants were male. Two patients (1.9%) were heterozygous *TPMT**1/*3C, the other

tested variants (*TPMT**2 and *3B) were not identified. The enzyme activity measurements showed a large group of patients with negative activity and we excluded all samples with negative enzyme activity. Therefore, it might be that we exclude some patients that have no enzyme activity due to a (genetic) defect in TPMT. No statistically significant differences in *TPMT* activity in relation to gender and age in any of the group was identified. No significant difference was observed between the median of TPMT activity (mU/L) between the patients on maintenance therapy and those that completed therapy. However, TPMT enzyme activity (nmol6MTG/gHb/h) in the treatment group was significantly higher than the treatment completed group (32 (24.5-41.7) vs. 25.55 (20.05-33.75), $p = 0.018$, respectively). To account for this all subsequent analysis on TPMT enzyme activity in this paper are split in two groups, patients on treatment and patients that completed.

Table 1. Demographic characteristics and TPMT phenotype and genotype of the patients. Continuous variable expresses as median+ IQR and categorical variable as number and percentage.

		During maintenance therapy (N=73)	Completed treatment (n=32)	p-value
Age (years)		6.5 (4-9)	8 (4-11)	0.16
Gender	Female	28 (38.4%)	8 (25%)	0.184
	Male	45 (61.6%)	24 (75%)	
Hb (g/dL)		11.59 (10.95-13.2)	13.53 (12.88-14.65)	<0.001
TPMT activity (nmol6MTG/gHb/h)		32 (24.5-41.7)	25.55 (20.05-33.75)	0.018
TPMT activity (mU/L)		60.3 (46.55-85.0)	57.05 (43.5-73.6)	0.506
*TPMT**1/*1		71 (97.2%)	32 (100%)	-
*TPMT**1/*2		0	0	-
*TPMT**1/*3B		0	0	-
*TPMT**1/*3C		2 (2.7%)	0	-

Hb = Hemoglobin

The correlation of TPMT activity in nmol6MTG/gHb/h and mU/L and Hb concentration are depicted in Table 2. TPMT activity expressed in mU/L is highly positively correlated with *TPMT* activity expressed in nmol6MTG/gHb/h in both treatment and treatment completed groups (r above 0.9 and $p < 0.001$). In both groups, a small (not significant) correlation between the TPMT activity and Hb levels were observed. The enzyme activity in nmol6MTG/gHb/h was negatively correlated with the Hb levels, while TPMT activity in mU/L was positively correlated with the Hb levels, though the correlations were not statistically significant. Besides, in the treatment group *TPMT* activity (nmol6MTG/gHb/h) was significantly higher in individuals with Hb < 12 g/dL compared to individuals with Hb ≥12 g/dL (34.05 (48.0-26.25) vs 27 (38.85-21.00), $p = 0.037$).

This analysis could not be performed for the group that completed treatment as almost all patients had a Hb value above 12.

Table 2. Correlation of *TPMT* activity in nmol6MTG/gHb/h and mU/L with Hb concentration

During maintenance therapy (n=73)		
	TPMT activity (mU/L)	Hb
TPMT (nmol6MTG/gHb/h)	r = 0.933 p < 0.001	r = -0.209 p = 0.076
TPMT activity (mU/L)	1	r = 0.131 p = 0.269
Completed treatment (n=32)		
	TPMT activity (mU/L)	Hb
TPMT (nmol6MTG/gHb/h)	r = 0.962 p < 0.001	r = -0.227 p = 0.212
TPMT activity (mU/L)	1	r = 0.014 p = 0.939

Hg = Hemoglobin, *TPMT* = Thiopurine S-methyltransferase

The distribution of TPMT enzyme activity (nmol6MTG/gHb/h) in the treatment group (n=73) is depicted in Figure 1. The activity ranged from 15 to 77 nmol6MTG/gHb/h (median value of 32) and from 27 to 144 mU/L (median value of 60.3). Around 8.2% of the treatment group had *TPMT* activity less than 18 nmol6MTG/gHb/h in whole blood. Most of the treatment group (91.8%) had an enzyme activity between 18-77 nmol6MTG/gHb/h. The cut-off value between normal and intermediate metabolizers was set to be 18 nmol6MTG/gHb/h or 37 mU/L based on ROC and previously published data (Adehin and Bolaji, 2018). But a well-defined cut-off value could not be set due to the lack of sufficient heterozygous genotypes in this study cohort. The median *TPMT* activity of the intermediate metabolizer was 16.15 nmol6MTG/gHb/h, while the normal metabolizer had a median enzyme activity of 33.1 nmol6MTG/gHb/h.

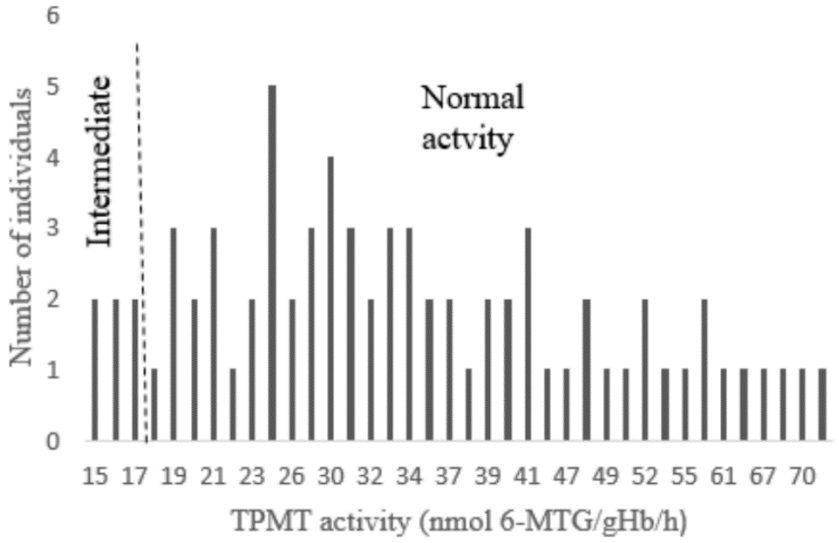

Figure 1. Frequency distribution of thiopurine *S*-methyltransferase activity (nmol6MTG/gHb/h) during maintenance therapy

The relationship between genotype and phenotype according to the SNPs in the treatment group is demonstrated in Table 3. Most of the patients that did not carry a variant in TPMT had a normal enzyme activity (93%). Only one of the two patients with the TPMT*1/*3C genotype had a decreased enzyme activity. While the other patient had an activity of 32 nmol6MTG/gHb/h, which is higher than the cut off value.

Table 3. Concordance between TPMT genotype and phenotype (n=73) during maintenance therapy

		TPMT (nmol6MTG/gHb/h)	
		Normal	Intermediate
TPMT genotype	*1/*1	66 (93%)	5 (7%)
	*1/*3C	1 (50%)	1 (50%)
Total		67 (100%)	6 (100%)

4. Discussion

In the present study, both TPMT phenotype and genotype were determined in children with ALL on 6-MP based maintenance therapy and in children who completed ALL therapy. Whole blood TPMT activity in the children with ALL on 6-MP based maintenance therapy were significantly higher than in those who completed ALL therapy (median difference of 6.45 nmol6MTG/gHb/h, $p < 0.018$). Similarly, *TPMT* activity (mU/L) in the children on 6-MP were higher than in those

who completed ALL therapy. This increase has been previously reported by Lennard *et al*., [27] who showed that the *TPMT* activity in children on chemotherapy was significantly higher than in long-term survivors of ALL and normal control children. A couple of other studies also indicated that TPMT activity during chemotherapy increase to levels well above the range recorded for healthy children and children at ALL diagnosis [22, 23, 28].

Gender is a significant factor affecting TPMT enzyme activity, where a male has higher TPMT enzyme activity than a female [5, 29]. In the current study, however, there was no effect of gender on TPMT activity. Similarly, also other studies did not detect an effect of gender on TPMT enzyme activity [23, 28].

It is well known that TPMT activity follows a trimodal distribution, however, this was not observed in this study [30]. Several other studies also did not observe the trimodal frequency distribution of *TPMT* activity [31–33]. This is probably due to the lack of individuals with genetic variants linked to decreased enzyme activity. The observed allele frequency of the common variants in *TPMT* in the present study is low as compared to allele frequency of Ethiopian Jews (3%) [34] and Nigerians (5.3%) [35].

Schaeffeler *et al*., [5] summarized genotype-phenotype concordance studies and reported an overall concordance of 76-100%; the concordance in the intermediate metabolizer group was lower (50-95.2%) in most studies. Kahlin *et al*., [10] also reported a lower rate (64.4%) of intermediate metabolizer genotype-phenotype concordance. Also, in the present study, there was good genotype-phenotype concordance in the group without a genetic variant. While a lower concordance rate (50%) between heterozygous genotype and phenotype was observed.

Patients with anemia can have high Hb-corrected TPMT activity, this can be overcome by expressing TPMT activity in mU/L. This misleading high TPMT activity could lead to inappropriate treatment [36, 37]. The result of this study is also in agreement with these findings as individuals with a low Hb value had higher TPMT activities than individuals with a normal Hb value when TPMT activity is expressed in nmol6MTG/gHb/h.

Neither phenotype nor genotype alone can give a 100% guarantee to identify individuals with a decreased TPMT activity. In most populations 95% of inactivating TPMT variant alleles can be covered by genotyping *TPMT*2* and the *TPMT*3* family variants. Phenotyping can be used to double-check to make sure that no variants have been missed, this is estimated to occur in 1 in

7,416 *TPMT* heterozygote individuals due to a rare/novel variant alleles [38]. However, compared to genotyping, the risk of misclassifying a TPMT deficient individual as one with intermediate activity is higher when phenotyping is used as the initial test [31]. 6-MP metabolite concentration measurement in pediatric ALL patients with intermediate TPMT activity showed that genotyping outperforms phenotyping to determine the activity. Similar TGN concentrations were observed in persons without a *TPMT* variant that were either intermediate or normal metabolizers [23].

This study had several limitations. The number of samples is relatively small though this is one of the largest studies with a cohort of Ethiopian pediatric ALL patients. We observed a relatively high drop-out for the enzyme activity measurements this can be for a large part be related to one sample batch. Thus it might be that we wrongly excluded patients with a very low enzyme activity, however the two patients with a genetic variant were both included in the enzyme analysis. Unfortunately, we are unable to sequence the *TPMT* gene for possible identification of variants, which possibly affect enzyme activity, other than the common variants.

Conclusion

This study showed that the commonly genotyped variants in TPMT are rare in this cohort of pediatric ALL patients from Ethiopia. Heterozygote patients are at risk of misclassification when only phenotyping is used for *TPMT* status evaluation. Therefore, genotyping is recommended for the pre-treatment classification of the patients before initiation of thiopurine therapy.

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ORIGINAL RESEARCH

Evaluating the Frequencies of *CNOT3*, *GRIA1*, *NFATC2*, and *PNPLA3* Variant Alleles and Their Association with L-Asparaginase Hypersensitivity in Pediatric Acute Lymphoblastic Leukemia in Addis Ababa, Ethiopia

Awol Mekonnen Ali[1], Haileyesus Adam[2], Daniel Hailu[2], Rawleigh Howe[3], Teferra Abula[1], Marieke JH Coenen[4]

[1]Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia; [2]Department of Pediatrics and Child Health, School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia; [3]Armauer Hansen Research Institute, Addis Ababa, Ethiopia; [4]Department of Human Genetics, Radboud Institute for Health Sciences, Radboud University Medical Center, Nijmegen, the Netherlands

Correspondence: Awol Mekonnen Ali, Addis Ababa, Ethiopia, Tel +251 913914432, Email awolalim@gmail.com

Introduction: L-asparaginase is a vital component for the treatment of childhood acute lymphoblastic leukemia (ALL); however, hypersensitivity reactions and hepatotoxicity hinder its anti-neoplastic efficacy. Previous reports indicated that genetic variants in *CNOT3, GRIA1*, and *NFATC2* genes might be associated with hypersensitivity reactions and *PNPLA3* with liver function.

Objective: In this study, it was investigated whether this association also exists in a pediatric ALL cohort from Ethiopia.

Methods: Three variants *GRIA1* rs4958351, *CNOT3* rs73062673, and *NFATC2* rs6021191 were genotyped in a cohort of 160 patients. Association analysis to investigate the association with hypersensitivity reactions was performed using logistic regression analyses. Besides these variants, a variant in *PNPLA3* (rs738409) was genotyped to assess the association with liver function.

Results: Genotype frequencies of *GRIA1* rs4958351, *CNOT3* rs73062673, and *NFATC2* rs6021191 were higher/lower than previously reported. One hundred and forty-four patients were included in the association analysis of which, 18 (12.5%) developed L-ASP hypersensitivity. Though the frequency of hypersensitivity was higher in patients that carried the risk alleles of the three investigated genes, no statistically significant differences were observed. Association analysis between *PNPLA3* rs738409 and liver function could not be investigated due to a lack of clinical information.

Conclusion: In conclusion, none of the tested genes did predict L-asparaginase hypersensitivity in an Ethiopian pediatric ALL patients.

Keywords: acute lymphoblastic leukemia, L-asparaginase, hypersensitivity

Introduction

L-asparaginase (L-ASP) is the first therapeutic enzyme with antineoplastic properties.[1] It has been the backbone of acute lymphoblastic leukemia (ALL) treatment protocol for nearly 40 years.[2] All pediatric regimens for the treatment of ALL and the majority of adult protocols consist of L-ASP in remission induction and intensification treatment protocols.[3] Currently, three L-ASP preparations are available; the native asparaginase derived from *E. coli*, a PEGylated form of this enzyme (PEG-asparaginase), and one isolated from *Erwinia chrysanthemi*.[4] All L-ASP preparations share the same mechanism of action.[5] They deplete serum asparagine levels and subsequently inhibit protein synthesis leading to cytotoxicity.[6]

Unfortunately, L-ASP treatment can be accompanied by side effects such as hypersensitivity, immunosuppression, hepatotoxicity, pancreatitis, and coagulation dysfunction.[7] Hypersensitivity is characterized as an allergic reaction with

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signs and symptoms consistent with an immune response to a known antigen.[8] Hypersensitivity reactions develop in 10–30% of the patients during L-ASP treatment.[8,9] Factors that may affect the incidence of hypersensitivity include the L-ASP preparation, intensity of dosing, route of administration, concurrent chemotherapy, and time point in therapy.[10] Several studies indicate that specific genetic variants might also be linked to L-ASP hypersensitivity, eg, variants in Glutamate Ionotropic Receptor AMPA Type Subunit 1 (*GRIA1*), CCR4-NOT Transcription Complex Subunit 3 (*CNOT3*), and Nuclear Factor of Activated T Cells 2 (*NFATC2*).[11] The intronic variant rs4958351 in the *GRIA1* gene appeared to be a strong risk factor for L-ASP hypersensitivity both in genome-wide and candidate gene studies.[12,13] Another genome-wide association study (GWAS) led to the identification of an association between L-ASP hypersensitivity and a SNP on chromosome 19 (rs73062673) in the non-coding region close to *CNOT3*. This gene regulates the transcription of *HLA* genes.[14] The *NFATC2* rs6021191 variant has been found to be significantly associated with the risk of developing L-ASP hypersensitivity in children with ALL.[15] Mice studies show that *NFATC2* knockout significantly reduces the risk of developing L-ASP hypersensitivity.[16]

Another candidate gene is *PNPLA3*. Several genetic studies showed that the common nonsynonymous variant c.444C>G (rs738409) of this gene is the key genetic determinant of fatty liver disease severity in pediatric and adult patients.[17,18] In a study focusing on patients with pediatric ALL, this variant showed an association with hepatotoxicity during the induction phase of ALL therapy.[19]

So far, GWAS and candidate gene studies have identified several genes that could be associated with L-ASP hypersensitivity reactions and hepatotoxicity. To the best of our knowledge, there have been no previous reports of the frequency of variants that are linked to L-ASP toxicity in Ethiopia, with a population of over 100 million people. Therefore, the present study was designed to evaluate the frequency of variants that are previously linked to L-ASP hypersensitivity and liver toxicity in a cohort of pediatric ALL patients from Ethiopia. In addition, it was investigated if the variants are associated with hypersensitivity reactions.

Method

Patients and Treatment Protocol

This study was conducted at Tikur Anbessa specialized referral teaching hospital, Addis Ababa University, Addis Ababa, Ethiopia. A total of 160 pediatric ALL patients were enrolled. ALL patients are stratified into standard risk (SR), intermediate risk (IR), and high-risk group (HR) based on physical examination, age, initial white blood cell count (WBC), central nervous system status, and early prednisolone response. Informed consent was obtained from all participants' caregivers before study enrolment. Patients with liver and kidney problems were excluded. This study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the College of Health Sciences, Addis Ababa University (AAU) (021/18/Pharma), the Armauer Hansen Research Institute Ethical Review Committee (PO51/18), and the Ethiopian National Research Ethics Review Committee.

Asparaginase in ALL Protocol

Patients were treated according to the standard ALL treatment protocol for low and middle income countries.[20] Intravenous (IV) native *E. coli* L-ASP is part of the induction, consolidation, and delayed intensification phases of this treatment protocol. It is administered nine times at a dose of 6000 U/m^2 both in the induction and delayed intensification phase irrespective of the risk group. Patients in the HR group receive additional L-ASP at a dose of 10,000 U/m^2 for eight times in the consolidation phase of the protocol.

L-ASP Hypersensitivity Phenotyping

The baseline data, demographic, clinical presentation during diagnosis, complete blood count (CBC) at diagnosis, peripheral morphology, peripheral and bone marrow blast, liver and kidney function test, risk group, and L-ASP hypersensitivity reaction were collected from medical records.

Sample and Genotyping

EDTA whole blood samples were collected from ALL patients aged less than 12 years old at the time of diagnosis. Genomic DNA was isolated from peripheral leukocytes in 1mL of whole blood using QIAamp Blood Midi Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The DNA quality was assessed by gel electrophoresis and NanoDrop™ ND-2000c Spectrophotometer (Thermo Scientific, Isogen, the Netherlands). Genotyping of SNPs rs4958351 in *GRIA1* and rs738409 in *PNPLA3* was performed using a KASPar-On-Demand (KOD) assay (LGC Genomics, Hoddesdon, UK) as described previously with little modification.[21] The final volume for each reaction was 5 µL containing 1 µL of DNA (10 ng/µL), 2.5 µL of KASP 5000 V4.0 Low ROX (2x; LGC Genomics), 0.0625 µL of the KASPar assay (40x), and 1.44 µL of MilliQ grade water. The PCR conditions consisted of an initial denaturation at 94°C for 15 min, followed by 10 cycles of denaturation at 94°C for 20s and annealing/extension at 61°C for 60s, including a drop of 0.6°C for each cycle. This was followed by 26 cycles of denaturation at 94°C for 10s and annealing/extension at 55°C for 60s, followed by 12 cycles of denaturation at 94°C for 20s and annealing/extension at 57°C for 60s. *CNOT3* rs73062673 and *NFATC2* rs6021191 variants were genotyped using TaqMan® SNP Genotyping Assays (Assay ID number C__98092291__20 for rs73062673, C_29689803_10 for rs6021191) as described before.[22] The final reaction volume of 5 µL was prepared by mixing 1 µL of DNA (10 ng/µL), 2.5 µL of TaqMan® Universal PCR Master Mix (2x; Applied Biosystems by Thermo Fisher scientific, Warrington, UK), 0.0625 µL of TaqMan® SNP Genotyping Assay (40x; Applied Biosystems by Thermo Fisher Scientific), and 1.44 µL of MilliQ grade water. The PCR conditions included an initial stage at 95°C for 12 min and followed by 50 cycles with step 1 at 92°C for 15s followed by step 2 with 60°C for 90s. MilliQ grade water and positive controls were included for quality control in both TaqMan® and KASP assay. The genomic DNA samples were amplified in Veriti™ 96-well Fast Thermal Cycler PCR (Applied Biosystems, Singapore) and results were analyzed using QuantStudio™ 3 v1.5. software (Applied Biosystems by Thermo Fisher Scientific, Singapore).

Statistical Analysis

Observed minor allele frequencies in this cohort were compared with minor allele frequencies reported in dbSNP. All statistical analyses were conducted using SPSS version 26. The Hardy-Weinberg equilibrium was assessed using the chi-square test. Demographic characteristics, clinical profiles, and genotype frequencies of participants were analyzed using descriptive statistics. Bivariable logistic regression analyses were performed to determine the association between genetic variants and asparaginase hypersensitivity. Factors previously reported to be linked with asparaginase hypersensitivity (age, gender, risk group) were included as co-variates. Tests were considered significant when the *p*-value was lower than 0.05.

Results

Incidence of Asparaginase Hypersensitivity

The basic characteristics of the enrolled patients are shown in Table 1. A total of 160 patients participated in this study, of which 144 (90%) were included in the final L-ASP hypersensitivity association analysis as data on (possible)

Table 1 Socio-Demographic Characteristics and Frequency of Asparaginase Hypersensitivity (N = 144)

		n (%)
Child's sex	Male	94 (65.3)
	Female	50 (34.7)
Child's age (years)	≤10	122 (84.7)
	>10	22 (15.3)
Hepatosplenomegaly	Hepatomegaly	83 (59.7)
	Splenomegaly	76 (54.7)
Risk group	Standard risk	67 (46.5)
	High risk	77 (53.5)
L-ASP hypersensitivity	Yes	18 (12.5)

hypersensitivity was missing for 16 patients. Fifty (34.7%) of the patients were girls, a bit more than half of the patients presented with hepatomegaly (59.7%) and splenomegaly (54.7%) in isolation or combination. One hundred and twenty-two (84.7%) of the study participants were 10 years and younger. 53.5% of the patients belonged to the high-risk group and 46.5% were assigned to the standard risk group. Eighteen (12.5%) of the patients included in the final L-ASP hypersensitivity association analysis experienced hypersensitivity reactions.

Allele Frequencies

Genotypes were determined for all patients included in the study (n = 160) (Table 2). *GRIA1* rs4958351 minor allele frequency (17.8%) is low compared to African general frequency (25.2% dbSNP). The minor allele frequency (6.9%) of *CNOT3* rs73062673 is a bit higher as compared to the frequencies of the African population reported in dbSNP (4.6%). A low minor allele frequency (5.6%) of *NFATC2* rs6021191 is observed in the present study compared to African general frequency (15.2% dbSNP). The minor allele frequency (16.9%) of *PNPLA3* rs738409 is higher compared to the reported African general allele frequency (8.9% dbSNP).

Association Analysis for L-ASP Hypersensitivity

Association analysis for L-ASP hypersensitivity was performed for 144 patients with complete information on hypersensitivity. Bivariable logistic regression analyses revealed that none of the patient profiles (age, gender, and risk group) or genetic factors showed a significant association with L-ASP hypersensitivity. A comparison of L-ASP hypersensitivity frequency in relation to the patient's genotype is presented in Figure 1. The frequency of L-ASP hypersensitivity is higher in patients with a variant allele compared to a patient without a variant allele, though not statistically significant. Due to a lack of clinical data on hepatotoxicity, no association analysis was performed for *PNPLA3* rs738409.

Discussion

Patients who develop L-ASP hypersensitivity reactions have increased L-ASP clearance leading to suboptimal concentrations of the drug in the serum.[23,24] Failure to receive the full course of L-ASP treatment due to toxicities has been linked with poor outcomes in ALL.[25,26] We investigated genetic variants in genes previously associated with L-ASP hypersensitivity.

The incidence of L-ASP hypersensitivity in this cohort (12.5%) is very low compared to incidence reports from Spain (55%),[27] America (41%),[12,28] and Slovenia (49.3%).[13] There is no clear explanation for this discrepancy; however, the frequency of L-ASP hypersensitivity has been associated with the L-ASP preparation, intensity and consistency of dosing, route of administration, concurrent chemotherapy, a time point in therapy, racial ancestry, and patient genetics.[10,12,15] Racial ancestry may play a major role for lower incidence of L-ASP hypersensitivity, as the rate of hypersensitivity is lower in the black population.

Table 2 Genotype and Allele Frequencies of Candidate Gene SNP (N = 160)

	Genotype	Genotype Frequency (%)	Minor Allele Frequency of this Study	dbSNP Minor Allele Frequency (African Population)
GRIA1 rs4958351	GG	108 (67.5)	17.8% (Allele A)	25.2% (Allele A)
	GA	47 (29.4)		
	AA	5 (3.1)		
CNOT3 rs73062673	TT	140 (87.5)	6.9% (Allele C)	4.6% (Allele C)
	TC	18 (11.3)		
	CC	2 (1.3)		
NFATC2 rs6021191	AA	142 (88.8)	5.6% (Allele T)	15.2% (Allele T)
	AT	18 (11.3)		
PNPLA3 rs738409	CC	111 (69.4)	16.9% (Allele G)	8.9% (Allele G)
	CG	44 (27.5)		
	GG	5 (3.1)		

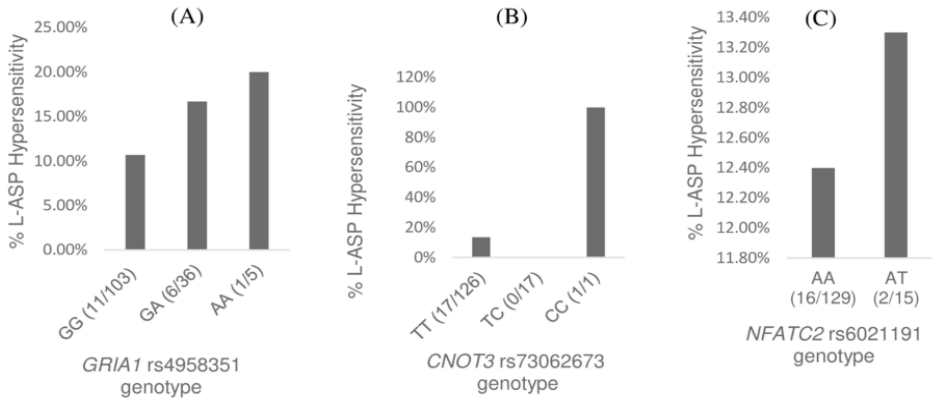

Figure 1 Frequency of L-ASP hypersensitivity in relation to the investigated SNPs. For each SNP the reference genotype is depicted in the first bar. (**A**) *GRIA1* rs4958351 (**B**) *CNOT3* rs73062673 (**C**) *NFATC2* rs6021191.

In this study, previously reported genetic variants in *GRIA1, CNOT3, NFATC2*, and *PNPLA3* were investigated. Lower allele frequencies as compared to the general African population were observed for the variants in *GRIA1* and *NFATC2*. In addition, the minor allele frequency of *GRIA1* rs4958351 (17.8% in this study) is lower than reported in America (27%),[12] Hungary (34%),[29] and Caucasian (36.9%).[13] The minor allele frequency of *NFATC2* rs6021191 (5.6%) was higher than a study performed in the USA (2.6%) but lower than the minor allele frequency of *NFATC2* rs6021191 in African Americans (14.2%).[15] Higher allele frequencies of *CNOT3* and *PNPLA3* were observed as compared to the general African population. Besides, the minor allele frequency of *CNOT3* rs73062673 was lower than previous findings (6.9% versus 10%).[14] Also, the minor allele frequency of *PNPLA3* rs738409 (16.9%) was lower compared to those from other studies done in North India (29.7%),[30] Germany (31.3%),[31] and Brazil (43.3%).[32] However, the minor allele frequency was higher than that of African Americans (13.9%).[33]

Due to a lack of complete clinical data, no association analysis was conducted for *PNPLA3*. However, several previous studies showed that this SNP was significantly associated with nonalcoholic fatty liver disease,[33,34] and L-ASP induced liver toxicity.[19] The associations of *GRIA1* rs4958351, *CNOT3* rs73062673, and *NFATC2* rs602119 with L-ASP hypersensitivity were not replicated in this cohort. GWAS study by Chen et al,[12] and candidate gene studies by Rajić et al,[13] and Fernandez et al,[15] identified a significantly higher incidence of L-ASP hypersensitivity in patients with *GRIA1* rs4958351 risk allele carrier. However, our study is in line with a report by Kutszegi et al,[29] who also showed no association between *GRIA1* rs4958351 and *E. coli*-ASP hypersensitivity. The first study showing association between *CNOT3* and PEG-asparaginase hypersensitivity was a genome-wide association study.[14] To the best of our knowledge, we are the first to perform a replication study, unfortunately, we were unable to show association between the *CNOT3* SNP rs73062673 and L-ASP hypersensitivity. In a previous study, Fernandez et al[15] found that the rs6021191 in the *NFATC2* was associated with L-ASP hypersensitivity. But, this is not replicated in the present study. Likewise, no association was found between *NFATC2* rs6021191 and PEG-asparaginase hypersensitivity in a study done by Liu et al.[35]

In general, it is interesting to note that the frequency of L-ASP hypersensitivity is higher in variant carriers compared to non-risk allele carriers for all three genotyped SNPs. Maybe an association might still exist; however, a larger population will be necessary to prove this.

Non-genetic variables like gender, age, racial ancestry, and risk arm have previously been associated with the risk of L-ASP hypersensitivity.[12,15] But other reports indicate that age and gender were not linked to L-ASP hypersensitivity.[13] The present study also investigated non-genetic factors (age, gender, risk group) for association with L-ASP hypersensitivity. We were unable to identify an association with these factors. In the current study, a possible reason for the lack of association between the predicting factors and L-ASP hypersensitivity could be a lesser incidence of the hypersensitivity reactions compounded by a smaller sample size.

The major limitation of this study is the small sample size although it is the largest group of pediatric ALL patients from Ethiopia. Unfortunately, the data on liver function were largely lacking and therefore we could not address all research questions. Besides, it might be of interest to know the asparaginase enzyme activity as this would allow for detecting subclinical L-ASP hypersensitivity. The strength of our study is that we investigated a group of patients from a large African country for which knowledge on allele frequencies and information on whether previously reported associations are translatable to this population is often missing.

Conclusion

In conclusion, the incidence of L-ASP hypersensitivity among pediatric ALL patients in Tikur Anbessa, Addis Ababa, Ethiopia is low. In the current study, there was no significant association between hypersensitivity and three previously investigated candidate genes, though the frequency of hypersensitivity is high in patients carrying the risk allele.

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Disclosure

The authors report no conflicts of interest in this work.

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