Cell lines and animal model in the analysis of pharmacogenomics markers in childhood acute lymphoblastic leukemia

par

BAHRAM SHARIFASKARI

Département de Pharmacologie
Faculté de médecine

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**Cell lines and animal model in the analysis of pharmacogenomics markers in childhood acute lymphoblastic leukemia**

présentée par:

**BAHRAM SHARIFASKARI**

a été évaluée par un jury composé des personnes suivantes:

Dr. Guy Rousseau  
président-rapporteur

Dr. Maja Krajinovic  
directeur de recherche

Dr. Elie Haddad  
co-directeur de recherche

Dr. Noël J-M Raynal  
membre du jury

Dr. Francine Durocher  
examinateur externe

Dr. Devendra Amre  
représentant du doyen
Abstract

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy of childhood. It is the principal cause of cancer–related mortality in children due to a persistent group of patients who does not respond to standard anti-cancer treatment. Susceptible patients may also suffer from number of toxicities associated with intensive chemotherapy treatment. Pharmacogenetic studies of our group, showed that particular genetic variants of the folate dependent enzymes, particularly, dihydrofolate reductase (DHFR) and thymidylate synthase (TS), major targets of methotrexate (MTX), correlate both individually and combined with increased risk of relapse in patients with childhood ALL. Furthermore, variations of \(ATF5\) gene involved in asparagine synthetase (\(ASNS\)) regulation and of \(ASNS\) gene were associated with higher risk of ALL relapse or with ASNase related toxicity in patients who received E.coli asparaginase (ASNase). The major goal of my doctoral research project was to further understand from the functional point of view the role of genetic variations underlying therapeutic responses of childhood ALL, by focusing on two major components of ALL treatment, MTX and ASNase.

My specific goal was to analyze associations found in clinical setting using cellular proliferation assay in lymphoblastoid cell lines (LCLs, n=93) and xenograft mice model of ALL. Genetic variation in \(TS\) polymorphism (homozygosity for triple repeat allele, 3R) and of \(DFHR\) haplotype *1b (defined by particular allelic combination derived from six polymorphic sites in the major and minor promoter of \(DHFR\)), on MTX sensitivity was assessed using cellular proliferation assay. Similar \textit{in vitro} assay in response to E.coli ASNase was used to access the \(T1562C\) variation in the \(ATF5\) 5’UTR and particular haplotypes of \(ASNS\) gene (defined by two genetic variation and arbitrarily named
haplotype *1). Xenograft mouse model was used to access the effect of TS 3R3R genotype.

Analysis of additional polymorphisms in ASNS gene revealed diversification of haplotype *1 of ASNS gene in 5 subtypes, two polymorphisms (rs10486009 and rs6971012,) defining particular subtypes correlated with in vitro sensitivity to ASNase and one of them (rs10486009) seems particularly important for reducing sensitivity to ASNase in vitro, possibly providing mechanistic explanation for lower sensitivity of haplotype *1 observed in clinical setting. No association between ATF5 T1562C variation and cellular proliferation assay in response to E.coli ASNase was found.

We did not observe genotype-related association when in vitro sensitivity to MTX in LCLs was analyzed. In contrast, in vivo results using xenograft mouse model demonstrated the relationship between the TS 3R/3R genotype and the resistance to MTX treatment in dose-dependent manner. Obtained results provided function explanation for the significantly higher risk of relapse seen in 3R/3R ALL patients and suggest that these patients might benefit from increase dose of MTX. Through these experiments we also showed that xenogeneic mice model can serve as a preclinical tool to explore individualized treatment options.

In conclusion, the knowledge acquired through my doctoral work confirmed and/or identified some functional variants in MTX and ASNase action pathway which may facilitate dose individualization strategies, allowing for optimal treatment selection or tailoring childhood ALL therapy based on individual genetics.
Keywords: Pharmacogenomics, dihydrofolate reductase (DHFR), thymidylate synthase (TS), polymorphisms, methotrexate (MTX), asparaginase (ASNase), asparagine synthetase (ASNS), childhood leukemia, animal model.
Résumé

La leucémie aiguë lymphoblastique (LAL) est le cancer pédiatrique le plus fréquent. Elle est la cause principale de mortalité liée au cancer chez les enfants due à un groupe de patient ne répondant pas au traitement. Les patients peuvent aussi souffrir de plusieurs toxicités associées à un traitement intensif de chimiothérapie. Les études en pharmacogénétique de notre groupe ont montré une corrélation tant individuelle que combinée entre les variants génétiques particuliers d’enzymes dépendantes du folate, particulièrement la dihydrofolate réductase (DHFR) ainsi que la thymidylate synthase (TS), principales cibles du méthotrexate (MTX) et le risque élevé de rechute chez les patients atteints de la LAL. En outre, des variations dans le gène ATF5 impliqué dans la régulation de l’asparagine synthetase (ASNS) sont associées à un risque plus élevé de rechute ou à une toxicité ASNase dépendante chez les patients ayant reçu de l’asparaginase d’E.coli (ASNase). Le but principal de mon projet de thèse est de comprendre davantage d’un point de vue fonctionnel, le rôle de variations génétiques dans la réponse thérapeutique chez les patients atteints de la LAL, en se concentrant sur deux composants majeurs du traitement de la LAL soit le MTX ainsi que l’ASNase. Mon objectif spécifique était d’analyser une association trouvée dans des paramètres cliniques par le biais d’essais de prolifération cellulaire de lignées cellulaires lymphoblastoïdes (LCLs, n=93) et d’un modèle murin de xénogreffe de la LAL. Une variation génétique dans le polymorphisme TS (homozygosité de l’allèle de la répétition triple 3R) ainsi que l’haplotype *1b de DHFR (défini par une combinaison particulière d’allèle dérivé de six sites polymorphiques dans le promoteur majeur et mineur de DHFR) et de leurs effets sur la sensibilité au MTX ont été évalués par le biais d’essais de
prolifération cellulaire. Des essais *in vitro* similaires sur la réponse à l’ASNase de E. Coli ont permis d’évaluer l’effet de la variation *T1562C* de la région 5’UTR de *ATF5* ainsi que des haplotypes particuliers du gène *ASNS* (définis par deux variations génétiques et arbitrairement appelés haplotype *1*). Le modèle murin de xénogreffe ont été utilisé pour évaluer l’effet du génotype *3R3R* du gène *TS*.

L’analyse de polymorphismes additionnels dans le gène *ASNS* a révélé une diversification de l’haplotype *1* en 5 sous-types définis par deux polymorphismes (*rs10486009* et *rs6971012*,) et corréle avec la sensibilité *in vitro* à l’ASNase et l’un d’eux (*rs10486009*) semble particulièrement important dans la réduction de la sensibilité *in vitro* à l’ASNase, pouvant expliquer une sensibilité réduite de l’haplotype *1* dans des paramètres cliniques. Aucune association entre *ATF5 T1562C* et des essais de prolifération cellulaire en réponse à ASNase de E.Coli n’a été détectée.

Nous n’avons pas détecté une association liée au génotype lors d’analyse *in vitro* de sensibilité au MTX. Par contre, des résultats *in vivo* issus de modèle murin de xénogreffe ont montré une relation entre le génotype *TS 3R/3R* et la résistance de manière dose-dépendante au traitement par MTX. Les résultats obtenus ont permis de fournir une explication concernant un haut risque significatif de rechute rencontré chez les patients au génotype *TS 3R/3R* et suggèrent que ces patients pourraient recevoir une augmentation de leur dose de MTX. À travers ces expériences, nous avons aussi démontré que les modèles murins de xénogreffe peuvent servir comme outil préclinique afin d’explorer l’option d’un traitement individualisé.

En conclusion, la connaissance acquise à travers mon projet de thèse a permis de confirmer et/ou d’identifier quelques variants dans la voix d’action du MTX et de
l’ASNase qui pourraient faciliter la mise en place de stratégies d’individualisation de la dose, permettant la sélection d’un traitement optimum ou moduler la thérapie basé sur la génétique individuelle.

**Mots clés:** Pharmacogénomique, dihydrofolate reductase (DHFR), thymidilate synthase (TS), polymorphismes, méthotrexate (MTX), asparaginase (ASNase), asparagine synthétase (ASNS), leucémie pédiatrique, modèle animal.
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Abbreviations and Acronyms

ALL: Acute Lymphoblastic Leukemia

ABC: ATP-binding cassette transporters

ABCG2: ATP-binding cassette superfamily G member 2

ATF5: Activating transcription factor 5

AICART: Aminoimidazole Carboxamide Ribonucleotide Transformylase

AML: Acute Myeloid Leukaemia

AML1: Acute myeloid leukemia 1 protein

ASO: Allele-specific Oligonucleotide

ASS1: Argininosuccinate Synthase 1

ASNase: Asparaginase

ASNS: Asparagine Synthetase

AVN: Avascular necrosis

BCRP: Breast Cancer Resistance Protein

BCR-ABL1: Breakpoint cluster region–ABL1

Bcl-XL: B-cell lymphoma-extra large

Bcl-2: B-cell lymphoma 2

BH3: Bcl-2 homology 3

BIM: BCL-2-interacting mediator of cell death

BM: Bone Marrow

CB-CIK: Cord blood cytokine-induced killer
CCND1: Cyclin D1
CD: Cluster of differentiation
CEPH: Centre d’Etude du Polymorphisme Humain
CML: Chronic Myeloid Leukaemia
CRLF2: Cytokine Receptor-Like Factor 2
CRPC: Castration-resistant prostate cancer
DFCI: Dana-Farber Cancer Institute
DHF: Dihydrofolate
DHFR: Dihydrofolate reductase
DFS: Disease Free Survival
DNA: Deoxyribonucleic acid
dTMP: Deoxythymidine mono phosphate
dUMP: Deoxyuridine mono phosphate
EBV: Epstein-Barr virus
E. coli ASNase: Escherichia coli Asparaginase
EFS: Event Free Survival
ERG: ETS-related gene
ETP: Early T-cell precursor
ETV6 RUNX1: Ets Variant Gene 6 Runt-related transcription factor 1
FPGS: Folylpolyglutamate Synthetase
GARTF: Glycinamide ribonucleotide transformylase
GC: Glucocorticoids
GGH: γ-glutamyl Hydrolase
GVHD: Graft-Versus-Host Disease
GWAS: Genome Wide Association Study
HDMTX: High dose Methotrexate
hIFN-α: Human interferon-alpha
HR: High risk
iAMP21: intrachromosomal amplification of chromosome 21
IBD: Inflammatory bowel disease
IC50: Half maximal inhibitory concentration
IG: Immunoglobulin
IHC: Immunohistochemical
IL-27: Interleukin (IL)-27
IKZF1: Ikaros family zinc finger protein 1
LCL: Lymphoblastoid Cell Line
LDMTX: Low dose Methotrexate
LICs: Leukemia-Initiating Cells
LYL1: Lymphoblastic leukemia derived sequence 1
MTHFR: Methylene tetrahydrofolate reductase
MLL: Mixed Lineage Leukemia
MRD: Minimal Residual Disease
MRPs: Multidrug Resistance-related Proteins
MSCs: Mesenchymal Cells
MTX: Methotrexate
NOD/SCID: NonObese Diabetic Severe Combined Immune Deficient
NSG mice: NOD/LtSz-scid IL-2Rgc null mice

NTD: Neural Tube Defects

OS: Overall survival

PBX1: Pre-B-cell leukemia homeobox 1

PCR: Polymerase Chain Reaction

PDNS: Purines de novo synthesis

PEG-ASNase: Polyethylene glycol–conjugated asparaginase

P-gp: P-glycoprotein

RFC1: Reduced Folate Carrier 1

RUNX1: Runt-related transcription factor 1

2R and 3R: Double and Triple repeat polymorphisms

SLC19A1: Solute Carrier family 19 member 1

SNPs: Single Nucleotide Polymorphisms

SR: Standard risk

TAL1: T-cell acute leukemia 1

TCF3 PBX1: Transcription factor 3 Pre-B-cell leukemia homeobox 1

TCR: T-cell receptor

THF: Tetrahydrofolate

TLX1: T-cell leukemia homeobox 1

TLX3: T-cell leukemia homeobox 3

TMP: Trimethoprim

TMQ: Trimetrexate

TPMT: Thiopurine Methyltransferase
TRAIL: TNF-related apoptosis-inducing ligand

TS: Thymidylate Synthase

3′UTR: 3′untranslated region

5′UTR: 5′untranslated region

VHR: Very high risk
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Chapter I
Introduction

Part I

Acute lymphoblastic leukaemia:

Acute lymphoblastic leukaemia (ALL) is the most common malignancy of childhood and the most common cause of cancer related mortality affecting children from 0 to 14 years. About approximately 57,000 cases reported worldwide, particularly, 6000 and 2500 new ALL cases are diagnosed every year only in the USA and Canada respectively [1-4]. B-cell precursor ALL is a main morphologic and histological subtype of childhood leukemia with approximately 75-80% of cases. It is one of the most common malignant disorders of lymphoid progenitor cells in children with peak prevalence of 3-5 years [5-8]. During last four decades introduction of multi-agent treatment protocols and risk-guided treatment changed outcome for children with ALL. Currently, 5-year event-free survival (EFS) rates for childhood ALL is more than 80-85% in the developed countries [9]. The other two important childhood leukemia include myelogenous leukemia (AML) with frequency of 20% to 25% and chronic myelogenous leukemia (CML) which account for 5% of leukemia cases [7]. Although ALL occurs in children and adults, children showed better prognosis compared to adults [6, 10]. It has been shown that 95% of children achieve complete remission after phase of treatment conducted with several intensive multi-agent chemotherapy and about 70% are long-term survivors. Nevertheless overall relapse rate is still 20% and survival after relapse remains poor [11].
Etiology and epidemiology of childhood leukemia:

Childhood ALL might arise from interactions between exogenous (e.g. infection, environmental exposure) or endogenous exposures (e.g. inflammation, oxidative stress) and genetic (inherited) susceptibility increasing the chance of a mutation in relevant oncogenes/tumor suppressor genes and chromosome alterations (Figure 1) [3]. There are several known risk factors including ionizing radiation and congenital or genetic syndromes (such as Down’s, neurofibromatosis, Shwachman syndrome, Bloom's syndrome, and ataxia telangiectasia, and Fanconi’s anaemia) which are associated with ALL (collectively <5% of ALL) [4, 7, 8]. Children with Down syndrome (trisomy 21) are 15 times more likely to develop leukemia compared with children without trisomy 21 [4, 7]. Diet of the mother and child, parental smoking, pesticides and household chemicals, traffic fumes are environmental exposures suggested to contribute to ALL development. The incidence rate of disease increased by 1% during last two decades. Furthermore, immune modulators through for example infections and vaccinations, can as well play a role in onset of lymphoblastic leukemia in particular the pre-B cell childhood ALLs [4]. A large number of risk factors including genetic component of ALL remains still undefined.
Figure 1: Different causes of childhood ALL (Inaba 2013).

Figure 2: Morphologic and immunohistochemical (IHC) pictures of B-ALL patients: A, The blasts with azurophilic granules. B, Diffuse infiltrate replacing bone marrow medullary space. Wright-Giemsa–stained bone marrow (Zhou 2012).
Genetic alterations in ALL:

Based on evidences commonly mutated genes in ALL patients are those involved in the development and differentiation of various types of blood cells, and these genes vary according pre-B or T cells leukemia [12]. ALL also has been classified into B-acute lymphoblastic leukemia (B-ALL) and T-cell lineage ALL (T-ALL). Furthermore, ALL has been traditionally classified into precursor B, B-cell and precursor T (or T-cell) phenotypes. According karyotypic abnormalities, ploidy and translocations, they will be further subdivided [13]. B-ALL is derived from B-cell progenitors (Figure 2). B-ALL accounting for 80%, while T-ALL comprises 12%-15% of all newly diagnosed paediatric childhood ALL. T-cell ALL is associated with a poor prognosis with only 7–23% of patients surviving for 3 to 5 years [10, 14]. Patients with B-ALL can be classified to \( ETV6-RUNX1 \) (\( TEL-AML1 \)) B-ALL, \( TCF3-PBX1 \) (\( E2A-PBX1 \)), \( BCR-ABL1 \) ALL, MLL-rearranged B-ALL according to specific genetic abnormality [15, 16] (Figure 3). \( ETV6-RUNX1 \) involves \( ETV6 \), previously known as \( TEL \), on chromosome 12p13 and \( RUNX1 \), located on chromosome 21q22, previously known as AML1 which is a transcription factor required for hematopoietic development and differentiation in embryonic and adult stages. \( TEL-AML1 \) translocation occurs in 20% to 25% of childhood ALL cases and confers a favorable prognosis [10, 12, 15, 17]. Another most common genetic abnormality is \( t(1;19) \) (q23;p13) which results in the fusion of the TCF3 (formerly E2A) gene with the \( PBX1 \) gene which occurs in 3-5% of B-ALL patients and is usually associated with favorable outcome, but in adult ALL the prognosis of \( t(1;19) \) patients with French and Italian studies reporting poor outcomes [10, 18]. High risk, includes...
rearrangements of the mixed lineage leukemia (MLL) gene; and very high risk includes
translocation t(9:22) which generate the BCR-ABL1 fusion gene, known as the
Philadelphia chromosome, and hypodiploidy [15, 16, 19, 20]. The frequency of
Philadelphia chromosome is about 3% to 6% among childhood ALL, but occurs more
frequently (around 25%) among adults [15]. Based on recent studies of genome-wide
profiling using microarrays, candidate gene, and second-generation sequencing helped to
identify other genetic alterations that define new ALL subtypes. Deletion of the IKZF1
(IKAROS) is common in BCR-ABL1 lymphoid leukemia but is rarely present in CML at
chronic phase. IKAROS is a zinc finger transcription factor required for the development
of all lymphoid lineages. IKZF1 alteration cooperate with BCR-ABL1 in the induction of
lymphoblastic leukemia and subsequent resistance to therapy in recent experimental
models of BCR-ABL1 ALL [15]. ALL patients carrier IKZF1 deletion had lower 5 years
EFS (30%) comparing those without this genotype (51%) and they showed very poor
outcome in spite of haemopoietic stem-cell transplantation [14]. Deletion in IKZF1 gene
which involved in regulating B-cell differentiation at relapse, commonly occur in the
BCR-ABL1-like ALL subtype, BCR-ABL1-like ALL include more than 15% of childhood
B-ALL cases which exhibit a gene expression profile similar to that of BCR-ABL1–
positive ALL, often have deletion/mutation of IZKF1, and associated with a very poor
outcome [14, 15].
Figure 3. A) Genetic subtypes in childhood B-ALL and B) Distribution of acute lymphoblastic leukemia subtypes by age. (Inaba 2013 and Wiemels 2012).
**Genetic profile and immunophenotypic features of B-ALL:**

To differentiate cancer arises from precursors of B or T cell, the bone marrow smear undergoes cytochemical staining for distinguishing between lymphoid and myeloid blasts. To further classify the leukemia, the blasts undergo immunophenotypic classification that measures the expression of cytoplasmic and surface antigens, which can discriminate B-cell, early pre-B-cell, pre-B-cell, or T-cell leukemia [10, 20]. In this regard, early pre–B lymphoblasts express CD19 (100% of patients), cytoplasmic CD22, and CD34 and later pre–B cells are positive for CD10, CD45 and CD20. Likewise T-cell leukemia is positive for CD3, CD5, CD7, CD10 and CD34 [10, 21].

**Ploidy within a leukemia blast cell population:**

Determination of the modal number of the leukemia chromosomes is one of the prognostic factors for B-cell precursor ALL patients. Chromosome number (ploidy) within a leukemia blast cell population includes hyperdiploidy with 51–68 chromosomes, low hyperdiploidy with 47-50 chromosomes, pseudodiploidy with 46 chromosomes, diploid or normal with no cytogenetic abnormalities and hypodiploidy with ≤45 chromosomes which occur respectively 25% to 30%, 11%, 28%, 30% and 7%–8% of childhood ALL cases [15, 20, 22]. Hyperdiploid leukemia confers a favourable prognosis, but hypodiploid leukemia with a chromosome number less than 46 is associated with poor prognosis [15, 20].
Molecular Monitoring and Response to Therapy:

The significance of the morphological examination is now being replaced by the detection of minimal residual disease (MRD), through flow cytometric detection of leukemia-associated immunophenotypes or PCR amplification of fusion transcripts, immunoglobulin (IG) and T-cell receptor (TCR) gene [23-25]. MRD is the single most powerful prognostic marker for patients in all risk groups, which can determine the response to treatment in ALL patients more precisely than morphological screening of bone marrow smears. Likewise, MRD now become the new definition for leukemia remission and is measured concurrently with the morphological examination. The other advantages and applications of MRD detection in patients with ALL are determining remission status before and after transplantation, and detecting early relapse. Accordingly, measuring MRD levels during remission induction therapy provide important prognostic information [25]. MRD level inversely correlates with 5 years event free survival (EFS) rate. Based on obtained results, the EFS of 88% is correlates to MRD level less than 0.01% and, EFS of 59%, 49% and 30% are correlates with MRD level more than 0.01%, 0.1%, and 1% respectively. Now, cytogenetic and MRD statuses are the 2 most important prognostic factors both in childhood B-ALL and adult B-ALL[10]. Based on Dana-Farber Cancer Institute (DFCI) protocol and investigation, end-induction MRD level can be considered as a significant independent predictor of outcome [26]. According to DFCI treatment protocol, children with high end-induction MRD (≥0.001) had a 10.5-fold greater risk of relapse than those with low MRD [26]. Considering MRD levels in the BM at day 15 were well correlated with risk of relapse, determining MRD levels at day 15 is an informative checkpoint to test the in vivo sensitivity of the leukemia
in the individual precursor B-cell ALL [27]. Likewise, patients presenting high levels of MRD at day 33 (at the end of induction phase) and no MRD detectable at day 78 (at the end of intensification phase) had a favourable outcome, but those with detectable MRD at day 78, showed significant increase risk of relapse [28].

**Treatment:**

Treatment of paediatric ALL has greatly improved due to the introduction of effective combination risk-adapted therapies. DFCI Consortium treatment protocol and their risk group classification for childhood ALL, is one of the referent protocol currently used for childhood ALL management and treatment (Table 1, II) [26]. DFCI ALL Consortium has been conducting multi institutional clinical trials in the United States and Canada in childhood ALL since 1981. DFCI Consortium treatment protocol assigned as a research protocols and in term of clinical research always addressing particular research questions through randomization. The major goal of DFCI ALL Consortium Protocol 91-01 conducting randomized clinical trials in childhood ALL and continuing focusing on improving efficacy and outcome of newly diagnosed ALL children while minimizing acute and late toxicities [26, 29]. Based on DFCI results, 1457 children aged 0–18 years were treated on four consecutive protocols among 1985–2000 [26]. The 2 year DFCI treatment program consisted of four phases; multi-agent remission induction therapy (4 weeks); CNS-directed treatment (3 weeks); intensification (20-30 weeks) and therapy will be continued until the completion of 24 months of complete remission [26, 29]. Treatment protocols vary based on risk group classification determined at the time of diagnosis, include; standard risk (SR), high risk (HR) and very high risk (VHR). CNS treatment for SR and HR patients began on the day that complete remission was attained.
The goal of remission induction treatment (also called induction therapy) for ALL is to restore normal hematopoiesis in 96–99% of children and 78–92% of adults with ALL and bring about a complete remission with as few toxic adverse effects as possible [3, 30, 31]. The speed rate and depth of the early response to remission induction therapy recognise as a major index of subsequent therapy in many protocols worldwide [32]. Remission induction is 4 weeks duration phase and patients received a vincristine, corticosteroids such as prednisone and dexamethasone, doxorubicin, cytarabine, ASNase and MTX. The goal of central nervous system (CNS) directed therapy is critical to maximize and improved survival rates. CNS therapy which elongated three weeks includes all remission induction phase drugs plus 6-mercaptopurine [26, 33]. CNS treatment for infants was delayed until the age of 12 months [33]. Intensification therapy is a critical phase of lymphoblastic leukemia treatment protocol to eradicate residual leukaemic cells [3, 26, 33]. Although about two-thirds of lymphoblastic leukemia cases can be treated successfully only with 12 months of treatment. Patients with childhood leukaemia need continuation treatment in order to prevent possible relapse [34]. Thus continuation therapy is beginning after intensification, and continued until the completion of 24 months of complete remission [26, 33].

**Drugs used in ALL:**

Protocol phases and related drugs based on risk group presented in table 1 and 2 [26, 33].

**Cytosine arabinoside** (Cytarabine, Ara-C) is an antimetabolite widely used in acute leukemia. Cytarabine must be phosphorylated to cytotoxic metabolite Ara-CTP (Ara-C three phosphates) and exert its cytotoxicity during or after incorporation into the DNA where they interfere with DNA strand elongation, replication, or repair processes [35].
Cytarabine therapy is associated with several adverse side effects, including myelosuppression, infections, mucositis, neurotoxicity, and acute pulmonary syndrome [36].

**Doxorubicin** is an anthracycline antibiotics commonly used in the treatment of leukemias. The major doxorubicin related concern is well-known dose-related cardiotoxicity [36]. Doxorubicin attack cancer cells by multiple mechanisms; they work primarily by DNA intercalation and inhibiting replication which lead to DNA damage and cell death. Also doxorubicin binds to mitochondrial DNA, inhibiting other cellular functions. It can inhibit the action of topoisomerase II, an enzyme that unzips the DNA molecule for replication [37, 38].

**6-Mercaptopurine (6-MP)** is an antimetabolite and an analog of purine base. It is important component of ALL treatment administered during intensification and maintenance treatment phases. Upon entry into the leukemic cells it is metabolized to 6-thioguanine nucleotides (6-TGN) by thiopurine methyltransferase (TPMT). 6-MP exerts its cytotoxic effect by inhibition of DNA synthesis and by interfering with the activity of nucleic acid-processing enzymes. More precisely, TGN incorporation into the DNA and RNA results in DNA strand-breaks, chromatid damage and subsequent cell cycle arrest and apoptosis. 6-TGN is more potent than 6-MP; based on *in vitro* and *in vivo* data, lymphoblasts are more sensitive to 6-TGN and exert more toxicity compared to 6-MP. 6-TGN is no longer used for continuation treatment, because it has been associated with thrombocytopenia, an increased risk of death, and hepatic veno-occlusive disease upon prolong use of thioguanine at a dose more than 40 mg/m2 [13, 19]. 6-TGN is active
metabolite of 6-MP which has cytotoxic and immunosuppressive properties [39, 40]. TPMT genotyping may be useful to predict this risk prior to drug administration [36, 40].

**Vincristine** is important vinca alkaloids known as antimicrotubule agent. The most frequent and clinically important side-effect of vincristine is dose-limiting autonomic and peripheral sensory-motor neuropathy [36, 41]. Vincristine induces cytotoxicity by interacting with and disrupting microtubules polymerization, especially those comprising the mitotic spindle apparatus so microtubules do not form. Vincristine also binds to neuronal tubulin, lead to disrupting axonal microtubules and causing neurotoxicity [42].

**Corticosteroids** induce apoptosis in the malignant lymphoid cells and are critical component of combination therapy for ALL. Glucocorticoids exert their cytotoxicity effects by binding with glucocorticoid receptors (GR). After binding, GR can homodimerize and translocate to the nucleus, and interact with glucocorticoid response elements to transactivate gene expression, resulting expression alteration in various oncogenes, cell cycle arrest and apoptosis [43][44]. Dexamethasone and prednisone may cause avascular necrosis (AVN) or osteonecrosis as a chronic complication of leukaemia treatment [45]. However, dexamethasone has a longer half-life and better central nervous system (CNS) penetration, appears to yield better CNS control [43].

Mechanism of action and pharmacogenomics of MTX and ASNase as two major components of DFCI protocol precisely presented in future sections.

<table>
<thead>
<tr>
<th>Induction (4 weeks)</th>
<th>CNS therapy (3 weeks)</th>
<th>Intensification (20–30 weeks) Every 3 week cycles</th>
<th>Continuation (78 weeks) Every 3 week cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>prednisone daily, 40mg/m² (days 0–28) IV (SR)</td>
<td>Vincristine 2 mg/m² IV (day 1) (SR)</td>
<td>Prednisone daily 40 mg/m² orally days 1–5 or (SR) Dexamethasone daily 6 mg/m² for 5 days (SR)</td>
<td>Prednisone daily 40 mg/ m² for 5 days within 3- weeks cycle (SR) or Dexamethasone 6-18 mg/m² (SR &amp; HR)</td>
</tr>
<tr>
<td>Hydrocortisone 9-15 mg (SR)</td>
<td>Vincristine 2.0 mg/m² IV day 1 (SR)</td>
<td>Doxorubicin 30 mg/m2 day 1 of each cycle (HR)</td>
<td>Vincristine 2.0 mg/m² IV day 1 (SR)</td>
</tr>
<tr>
<td>Cytarabine intrathecal 1 dose according to age (day 0) (SR)</td>
<td>Cytarabine intrathecal twice weekly × 4 doses (SR)</td>
<td>Methotrexate 2.0 mg/m² IV day 1 (SR)</td>
<td>Methotrexate weekly 30 mg/m² (SR)</td>
</tr>
<tr>
<td>Doxorubicin 30 mg/m²/dose (days 0 and 1) IV (SR)</td>
<td>Methotrexate intrathecal 4 doses of 6-12 mg</td>
<td>Methotrexate 30 mg/m² IV or IM days 1, 8, 15 (SR)</td>
<td>6-Mercaptopurine daily 50 mg/m² orally days 1–15 (SR)</td>
</tr>
<tr>
<td>Methotrexate one dose of 4g/ m² with leucovorin (day 3) IV (SR)</td>
<td>6-Mercaptopurine daily 50 mg/m² orally days 1–15 (SR)</td>
<td>Asparaginase E.coli ASP 25,000 IU/m² weekly or PEG ASP 2500 IU/m² every 2-weeks (SR)</td>
<td></td>
</tr>
<tr>
<td>Asparaginase E.coli or Erwinia ASP 25,000 IU/m2 × 1 dose (day 4) (SR)</td>
<td>Doxorubicin 30 mg/m2 day 1 of each cycle (HR)</td>
<td>6-Mercaptopurine daily 50 mg/m² orally days 1–15 for 14 days (SR)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Risk Group Classification on DFCI ALL Consortium Protocols (1985–2000)  
(Modified from Silverman 2010).

<table>
<thead>
<tr>
<th></th>
<th>Standard Risk: (All of the following)</th>
<th>High Risk: (Any of the following)</th>
<th>Very High Risk: (Any of the following)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell type</strong></td>
<td>B-precursor</td>
<td>T-cell</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical CNS disease</strong></td>
<td>Absent</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td><strong>TEL-AML1 (ETV6-RUNX1) - t(12:21)</strong></td>
<td>Present</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td><strong>Mixed Lineage Leukemia (MLL)</strong></td>
<td>Absent</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td><strong>BCR-ABL1-t(9;22) Philadelphia chromosome</strong></td>
<td>Absent</td>
<td>Present</td>
<td></td>
</tr>
</tbody>
</table>
Pharmacogenomics and importance of ALL:

Pharmacogenomics is the study of genetic variation of drug-metabolizing enzymes, receptors, transporters and targets, and talk about how these genetic variations (either inherited, acquired or both) affects drug-related phenotypes, such as drug response or toxicity. The major goal of pharmacogenomics is providing strategies to individualize therapy based on human genetic variability and its influence on drug response. Although pharmacogenetics and pharmacogenomics terms are often used interchangeably, the pharmacogenetics is the study of genetically determined variation in how individuals respond to drugs, but pharmacogenomics used to describe the study of the entire spectrum of genes that determine drug responses in relation to the diversity of the human genome sequence and its clinical consequences [46, 47]. Progress in pharmacogenomics studies provide significant potential for further clinical application in order to improve clinical outcomes in individual patients [46]. The treatment of paediatric ALL has greatly improved due to the introduction of effective combination risk-adapted therapies. However, therapy resistance is still a major obstacle to successful treatment, whereas intensive treatment has also important short-term side effects and long-term consequences. Unfavourable clinical pharmacokinetics and pharmacodynamic as well as cellular drug resistance play a major role in ALL treatment failure [48]. In this regard, treatment related toxicity is one of the major reasons for withdrawal or discontinuation, which might be increase risk of relapse despite of standard treatment approaches. On the other hand intensive treatment has short and long term consequences, such as myelosuppression, infection, and thrombosis, alteration in glucose metabolism, secondary malignancies, growth retardation, bone abnormalities, neurotoxicity or cognitive
impairments [36, 49-51]. Then, most important concern of oncologist is to recognize ALL patients before relapse due to fail in current treatment protocols [9]. Numerous pharmacogenetic studies have shown that polymorphisms in genes that encode drug metabolising enzymes and targets can affect enzymatic activity and modulate drug action [52]. Drug resistance and drug-related side effects can be due to polymorphisms in genes that encode enzymes involved in drug pharmacokinetics and pharmacodynamic. Polymorphisms in such genes often referred to as candidate genes are analyzed in pharmacogenetic studies for their capability to predict unfavourable treatment responses. Identification of such polymorphisms may lead to different treatment schedules, improving the efficacy of treatment and allowing for a reduction in drug side effects [50].

ALL was widely addressed in number of pharmacogenetic studies including candidate gene approach and Genome Wide Association Studies (GWAS). These studies increased our knowledge on genetic contribution to specific drug metabolism and signalling pathways [19]. One of the best examples of pharmacogenomics studies in ALL is 6-MP dosing based on TPMT genotypes and subsequent association of dose adjustment with better outcomes and less toxicity [19] TPMT enzyme is affected by functional polymorphisms which define the most prevalent mutant alleles associated with loss of catalytic activity and play an important role in metabolites pharmacokinetics variability affecting the balance between 6-MP efficacy and toxicity. These polymorphisms include three SNPs in the TPMT gene; TPMT*2 (238G > C), TMPT*3A (G460A), and TPMT*3C (719A > G) alleles, which account for 90% of the enzymatic deficiency in most populations. Due to higher TGN accumulation these polymorphisms have been associated with increased risk of myelosuppression [19, 36, 78] in ALL patients treated
with standard 6-MP doses. However in a case of treatment adjusted according to the TPMT genotypes. Based on TPMT genotyping, about 90% of the individuals have two wild-type \textit{TPMT} alleles (\textit{TPMT}*1), thus they have normal enzyme activity whereas individuals with two nonfunctional alleles (1 in 300) have very low or lack of TPMT activity, resulting high levels of TGN and acute hematopoietic toxic effects. Patients who carry two nonfunctional \textit{TPMT} alleles experience severe myelosuppression, if treated with standard doses of thiopurines. These patients require a reduction of more than 90% of the standard doses. Likewise administration of 50% of the standard dose is recommended for about 5-10% of individuals who carrier heterozygous alleles and have intermediate levels of enzyme activity. TPMT-deficient patients, upon thiopurine dose adjustment can tolerate full doses of their other ALL chemotherapeutic agents which associated with greater therapeutic efficacy [36, 78]. In addition to the analysis in clinical setting which addresses an association between polymorphisms and therapeutic responses, understanding functional effect of identified polymorphism makes also the part of pharmacogenetic studies spectrum and may as well give an indication for drug dose adjustments. This is of particular interest when treatment in composed of multiple therapeutics as in case of ALL.
Part II

Methotrexate (MTX) as a major anti-neoplastic component in ALL treatment protocol:

MTX is an important antifolate drug, designed to mimic a folate molecule which bind in the active site of folate-dependent enzymes and block its action (Figure 4). MTX is an essential drug used in the treatment’s protocol of several malignant and non-malignant diseases including ALL, choriocarcinoma, osteogenic sarcoma, seminoma, as well as lung, mammary gland and ovarian cancer [53-57]. Apart of antitumor effects, MTX play a major role in treatment autoimmune diseases, such as rheumatoid arthritis (RA) and moderate to sever psoriasis [58, 59], lupus erythematos and inflammatory bowel disease (IBD) [60] primary billiary cirrhosis and intrinsic asthma, as well as prevention of graft-versus-host disease (GVHD) [49]. MTX is used in induction, CNS relapse prevention, and maintenance therapy in ALL treatment protocol [26, 29]. Also MTX as a broad spectrum antitumor component used in treatment’s protocol of several malignancies such as accordingly, high-dose MTX (HDMTX) is commonly given in induction phase, and low-dose oral MTX (LDMTX) is given in maintenance therapy in ALL treatment’s protocols [61-63]. MTX uses the reduced folate carrier 1 (RFC1) or solute carrier family 19 member 1 (SLC19A1), for entering the cells [28, 36, 50, 57, 64], ABC proteins (specifically the ABCC1-4 and breast cancer resistant, ABCG2 proteins) for the transport out of the cell (Figure 5) [28, 50, 57, 63]. Overexpression of ABCC2 [(multidrug resistance-associated protein (MRP) 2] may lead to lower cellular MTX accumulation and subsequent moderate to high level of resistance [65, 66]. Impaired function of RFC1 has been recognized as a frequent mechanism of anti-folate resistance
Variations in this gene have been also showed to contribute to MTX response. RFC gene is located on human chromosome 21, Mostly investigated polymorphism is RFC1 80G>A. Patients with AA genotype showed higher MTX plasma level of MTX in their plasma due to impaired MTX influx [50, 56, 67, 68]. Some of chromosomal translocations that are typical for ALL cells may have altered level of MTX influx and efflux proteins. For example, E2A-PBX1 ALL patients showed significantly lower expression of RFC1 and TEL-AML1 ALL patients showed higher expression of ABCG2. Both conditions lead to decrease in MTX polyglutamates (MTXPG) level in the cells and subsequent resistance to MTX [56]. The solute carrier organic anion transporter 1B1 (SLCO1B1), which is mainly expressed in the liver, is important for hepatic uptake and thus for the pharmacokinetics of MTX [68]. It has been shown that variants in the SLCO1B1 gene are associated with MTX clearance and gastrointestinal (GI) toxicity in ALL patients [68]. After entering the cells (especially in hepatocytes, erythrocytes, and tumour cells), MTX undergoes polyglutamylation to MTX polyglutamates via folylpolyglutamate synthetase (FPGS) (Figure 5). Polyglutamation increases intracellular retention of MTX and subsequent affinity for the MTX-sensitive enzymes within the cell [55, 57, 63, 69]. In the other words, MTX gains its antileukemic effects through an accumulation of long-chain MTXPG [56, 57, 67]. The level and time-course of MTXPG formation in tumour cells is a key determinant of the cytotoxic activity exerted by MTX [65]. Pre B-ALL have more efficient accumulation of (long chain) MTXPGs compared to T-ALL and acute myeloid leukemia [69]. Reversed process is directly dependant on active transport of MTXPG into lysosome and further hydrolysis by γ-glutamyl hydrolase (GGH), which allows efflux of the drug out of the cell [50, 55, 57, 68]. Moreover,
MTXPG can inhibit glycinamide ribonucleotide transformylase (GARTF) and aminoimidazole carboxamide ribonucleotide transformylase (AICART), the two enzymes essential for *de novo* biosynthesis of purines [55, 68]. MTX and MTXPG acts as competitive inhibitors of several key enzymes in folate pathway such as dihydrofolate reductase (DHFR), thymidylate synthase (TS), 5,10-methylenetetrahydrofolate reductase (MTHFR) [28, 36, 64], and enzyme of purine synthesis resulting in DNA synthesis impairment and cell death [50, 55, 67]. In contrast to classic antifolates, nonclassical or novel antifolates, defined as antifolates that do not require active transport or polyglutamation, can passively diffuse into cells and do not depend on FPGS or the RFC. The most important nonclassical antifolates are trimethoprim (TMP), trimetrexate (TMQ) and piritrexim (PTX) [70-72].
Figure 4: MTX as an important antifolate drug;

MTX is designed to mimic a folate molecule, binds to the active site of the folate-dependent enzymes and blocks its action. Methotrexate has the same size and chemical composition as folate molecule. The similar structure of MTX and folate, suggests their competitive binding to same molecules (2014 David Goodsell & RCSB Protein Data Bank, http://www.rcsb.org/pdb/101/motm.do?momID=34).
Figure 5: Summary of the intracellular metabolism and targets of MTX:

MTX is transported actively into the cell by RFC, where it undergoes polyglutamylation and exerts its toxic effects by inhibiting DHFR, TS and de novo biosynthesis of purines (PDNS). DHFR, dihydrofolate reductase; DHF, dihydrofolate; THF, tetrahydrofolate; TS, thymidylate synthase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FPGS, folypolyglutamate synthetase; FPGH, folypolyglutamate hydrolase; MRPs, multidrug resistance-associated proteins; BCRP, breast cancer resistant protein; MTXGLs, MTX polyglutamates (Fotoohi 2008).
Pharmacogenetics of MTX and MTX-related toxicity and resistance:

MTX dosing has traditionally been adjusted based on several prognostic factors such as age, weight, body surface or side effects. However, despite dose adjustments of MTX, some ALL patients do not respond well to MTX treatment [68]. Based on evidences, MTX causes severe dose-limiting adverse events, including ulcerative stomatitis, nausea, abdominal distress, fever and myelosuppression [28, 64]. Treatment-related toxicity is not only life threatening but also is one of the main reasons for early drug withdrawal, and subsequent increase in relapse risk [11, 55, 63]. In vitro or in vivo studies have shown that polymorphisms in genes encoding folate dependent enzymes may affect response to MTX [52]. Two important groups of genes are involved in MTX resistance. First group are genes that encode drug metabolizing enzymes and transporters which control pharmacokinetic properties and the second group includes genes that encode drug target and affects pharmacodynamic properties of MTX [52]. In regard to the latter, increase in DHFR and TS activity, due to overexpression of DHFR and TS gene is a common mechanism underlying resistance to MTX [28, 55, 73, 74]. Other important determinants of MTX resistance include decreased intracellular retention (caused by decreased polyglutamylation and decreased affinity of DHFR for MTX), qualitative and/or quantitative alterations in influx and/or efflux transporters, and expansion of the tetrahydrofolate (THF)- pool size in cells [55, 65, 74]. The cellular THF- pool is an important index of polyglutamation; cells with low level of THF pool are more sensitive to antifolates and large THF pool is associated with resistance to MTX and other antifolates, which require polyglutamylation for their activity within the cell [55, 70, 74]. DHFR is a member of the reductase enzyme family, is ubiquitously expressed in all
organisms. One of the major and critical roles of DHFR is maintaining intracellular THF-cofactor pools at normal levels irrespective of the rate of synthesis of thymidylate [74]. Reduction of DHFR enzymatic activity diminishes the THF pool inside the cell affecting the level of folate coenzymes and thus purine and pyrimidine synthesis [53, 75, 76]. MTHFR is another folate dependent enzyme, is not directly inhibited by MTX and inhibited by metabolites of MTX. This enzyme is influenced by MTX due to effects of MTX on the intracellular folate pool. MTHFR by converting 5-10 methylene-THF into 5-methyl-THF, provide a methylene group for homocysteine methylation resulting maintenance of homocysteine level in plasma [48, 49, 68, 77]. Moreover, MTHFR, as a major enzyme in folate and homocysteine cycles, has two famous known single nucleotide polymorphisms (SNPs), 677C>T and 1298A>C, associated with reduced enzyme activity. Both have been shown to alter sensitivity to MTX. In the case of 677C>T polymorphism, it has been shown that the heterozygous 677CT have 65% of their enzyme activity whereas homozygotes 677TT have 30% of activity compared with the homozygous CC individuals (677CC). Likewise, homozygous CC1298 cells exhibit reduced enzyme activity [55]. Homozygosity for the 677T allele was associated with an increased risk of hepatotoxicity or myelosuppression adult ALL [78]. Based on association studies conducted in childhood ALL patients, there is no association of 677T allele with MTX-related toxicity. However based on recent studies, children with MTHFR polymorphisms developed more frequently myelosuppression and had higher creatinine levels [48, 79]. Beside folate dependent enzymes some other genes, like those involved in cell cycle regulation and apoptotic cascade, are also correlated with resistance to MTX [55] such as mutation in P53 gene [80] or apoptosis regulators Bcl-XL and Bcl-2
Cyclin D1 (CCND1) and retinoblastoma genes are key proteins involved in cell cycle regulation. They may as well play a role in MTX resistance, through increase in DHFR transcription. For example, CCND1 A870G polymorphism can modulate the ratio of CCND1 mRNA isoforms, favoring isoforms with longer half-life [82, 83]. The A870G polymorphism was associated with a significant increase in cancer risk including ALL and also affected response to treatment in ALL patients [84]. Overall, the molecular basis of resistance to antifolates, such as MTX, has been associated with target enzyme overexpression, change in the expression of a number of transporters and polymorphisms of genes affecting the activity of these proteins [65].

**Pharmacogenetic markers in MTX pathway of interest for this study:**

DHFR catalyzes the reduction of dihydrofolate (DHF) to THF which are needed for the action of folate dependent enzymes and thus are essential for DNA synthesis and methylation. DHFR inhibition by MTX diminishes the THF pool inside the cell affecting the level of folate coenzymes and thus purine and pyrimidine synthesis and finally causing cell death [36, 53, 68]. TS is a key enzyme in the nucleotide biosynthetic pathway that catalyzes conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (Figure 5). TS is inhibited by MTXPGs, which leads to inhibition of purine and pyrimidine synthesis and inhibition of cell proliferation [36, 55, 65].
1. **DHFR gene polymorphisms:**

The functional gene is mapped at chromosome 5q11.2-13.2. Multiple intronless pseudogenes or dihydrofolate reductase-like genes have been identified on separate chromosomes [53]. The changes in DHFR expression or activity can be partly due to the functional polymorphisms in the DHFR gene, thereby influencing a risk of folate-dependent diseases or affect response to antifolates. Association analysis performed in our group, identified particular DHFR haplotype that may affect the response to treatment in childhood ALL patients. The initial analysis of 15 DHFR minor promoter polymorphisms showed an association between homozygosity for A-317 and C-1610 alleles with lower event free survival (EFS) [85, 86]. Haplotype analysis identified an association with haplotype *1 harbouring both the A-317 and C-1610 alleles. Same haplotype was associated with higher DHFR expression, Further analysis performed in our lab, on the adjacent ~400-bp major DHFR promoter (located between minor and major transcript initiation sites) (Figure 6A) identified 6 polymorphisms, including 5 single nucleotide polymorphisms (SNPs) and one length polymorphism composed of variable number of 9-bp elements and 9-bp insertion/deletion [85]. The haplotype analysis revealed diversification of haplotype *1 into five subtypes, (*1a to *1e) (Figure 6B). Only *1b, conferred high transcriptional activity and was associated with lower EFS (Figure 6C). DFHR haplotype *1b is defined by particular allelic combination derived from three tag SNPs (C-1610G/T, C-680A and A-317G) in the minor promoter and three tag polymorphisms (C35T, G308A and compound length polymorphisms) in the major promoter [68, 85].
A

Minor promoter

~ 2 kb

Major promoter
and minor transcript
region

~ 400 bp

non-coding RNA
(minor)
coding RNA (major)

B

Minor promoter

~ 2 kb

Major promoter
and minor transcript
region

~ 400 bp

C

Minor promoter

~ 2 kb

Major promoter
and minor transcript
region

~ 400 bp

non-coding RNA
(minor)
coding RNA (major)

EFS

Time (month)

*p=0.01

209 (35)

47 (14)

*p=0.01

209 (35)

47 (14)
Figure 6: **DHFR risk haplotypes in major promoters:** A. Structure of the human DHFR gene. The region of interest (~400-bp) is located between the minor and major transcript initiation sites indicated by arrows. B. Haplotype *1b* includes six polymorphisms in the major promoter. Tagging polymorphisms of the major promoter are at positions 35, 63/91, and 308. Haplotype *1b* is composed of three polymorphisms (C35T, G308A and compound length polymorphisms composed of 9-base pair (bp) insertion at position 63 and triple 9bp element at position 91) in the major promoter region. In major promoter lower event-free survival was associated with an A allele of G308A polymorphism and with haplotype *1b*. C. The carriers of haplotype *1b* showed reduced EFS (Dulucq 2008 and Al-Shakfa 2009).

2. **TS gene polymorphisms:**

Thymidylate synthase (TS) is an essential enzyme in folate metabolism, and is encoded by the *TS* gene located on chromosome 18p11.32. It plays a vital role in maintaining a balanced supply of deoxynucleotides required for DNA synthesis and repair by catalyzes conversion of dUMP to dTMP in the presence of 5, 10-methylenetetrahydrofolate (methylene-THF) [36, 65, 87]. TS is an important target for various chemotherapeutic and anticancer drugs, such as MTX and 5-fluorouracil. Inhibition of TS by methotrexate polyglutamates leads to inhibition of pyrimidine synthesis, deoxythymidine triphosphate depletion, cytotoxicity through thymineless death, uracil misincorporation into DNA, chromosome breaks and consequently to an inhibition of cell proliferation and cell death [74, 88]. TS also is a key enzyme in the nucleotide biosynthetic pathway that folate metabolism alteration due to functional polymorphisms in TS, were associated with
increased risk of hematological malignancies [55, 89]. Likewise polymorphisms in TS gene may affect response to cytotoxic drugs targeting this enzyme, such as MTX and 5-FU [82, 88]. Three important polymorphisms have been well defined in TS gene; the variable number of 28-bp tandem repeats (VNTR) 2R or 3R polymorphism located on the 5’-UTR enhancer region of the TS promoter (thymidylate synthase enhancer region [TSER]), the G>C substitution at the 12th nucleotide in the second repeat of the 3R allele (3RG>3RC) and the 6-bp deletion in the 3’untranslated region (3’UTR) (Figure 7) [36, 73, 80, 90]. According molecular epidemiologic studies the majority of population harbour either a 2R or a 3R allele; however they have been shown four, five or nine repeats in African and Asian populations (Table 3). It was shown that increasing number of repeats would increase the amount of several transcription factors mainly the upstream stimulating factors (USF) which stimulate transcription of TS [82]. Based on several studies, each of these polymorphisms can alter TS expression and consequently drug resistance and toxicity [87, 91]. The presence of a triple versus double 28-bp repeat in the enhancer region of 5’-UTR, has been associated with an increased TS expression both in vivo and in vitro studies [80-82]. Based on several studies, the 3R/3R genotype has been identified as a predictor of poor clinical outcome to MTX-based chemotherapy in childhood ALL patients (Figure 8) [73, 92]. It might be hypothesized that inhibition of 3R/3R genotype, requires higher concentrations of MTX in comparison to other genotyping groups to efficient target inhibition [68]. In addition to tandem repeat sequence G to C polymorphism in the second repeat element of the 3R allele (3RC vs. 3RG) of TS gene, can influence gene expression and TS mRNA transcriptional and translational efficiency [73, 87, 93]. This SNP can further diversify the 3R allele into
either 3C or 3G, the 3G alleles seems to affect mRNA expression and catalytic activity of TS and also was associated with poor response to MTX in some studies [73, 80, 83]. Our group has studied the effect of substitution in 3RG allele on ALL outcome [73]. Although the 3RG3RG individuals had lowest EFS, we did not notice significant difference between 3R3R subgroups (Figure 9) [73]. A double repeats (2R) of 28-bp polymorphism in the TS promoter enhancer region is associated with lower expression of the gene and the individuals with this allele may be more prone to toxic effects as shown in several studies [94]. The 6-bp deletion in the 3’UTR of TS gene can play a role in mRNA stability and translation [90, 95, 96]. 6-bp deletion allele being associated with decreased TS mRNA stability and expression [80, 87, 95]. This polymorphism was show to affect sensitivity to anticancer drugs and prognoses of breast and gastrointestinal cancers [96, 97]. Krajinovic et al (2005), combined TS polymorphisms for the analysis, and found lower frequency of the 2R6 bp- haplotype among children with an event, whereas 3R homozygosity combined with 6 bp+ alleles, seems to potentiate lower EFS (Figure 10) [73].
Figure 7: TS gene important polymorphisms:

The variable number of 28-bp tandem repeats (2R or 3R) in the 5’untranslated region (5’UTR); the G>C substitution at the 12\textsuperscript{th} nucleotide in the second repeat of the 3R allele (3RG>3RC) and the 6-bp deletion in the 3’UTR (+6bp/-6bp 3’UTR) region (Modified from Lima 2013).
Table 3: *TS* polymorphisms characteristics:

2/3/4/5/9 represents the possible number of repeats. Del: Deletion; ins: insertion; VNTR: Variable number tandem repeat (Lima 2013).

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<td>(CCGCGCCACTTCGCT GCCTCCGTCCCG) 2/3/4/5/9</td>
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<td>5´-UTR</td>
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Figure 8: Event-free survival (EFS), for patients with and without TS 3R/3R genotype: ALL patients with 3R3R genotype had lower event-free survival (Krajinovic 2002).
Figure 9: Effect of 28-bp repeat variation genotypes following stratification of 3R allele on 3RC and 3RG: Impact of EFS for ALL patients: EFS curves are given for 2R carriers (2R*) and each 3R/3R subgroup (3RG/3RG, 3RC/3RG, 3RC/3RC). 3RG/3RG individuals have lower EFS however the difference was not significant when compared to other 3R3R subgroups (Krajinovic 2005).

Figure 10: EFS for ALL patients following stratification of 3R3R genotypes by 6 bp+/- polymorphism: The lower curve represents EFS for patients who are both homozygous for 3R variant and the carriers of 6 bp+ allele. The upper curve represents EFS for individuals without these TS genotypes. The genotype and the number of patients in each curve and numbers of individuals with an event are in the parenthesis (Krajinovic 2005).
Combined effect of *DHFR* and *TS* genes; impact of MTX metabolic pathway: Based on previous study in our lab, promoter haplotype *1b* of *DHFR* and homozygosity for 3R repeat of *TS* gene are relapse-predisposing (at risk) genotypes. Given that both DHFR and TS belong to the sub-pathway of MTX action, the relapse rate is further increased if these two at risk genotypes are combined (Figure 11) [73, 85, 86].

A)

![Diagram showing the metabolic pathway of DHFR and TS genes with DHFR promoter haplotype *1b* and 3R3R repeat of TS gene.]

B)

![Graphs showing Kaplan-Meier survival analysis for all patients and high-risk patients with different genotypes.](https://example.com)
Figure 11: Combined effect of DHFR haplotype *1b and TS 3R/3R genotypes on EFS in patients with ALL.

Haplotype *1b of DHFR and homozygosity for 3R repeat of TS gene are risk genotypes. Both risk genotypes are associated with higher expression of respective genes, possibly resulting in resistance to MTX, thus explaining reduction in EFS in patients with these genotypes. The EFS is further decreased if these two at risk genotypes are combined. All patients regardless of risk groups are presented in A and only high risk patients are presented in B. (Al-Shakfa 2009 and unpublished results).
Part III

L-Asparagines (ASNase) - standard component in the childhood ALL treatment:

ASNase is an essential and standard component of multi-agent chemotherapy in the childhood lymphoblastic leukemia treatment. ASNase is used in both induction and post-induction phases of ALL treatment protocols [3, 26, 98]. The logic behind the ASNase therapy is rapid efflux of cellular asparagine, needed for protein biosynthesis and cell survival. Most cells express sufficient asparagine synthetase (ASNS) except leukemic cells which are thus dependent on circulating asparagine levels that are in turn depleted by ASNase treatment. The mechanism underlying the activity of asparaginase relies on the conversion of asparagine into aspartate and glutamate [98-100]. Different kind of ASNase preparations are used in childhood ALL treatment protocols, including; Escherichia coli (E. coli), polyethylene glycol–conjugated (PEG) and Erwinia ASNase. E. coli ASNase and PEG- ASNase are derived from E. coli whereas Erwinia ASNase is derived from the Erwinia chrysanthemi bacteria [92, 99, 101]. E.coli derived asparaginase seems to have higher efficacy. Fewer doses requirement per treatment period and longer half-life as well as its lower immunogenicity are the advantages of using PEG- ASNase [99]. In vitro experiments conducted in leukemia cell lines and patient lymphoblasts, showed that asparaginase resistance in ALL can be related to an increase of ASNS activity [36, 102]. A significant improvement in overall survival (OS) and event free survival (EFS) was seen for children with ALL who received ASNase during post remission consolidation compared with those who did not receive ASNase.
Associations between treatment outcome and ASNase dose intensity and between an inferior outcome and the use of ASNase preparation with a shorter half-life have been reported. ASNase intolerance produces allergic reactions, pancreatitis, and abnormalities of haemostasis. These ASNase associated complications may also affect treatment outcome [102, 103].

**ASNase-related toxicity and resistance:**

Several genes are involved in antileukemic effect of asparaginase. Polymorphisms in the asparaginase synthetase gene (*ASNS*) and the basic region leucine zipper activating transcription factor 5 (*ATF5*), have been associated to lower event-free survival of ALL patients [102]. Differences in susceptibility to ASNase have long been attributed to variable levels of asparagine synthetase (*ASNS*) expression. *In vitro* experiments conducted in leukemia cell lines suggested that elevated ASNS levels might be a cause of ASNase resistance and that ASNS inhibitors may suppress a proliferation of resistant cell lines. It has been shown that leukemic cells can up-regulate *ASNS* gene expression under nutrient stress caused by ASNase [102]. Recently suggested mechanism of asparaginase resistance includes protection of leukemic lymphoblasts against asparaginase cytotoxicity by bone marrow mesenchymal cells (MSC) niches due to high asparagine levels and excess asparagine production, provided by higher activity of *ASNS* in these cells (Figure 12) [98, 104] *In vitro* studies have shown that ASNS plays a role in mediating drug resistance, especially in T-ALL cells and B cells with *ETV6-AML1* or hyperdiploidy anomalies. Based on evidences there is an inverse correlation between the ASNS protein
and ASNase-induced cell death. Using genome-wide expression profiling and patient samples, revealed that ASNase-resistant, \textit{TEL-AML1}–negative B cells had significantly higher \textit{ASNS} mRNA expression compared with sensitive ones [105]. An increase in \textit{ASNS} expression was predictive of inferior disease outcome. Whereas other groups reported an absence of association between \textit{ASNS} expression and \textit{in vitro} response to ASNase when patient lymphoblasts were analyzed [102]. Acute adverse effects of ASNase therapy such as allergic reaction and hypersensitivity [101, 106], pancreatitis [106], cerebrovascular accidents [107], hyperglycaemia secondary to hypoinsulinaemia, hypolipoproteinaemia and hypoalbuminaemia are a significant source of morbidity and may increase relapse risk in patients unable to receive all intended doses [101, 106]. The anti-leukaemia effect of ASNase is related to asparagine depletion. Prolonged asparagine depletion is also associated with the development of coagulation defects. Thus asparaginase-related thrombosis is a significant complication of ALL therapy in children and adults [106, 107]. Arginosuccinate synthase 1 (ASS1) is involved in catalyzing the conversion of aspartate and citrullin into argininosuccinate, which may affect level of aspartate needed for asparagine synthesis. \textit{In vitro} experiments and microarray analysis identified association between up-regulation of ASS1 in cell lines and resistance to ASNase and in turn, \textit{ASS1} mRNA suppression restores ASNase sensitivity in resistant cell lines [102, 108].
Figure 12: Protection of leukemic lymphoblasts with bone marrow MSCs; Impact of ASNS role in resistance to ASNase: Asparagine, produced in large amounts by bone marrow mesenchymal cells, protects leukemic blasts from the asparagine-depleting effects of ASNase treatment (Williams 2007).
Pharmacogenetic markers in ASNase pathway of interest for this study:

ASNS, ATF5 and ASS1 have been shown to mediate the antileukemic effect of ASNase by providing variable expression between leukemia cells that are resistant and sensitive to treatment [102, 103]. Microarray analysis identified up-regulation of ASS1 in cell lines with resistance to asparaginase. Recent genome-wide analysis identified an association of \textit{ASS1} polymorphisms with \textit{in vitro} asparaginase sensitivity in ALL patient samples and HapMap cell lines. Although, GWAS showed implication of ASS1 in \textit{in vitro} sensitivity, but there is no data showing association of ASS1 with EFS [102]. Rousseau et al, recently analyzed the genes in asparaginase pathway and found that \textit{C1562T} allele in the 5’UTR of the gene coding for transcription factor \textit{ATF5}, which regulates ASNS levels, is associated with higher promoter activity and confers higher risk of ALL relapse in patients who received E.coli ASNase (Figure 13A) [102]. ATF5 involved in several cell regulatory process such as cellular differentiation, cell cycle, and apoptosis [102]. ATF5 as a stress response transcription factor and responds to amino acid limitation. In the case of nutrition deprivation, ATF5 can bind to the nutrient-sensing response unit of the \textit{ASNS} gene and acts as a transcriptional activator of ASNS [102, 109]. Several haplotypes have been distinguished in \textit{ATF5} gene located in the regulatory region (including the 5' UTR and the 2-kb element upstream from the transcription start site), which can affect promoter activity and mRNA levels. Also several haplotypes were shown by luciferase assay to affect promoter activity [102]. Based on Rousseau et al, among these haplotypes, only haplotype tagged by \textit{T1562} alleles was associated with reduced EFS in ALL in two independent patient cohorts (Figure 13B). This association was limited to ALL patients that received \textit{E coli} asparaginase, which might be related to higher activity,
of this drug formulation [102]. According in vitro experiments conducted in leukemia cell lines and patient lymphoblasts, one of the main factors of ASNase resistance is elevated ASNS activity. Based on Rousseau et al. data, promoter variant of transcriptional factor ATF5 involved in ASNS regulation is associated with higher promoter activity and confers higher risk of ALL relapse in patients who received E.coli ASNase. ASNS gene has tandem repeated sequences as a polymorphism of the ASNS gene and it can function as a transcriptional enhancer element; as insertional sequences or increased number of tandem repeat might be involved in gene expression and increased activity [102, 105]. Two 14-bp tandem repeat (2R) sequences in the promoter region of the ASNS gene is located in the first intron of the gene. Approximately 75% of ALL patients carry 2R sequence in both allele, whereas 20% and 3% ALL patients had 3R and 4R, tandem repeats in one allele, respectively [105]. Our association analysis between polymorphism in ASNase pathway and ASNase-related acute complications (allergies, pancreatitis and thrombotic events), showed an association of 3R3R genotype of ASNS gene, with a higher frequency of ASNase–related adverse reactions. The protective role of haplotype harbouring 2R allele against ASNase toxicities has been also found (Figure 14) [103].
Figure 13: *ATF5* gene polymorphisms and EFS for patients with ALL according to *ATF5* genotypes:  
**A:** Tag SNPs (sufficient to define common haplotypes) with frequency ≥5% in patients comprising 8 SNPs includes *C1562T* polymorphism (red arrow) in *ATF5* promoter.  
**B:** EFS curves are shown for patients who were carriers (T+; dark gray) or not (T-; light gray) of the *T1562* allele. EFS curves are presented for all HSJ and DFCI patients who were assigned to *E coli* asparaginase. The number of all patients in each curve (with the number of cases with an event in brackets) is indicated next to the curve. (Rousseau 2011).
Figure 14: Tandem repeat polymorphism of ASNS gene in relation to allergies and pancreatitis in ALL patients of discovery cohort:

The frequency of individuals with genotypes of tandem repeat polymorphism in patients with (+, dark gray bars) and without (−, light gray bars) allergies and pancreatitis (left and right side panel, respectively) (Ben Tanfous 2014).
References:


Chapter II
Hypothesis and Objectives:

ALL is the most frequent malignancy of childhood. The relapse and drug-related adverse events may hamper the success of treatment and may be modulated among other factors by patient genetic background. ALL treatment protocols include several chemotherapeutics imposing the question whether an association observed in clinical setting results from an association between a given polymorphism and a single drug or it results from interaction with other chemotherapeutics. Understanding the mechanism governing associations found in clinical setting is essential to define how to best tailor the treatment according to patient genotype. The work presented in this thesis builds on the finding of our research group interrogating relationship between gene variants of MTX and ASNase pathway and therapeutic responses in ALL patients [1-5].

These finding include:

i) Association between genetic variations of MTX action pathway and ALL outcome indicated a higher risk of relapse in patients who are homozygous for triple repeat (3R) allele of *TS* gene and/or have *1b* haplotype defined by particular allelic combination of polymorphisms in the promoter of DHFR gene. Both variations are associated with an increase in respective gene expression, suggesting that lower sensitivity to MTX associated with these variations might be a cause of higher risk of relapse and reduced disease free survival in patient with ALL [1-3]. Higher *DHFR* expression associated with haplotype *1b* results in higher tetrahydrofolate levels, whereas higher *TS* expression associated with 3R allele might produce enough nucleotides required for DNA synthesis preventing initiation of a DNA damage response pathway and apoptosis [6, 7]. This
would suggest that patients with particular \textit{DHFR} or \textit{TS} genotypes might need higher doses of MTX to effectively inhibit DHFR and/or TS targets.

ii) Promoter variant of transcriptional factor \textit{ATF5} involved in \textit{ASNS} regulation, is associated with higher promoter activity and confers higher risk of ALL relapse in patients who received E.coli ASNase [4] Polymorphisms of \textit{ASNS} gene located in enhancer repeat element affected risk of relapse and frequency of ANSase-related complication. Differences in sensitivity to ASNase have been attributed to variable levels of \textit{ASNS} expression which may counteract the ASNase effect and underlie the resistance to treatment, or, may mediate higher sensitivity to treatment and possibly higher frequency of ASNase-related complications [5].

\textbf{Hypothesis:} The observed associations between genetic variations and increased risk of ALL relapse and or drug-related toxicity observed in clinical setting result from genetic driven change in sensitivity to particular components of multi-agent treatment protocol.

Major goal of my PhD project was to assess the functional role of genetic variations of MTX and ASNase action pathway that were found in clinical setting to affect the treatment response in childhood ALL, using lymphoblastoid cell lines and xenograft mouse model of ALL.

The specific aims of this study were:

i) To analyze the effect of \textit{TS} polymorphism on MTX sensitivity using cellular proliferation assay and xenograft mouse mice model of ALL.
ii) To analyze the effect of $DHFR$ polymorphism on MTX sensitivity using cellular proliferation assay.

iii) To analyze the effect of $ATF5$ and $ASNS$ genes polymorphisms on sensitivity to $E.coli$ ASNase using $in vitro$ proliferation assay.
Materials & Methods:

**A. In vitro proliferation assay in response to MTX and ASNase**

**A.1. Cell viability test:**

Lymphoblastoid cell lines (LCLs) originating from CEPH (Centre d’Étude du Polymorphisme Humain) families were used. CEU cell lines (each originating from different individual of Northern and Western European ancestry), were purchased from Coriell Cell Repositories. In vitro sensitivity to MTX and ASNase were assessed in 93 and 89 LCLs respectively. LCLs (5 x10⁴ cells) were incubated with different drug concentrations of MTX ranging from 0.001- 100 μM and *E.coli* ASNase from 0.01-10 IU in 96-well plate, followed by addition of a cell proliferation reagent WST-1 [sodium 5-(2, 4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H tetrazolium inner salt], which is reduced to coloured formazan by living cells only. The amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. The formazan production is quantified using a spectrophotometer at 450 nm. The optical density (OD) is linearly related to the cell number. The drug concentration resulting in 50% inhibition of cell growth (IC₅₀) during 96h for MTX and 48h for ASNase incubation times was estimated (Figure 1). Cellular viability was calculated at each drug concentration by the equation: (OD treated well/OD control wells) x 100%. IC₅₀ values were estimated using the GraphPad software by fitting sigmoid dose-response curves. Relationship between genetic variants of interest in *DFHR*, *TS*, *ATF5* and *ASNS* genes, and IC₅₀ values were assessed by ANOVA or nonparametric tests depending on data distributions and homogeneity. The cell lines were also grouped based on IC50 values in
groups with sensitive, resistant and intermediate phenotype and the correlation was performed by chi-square.

Figure 1: Lymphoblastoid cell lines treated with MTX and ASNase:
Lymphoblastoid cell lines were treated with different concentration of (A) MTX (0.001-100 μM) and (B) ASNase (0.01-10 IU). Left side graphs indicate an example of sensitive and right side of resistant cell lines. Cell viability test with cell proliferative reagent, WST-1 (10 μl/ well). IC50 values are indicated.
A.2. Genotyping:

DNA is extracted from these cells and genotyping performed to define presence or absence of genetic variant of interest (*1b of DHFR, 3R3R of TS, ATF5 T1562C substitution and ASNS polymorphism). DFHR haplotype *1b is defined by particular allelic combination derived from three polymorphic site (C-1610G/T, C-680A and A-317G) in the minor promoter and three polymorphisms (C35T, G308A and length polymorphisms at positions 63/91) in the major promoter. Genotyping of nucleotide substitutions is performed by polymerase chain reaction (PCR) followed by allele-specific oligonucleotide (ASO) hybridization. Presence or absence of TS triple repeat, 3R/3R genetic polymorphism and of ASNS tandem repeat were resolved by polymerase chain reaction (PCR) amplification followed by 3% agarose gel electrophoresis at 85V for 1h30 for TS (Figure 2), and 4% agarose gel electrophoresis at 70V for 2h30 for ASNS genetic polymorphisms. The genotypes of remaining ASNS polymorphisms for each CEU LCLs were obtained from HapMap site (http://hapmap.ncbi.nlm.nih.gov/).
Figure 2: *TS* triple repeat (3R) 28 bp genotyping:

Lymphoblastoid cell lines performed for *TS* triple repeat (3R) 28 bp genotyping.

The presence or absence of *TS* triple repeat, 3R/3R genetic polymorphism was resolved by polymerase chain reaction (PCR) amplification. PCR amplification was followed on 3% agarose gel electrophoresis at 85V for 1h30.
B. in vivo xenograft mouse model of human ALL:

B.1. Patient samples:

Bone marrow aspirates were obtained from newly diagnosed paediatric ALL patients treated at the CHU Sainte-Justine (Montréal, Canada). The patient’s samples were collected on heparinized tubes and bone marrow mononuclear cells (BMMC) were obtained after Ficoll-Hypaque (GE Healthcare) density gradient centrifugation. BMMC were cryopreserved in DMSO 10% for later use. The study has been approved by the Institutional Ethical Review Board in Research of the CHU Sainte-Justine and an informed consent was obtained from the patients and/or their parents.

B.2. Xenograft mouse model of human ALL:

NSG mice (NOD/LtSz-scid IL-2Rγc null, Jackson Laboratory) were bred and maintained under specific pathogen-free conditions in micro-isolator cages and provided with autoclaved food and water in the research animal facility of the CHU Sainte-Justine. BMMC samples were thawed and evaluated for phenotypic analysis of blasts content by flow cytometry. Patient’s blasts were expanded in a primary round of in vivo expansion by injecting between 5 x10^6 thawed ALL patient blasts in the lateral tail vein of one to three non-irradiated NSG mice. In order to deplete the T lymphocytes present in the sample that may have caused a xeno-Graft-versus-Host Disease (GvHD) in the primary injection, mice received anti-CD3 antibody. Mice were evaluated for leukemia development by detection of circulating human blasts in the blood by flow cytometry. The mice were sacrificed when human blasts were ranging from 30-90% of total CD45
and/or CD19 and/or CD10-positive lymphocytes. The spleen was then crushed on a 70μm strainer using PBS. Human blasts were phenotyped and enumerated by flow cytometry.

B.3. MTX treatment:

For the assessment of MTX sensitivity, after optimisation experiments (Figure 3), 7- to 10-week-old NSG mice were injected intravenously with $5 \times 10^6$ in vivo expanded pre-B patient’s leukemic blasts with and without TS 3R/3R genotype. When the percentage of human ALL blasts reached 1-5% of CD45+ and/or CD19+ and/or CD10+ cells in peripheral blood, mice were randomized to receive MTX (Accord Healthcare) intraperitoneally in groups of 5 individuals at either 0.25; 1 or 5 mg/kg/day or PBS (negative control) on 4 consecutive days, every 14 days for a maximum of 8 weeks when possible. Peripheral blood was weekly collected from the saphenous vein on Microvette-200 heparinized tubes (Sarstedt). Red blood cells were lysed using ammonium chloride 0.3 M solution and cells were then analyzed by flow cytometry analysis. B-cell-specific markers (as determined by flow cytometry) and genotyping of TS locus was used to evaluate blast phenotype and consistency of genotype after transplant. Mice were sacrificed at the end of MTX treatment or when they reached established limit points (hunch-back, weight loss and ruffled fur, performed in blinded fashion as to assigned treatment). Limit points were assessed every day and determined by our internal committee for good animal practices in research.
B.4. Flow cytometry analysis:

Flow cytometry analyses of xenograft leukemia cells in peripheral blood and spleen were performed using monoclonal antibodies specific for murine CD45 conjugated with fluorescein isothiocyanate (FITC), anti-human CD45 conjugated with allophycocyanin (APC), anti-human CD19 conjugated with phycoerythrin (PE), anti-human CD3 conjugated with BD Horizon V500, and anti-human CD10 conjugated with phycoerythrin-cyanine7 (PE-Cy7) (from BioLegend). Dead cells were excluded by 7-amino actinomycin D (7-AAD) staining (BD Biosciences). Count Bright Beads (Life Technologies) were added in each tube for absolute counts determination. Flow cytometry analysis was performed using an LSRFortessa flow cytometer (BD Biosciences). The development of leukemia in mice was determined by estimating the proportion of human cells among total lymphocytes (human + murine) and by estimating the absolute counts of blast cells. The results were analyzed using FCS Express 4 for Flow Cytometry RUO Edition (De Novo Software).

B.5. Statistical analysis:

Results were analyzed using GraphPad Prism 4.0 (GraphPad Softwares Inc., La Jolla, USA) and are shown as mean ±SEM. Elsewhere, two-tailed Mann-Whitney tests were used with significance set at $P \leq .05$. 
Figure 3: MTX efficacy assessment in NSG mice, optimization experiments: NSG mice showed expansion of leukemia cells about four weeks after receiving leukemia cells. MTX treated group showed significant reduction of leukemia cells in comparison with PBS control group, just one week after treatment with MTX. One week after drug withdrawal, MTX treated group showed increase of leukemic cells.

The results assessing *in vivo* approach using xenograft mouse model are presented in the article 2.
Chapter III
Article I

Dihydrofolate Reductase Gene Variations in Susceptibility to Disease and Treatment Outcomes

Bahram Sharif Askari,1 and Maja Krajnovic*,1,2,3

1Research Center, CHU Sainte-Justine, Montreal, QC, Canada;
2Department of Pediatrics, University of Montreal, Canada;
3Department of Pharmacology, University of Montreal, Canada
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AUTHORS’ CONTRIBUTION

In this review article, my contribution is:

- Design of the study
- Writing the article
- Revised this article

Contributions of other authors are: Maja Krajinovic contributed to design, writing and revision of this article.

Abstract:

Dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolate to tetrahydrofolate (THF). THF is needed for the action of folate-dependent enzymes and is thus essential for DNA synthesis and methylation. The importance of this reaction is demonstrated by the effectiveness of antifolate medications used to treat cancer by inhibiting DHFR, thereby depleting THF and slowing DNA synthesis and cell proliferation. Due to the pivotal role that DHFR plays in folate metabolism and cancer treatment, changes in the level of DHFR expression can affect susceptibility to a variety of diseases dependent on folate status such as spina bifida and cancer. Likewise, variability in DHFR expression can affect sensitivity to anti-cancer drugs such as the folate antagonist methotrexate. Alterations in DHFR expression can be due to polymorphisms in the DHFR gene. Several variations have recently been described in DHFR, including promoter polymorphisms, the 19-bp deletion allele and variations in 3’UTR. These polymorphisms seem to be functional, affecting mRNA levels through various interesting mechanisms, including regulation through RNA interference. Several groups have assessed the association of these polymorphisms with folate levels, risk of cancer and spina bifida as well as the outcome of diseases treated with MTX. The latter may lead to different treatment schedules, improving treatment efficacy and/or allowing for a reduction in drug side effects. This review will summarize present knowledge regarding the predictive potential of DHFR polymorphisms in disease and treatment.

Keywords: Gene, dihydrofolate reductase (DHFR), polymorphisms, disease susceptibility, methotrexate (MTX), therapeutic response
INTRODUCTION

Dihydrofolate reductase (DHFR) is a member of the reductase enzyme family, which is ubiquitously expressed in all organisms. At the transcriptional level, DHFR is governed by a TATA-less promoter that is controlled by numerous transcription factors, including Sp1 and E2F that are important for its regulation throughout the cell cycle [1, 2]. Levels of this enzyme peak at the G1/S cell cycle boundary. Autoregulation, through DHFR-RNA interactions, has also been reported [3, 4]. DHFR catalyzes the NADPH dependent reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) needed for several one-carbon transfer reactions in purine and pyrimidine synthesis [2]. DHFR is also needed for the intracellular conversion of synthetic folic acid, consumed in supplements and fortified foods, into the THF forms that can participate in folate/homocysteine metabolism. Reduction of DHFR enzymatic activity diminishes the THF pool inside the cell affecting the level of folate coenzymes and thus purine and pyrimidine synthesis [1]. This may as well influence homocysteine levels and methylation processes, since methyl-THF is needed for the remethylation of homocysteine to methionine, thereby ensuring the provision of S-adenosylmethionine (SAM) necessary for most biological methylation reactions [5]. DHFR inhibition is essential to the action of antifolate medications used to treat cancer and some inflammatory diseases [6-9]. The DHFR gene family includes the functional DHFR gene and four other intronless pseudogenes (DHFRP1–4) [10]. The functional gene is located at chromosome 5q11.2-13.2 and is expressed in three mRNA isoforms with alternatively spliced 3’UTR ends [11]. The changes in DHFR expression or activity can be partly due to the functional polymorphisms in the DHFR gene, thereby influencing a risk of folate-dependent diseases. Importantly, gene variations may also
affect therapeutic responses to antifolates, leading to lower treatment efficacy or higher adverse drug event frequency. This review will summarize several studies that analyzed whether DHFR gene polymorphisms may affect disease susceptibility or antifolate treatment outcomes.

**DHFR POLYMORPHISMS AND DISEASE SUSCEPTIBILITY**

Neural tube defects (NTD) are a group of common birth defects with a prevalence of approximately 1 per 1,000 in Europe [12]. It is well documented that low serum folate and high homocysteine levels are associated with an increased risk of NTD, explaining the preventive effect of folic acid supplement [13, 14]. Change in activity of the folate cycle enzymes may affect the folate levels and affect NTD development. It is, therefore, not surprising that polymorphisms of folate-dependent enzymes of both mother and child, such as methylene tetrahydrofolate reductase (MTHFR), have been shown to contribute to the risk of NTD [15, 16]. Neural tube closure occurs during a period of rapid cellular proliferation and DHFR activity may be a crucial factor in maintaining optimal DNA synthesis during this time [13]. The most extensively studied DHFR polymorphism is a 19bp insertion to deletion in the first intron that has been evaluated as a risk factor for NTD in several studies (Table 1). Johnson et al. 2004 [17], suggested that the deletion (del) allele could affect gene expression, since the Sp1 transcription factor binding site is located within the deleted sequence. The first, a small case-control study, provided evidence that the risk of having a child with spina bifida is higher for women with the
DHFR 19bp del/del genotype than it is for those with the remaining genotypes [17]. The same authors [18] reported a higher risk of pre-term delivery in patients with the del allele. Van der Linden et al., 2007 [19], reported an absence of association between del/del genotype and spina bifida risk, whereas Parle-McDermott et al., 2007 [13], observed a lower risk of having a child with NTD in women with the del allele. It has also been reported that individuals with the del/del genotype have lower homocysteine [20] and increased serum and red blood cell folate levels [14]. A nonsignificant increase in mRNA levels for homozygous del individuals [13], and subsequently a significant increase in DHFR expression with the number of del allele [21], were reported. This would support the possible maternal protective role of the 19-bp del allele in NTD by increasing the amount of DHFR available to reduce DHF to THF. However, others reported an absence of association between del allele and mRNA levels [19] or even suggested in the recent cross-sectional, population-based study [22] that the del/del genotype resulted in a diminished capacity of the enzyme to reduce folic acid. It seems that the role of DHFR polymorphisms in NTD risk still remains to be determined, since results regarding 19bp del allele are inconclusive. The abrogation of the Sp1 binding site by the deletion allele or its close location to the splice donor site (60bp) may suggest a functional role of this polymorphism, yet contradictory results have been obtained. The other polymorphisms that are in linkage disequilibrium (LD) with 19bp insertion to deletion variation may be responsible for the observed results or discrepancies. Indeed, the high extent of LD was noted in the DHFR gene with 19bp insertion to deletion being in LD with the promoter polymorphisms [23]. The same polymorphism is shared among
several haplotypes, showing the necessity of estimating the impact based on the analysis of both individual polymorphisms and haplotypes. In some instances the haplotypes may provide more precise information for predicting disease risk than individual polymorphisms [24]. DHFR is an important folate cycle enzyme required for nucleic acid synthesis as well as homocysteine remethylation, suggesting that DHFR polymorphisms may play a role in cancer susceptibility as well. It is possible that similarly to MTHFR, the DHFR gene variations may play a dual role. Polymorphisms associated with a higher expression of DHFR may protect against cancer, due to the higher levels of 5,10-methylene-THF needed for thymydylate synthesis, whereas a change in the 5-methyl-THF pool may affect methylation reactions and, consequently, increase cancer risk. Both genomic DNA hypomethylation and gene-specific promoter CpG island hypermethylation are important epigenetic mechanisms of carcinogenesis [5, 25]. Indeed, a protective role of the DHFR 19 bp del allele in adult acute lymphoblastic leukemia (ALL) patients has been reported [26], which was further potentiated when the del allele was combined with the TT677 genotype of MTHFR, previously shown in several studies to reduce the risk of ALL [27, 28]. Analysis of the DHFR 19-bp insertion to deletion polymorphism in relation to breast cancer susceptibility did not reveal a significant association with overall breast cancer risk [21, 29]. However, when analyses were performed following stratification according to multivitamin use [21], an association appeared significant in patients that used the multivitamin supplements. Individuals with the del/del genotype had a 50% increase in breast cancer risk compared with individuals without this genotype [21]. Although several cohort studies suggested that higher folate intake was associated with lower breast cancer risk, a recent large screening trial showed
that higher supplement intake may increase the risk of breast cancer in postmenopausal women [30]. The authors [30] further hypothesize that, because of the complexity of folate function, it is possible that both deficiency and abundance of folate may contribute to breast carcinogenesis at different stages of tumor development or in different tumor phenotypes. It is thus possible that genetic variations along with dietary intake of folate and methionine influence cellular one carbon metabolism and methyl-donor status, which may also affect susceptibility to other cancers such as colorectal carcinoma. Recent studies have shown the potential relationship between germline variants in methyl-group metabolism genes and promoter CpG island methylation in colorectal tumors [31], which seems to be a specific molecular pathway for colon carcinogenesis. Curtin et al. [32] looked into an association among genetic polymorphisms relevant to folate mediated one-carbon metabolism, including DHFR 19-bp insertion to deletion polymorphism and colon cancer risk, but did not find any association with the del allele [32]. In contrast, a large population-based study that analyzed 395 tag SNPs (a sufficient number of polymorphisms to define common haplotypes, as based on LD) in 15 folate-pathway genes identified two tag SNPs in the DHFR gene (intronic rs1677693 A to C and rs1643659 A to G polymorphisms) associated with lower colorectal cancer risk [33]. The analyzed population was on a folate fortified diet, but the observed protective effect was particularly obvious in non-multivitamin supplement users, confirming previous observations that the effect of folate cycle gene variants may be potentiated or abrogated by dietary folate intake.
**DHFR GENE POLYMORPHISMS AND RESPONSE TO TREATMENT**

The same gene variations that favour THF accumulation may both protect against cancer establishment (as shown in ALL patients [26]) and affect antifolate treatment being responsible for individual differences in terms of survival. This paradoxical dual and opposite effect was recently described as ‘‘false-friend allele’’ behavior [26], and has also been previously noted for MTHFR variant alleles [34]. Higher DHFR activity and THF accumulation can contradict a cytotoxic effect of antifolates, thereby reducing treatment efficacy. Methotrexate (MTX) is an important antifolate widely used in the treatment of several malignancies, including acute lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma and choriocarcinoma [35]. DHFR, as a major MTX target, plays an important role in the development of MTX resistance in ALL. In both experimental and clinical settings, altered levels of DHFR and DHFR gene amplification were found in relapsed leukemia patients and in leukemia and colon cell lines manifesting MTX-resistant phenotypes [8, 36-38]. Of note is that several mechanisms may contribute to the development of MTX resistance. This review is, nevertheless, limited only to DHFR gene variations and other mechanisms are reviewed in detail elsewhere [9]. Regarding the potential role of DHFR polymorphisms in response to MTX treatment (Table 1), Goto et al., 2001 [39], described one of the first variations in DFHR while analyzing the 3'-untranslated region (UTR) of the human DHFR gene transcript. They discovered C829T substitution located 223 base pairs downstream from the stop codon and positioned between the first and second polyadenylation site. This
polymorphism seems to influence DHFR expression, which increased with the number of $T$ alleles being highest in $TT$ individuals [39]. Consequently, the $C829T$-associated increase in DHFR expression may reduce MTX-related cytotoxicity. Indeed, reduction in sensitivity to MTX driven by this polymorphism was subsequently documented by Mishra et al., 2007 [40], who further elucidated the functional role of this variation. They showed that this SNP is located near the microRNA (miR)-24 3' UTR binding site and that it affects DHFR expression by interfering with miR-24 function. Analysis \textit{in vitro} using Chinese hamster ovary derived cell lines that lack DHFR showed that the cells with the $T$ allele bind miR-24 less efficiently, resulting in a 2-fold increase in DHFR mRNA half-life and, hence, higher DHFR mRNA and protein levels. This in turn affected sensitivity to MTX. The cells with the $T$ allele were 4-fold more resistant to MTX as compared with cells without this allele [40]. It is known that miRNAs play an important role in different biological processes, such as cell proliferation, cell death, stress resistance and fat metabolism through the regulation of gene expression [41]. They may also be differentially expressed in human cancers or may act as oncogenes and tumor suppressors by targeting key regulators of cell growth [42, 43]. Moreover, translational control mediated by miRNAs plays an important role in the mechanism of cellular resistance to anti-cancer drug treatment [44]. The work by Mishra et al. [40, 45, 46] was the first to show that genetic polymorphisms may affect drug sensitivity acting through this mechanism. Several groups reported that $C829T$ is non-polymorphic (or appears with very low minor allele frequency) in Caucasians [13, 20]. Nevertheless, it would be worth verifying whether this is also true at the transcriptional level and does not result from the difficulty in designing the primers for DNA amplification (i.e., pseudogenes and
repetitive elements). Changes in the level of DHFR expression and consequently in sensitivity to MTX can be also due to the genetic polymorphisms located in the promoter. The polymorphisms in the 2 kb region upstream of the first or minor transcription initiation site (corresponding to the minor $DHFR$ promoter) were recently analyzed in a cohort of childhood ALL patients [23]. Association of individual polymorphisms and resulting haplotypes with ALL outcome revealed that a reduction in relapse-free survival was associated with $A$ and $C$ alleles located at positions 317 and 1610 upstream from a minor transcription initiation site, respectively. The association was also noted with the haplotype harboring these alleles, arbitrarily named haplotype *1. Haplotype *1 conferred higher transcriptional activity, as shown by reporter gene assay and quantitative mRNA analysis, likely explaining a worse prognosis in patients carrying this haplotype. In addition to the minor promoter, the human $DHFR$ gene also has a downstream major promoter [3, 4]. This adjacent regulatory region is located between minor and major transcript initiation sites and has been shown to act as a non-coding interfering transcript, controlling the transcription of productive mRNA from the major promoter [4]. The polymorphisms of this region were recently defined [24] and tagging variations were subsequently analyzed in the same group of ALL patients [24]. The haplotype analysis revealed diversification of haplotype *1 [23] into five subtypes, and only one of those, *1b, was responsible for the lower relapse-free survival observed in ALL patients, defining more precisely the relapse predisposing variations of $DHFR$. This association seems to be confined to patients with high-risk of relapse, as defined by classical clinical prognostic criteria, and it was further validated in an additional cohort of ALL patients. The *1b subtype was characterized by a particular allele combination defined by allele $T$
and A at positions 35 and 308 from the first transcription initiation site, respectively, compound length polymorphisms composed of 9bp insertion at position 63 and triple 9bp element at position 91. The 9bp repeat element of the compound length polymorphism was initially observed by Fujii et al. [47] and resembles that described in DHFR 5’UTR [19] and a mismatch repair gene (hMSH3) overlapping DHFR [48], although the sequence alignment and the number of repeats were to a certain extent differently resolved. Importantly, *1b was the only haplotype *I subtype associated with higher mRNA levels and was predicted in silico to affect the structure of the major promoter [24]. It has been shown [3, 4] that the DHFR repression is regulated by a non-coding interfering transcript and that this regulation is due to the formation of a stable complex between noncoding RNA, which also acquires a different conformation, and the major promoter. It is, thus, possible that the *1b haplotype could affect the function of the non-coding transcript, resulting in an observed increase in mRNA levels and a higher risk of ALL relapse [24]. Low-dose MTX is considered the ‘gold standard’ of therapy for rheumatoid arthritis (RA) patients [49]. Nevertheless, there is considerable inter-individual variation in its clinical efficacy. A substantial number of patients do not respond to treatment, whereas others (10-30%) develop drug side effects requiring discontinuation of therapy [50, 51]. Wessels et al., 2006 [50], analyzed the association of genetic polymorphisms of the folate pathway with MTX efficacy, expressed as a disease activity score, and methotrexate toxicity, specifically respiratory, gastrointestinal, skin, mucosal and hepatic adverse drug events (Table 1). Among DHFR polymorphisms, they analyzed DHFR G-473A (rs1650697) replacement in 5’UTR and DHFR A35289G (rs1232027) substitution positioned relative to the translation initiation site. No
association was found, whereas in contrast, Chandran et al. [52] found an association of the A allele of A35289G polymorphism with MTX efficacy in patients with psoriatic arthritis. The study conducted in RA patients from northern India [53] addressed additional DHFR A/T polymorphism in 3’UTR (position 1171 of mRNA, rs7387) and revealed its contribution to MTX efficacy only in a multivariate model when analyzed together with other variations of folate dependent enzymes. Regarding the potential functional role of DHFR polymorphisms selected for rheumatoid and psoriatic arthritis study, little can be said. The G-473A variation in 5’UTR (rs1650697) corresponds to C35T substitution (given relative to the forward strand and first transcription initiation site) described in ALL patient analysis [24]. Individually this polymorphism does not seem to have an impact on mRNA levels. However the T allele is one of the alleles of haplotype *1b, which, as described above [24], seems to increase DHFR expression. The polymorphism in 3’ UTR such as rs7387 [53] may potentially affect mRNA stability. However, it is not clear what the functional role [54] of the A35289G variation located several Kb downstream from the 3’UTR would be (based on dbSNP data [55]). Another polymorphism in LD with this DHFR variation may possibly explain the positive association reported.
CONCLUSION

DHFR is a critical folate cycle enzyme targeted by antifolate medication used in the treatment of cancer and rheumatoid arthritis. The change in DHFR expression and activity caused by genetic polymorphisms may affect an individual’s predisposition to respond to the treatment in terms of efficacy and drug side effects. Due to the crucial role DHFR plays in the conversion of DHF to THF required for nucleic acid synthesis and methylation reaction, DHFR gene polymorphisms might affect diseases dependent on folate status, such as cancer and spina bifida. The genotype-phenotype relationships have only begun to unravel. The results obtained hold promise for the future. They are still scarce and sometimes contradictory requiring further analysis and replication. It is clear that other polymorphisms of the folate pathway, dietary intake and different genetic and epigenetic mechanisms beyond genetic polymorphisms may contribute toward the variability in treatment responses, the understanding of which would allow such information to be used in disease prevention and treatment tailored to individuals.
REFERENCES


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Table 1:

Summary of the studies associating *DHFR* polymorphisms with disease susceptibility or response to treatment.

<table>
<thead>
<tr>
<th>Location/Position</th>
<th>Polymorphism</th>
<th>Impact</th>
<th>Related Disorders</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron 1</td>
<td>19-bp insertion/deletion</td>
<td>Low-serum folate/high homocysteine, change in mRNA levels</td>
<td>NTD, Breast cancer</td>
<td>(13, 17) (21)</td>
</tr>
<tr>
<td>Intron 3</td>
<td>A10372C/A8890G</td>
<td>unknown</td>
<td>Colon cancer</td>
<td>(33)</td>
</tr>
<tr>
<td>3'-UTR</td>
<td>C829T</td>
<td>Interfering with miR-24 function, higher DHFR mRNA and protein levels</td>
<td>MTX resistance</td>
<td>(40)</td>
</tr>
<tr>
<td>Minor promoter*</td>
<td>C-1610G or T A-317/G, G308A C35T</td>
<td>Higher DHFR expression</td>
<td>Higher risk of relapse in ALL</td>
<td>(23)</td>
</tr>
<tr>
<td>Major promoter*</td>
<td>G308A C35T, Length polymorphism 63/91: 9-bp insertion deletion/9-bp repeat</td>
<td>Higher DHFR expression</td>
<td>Higher risk of relapse in ALL</td>
<td>(24)</td>
</tr>
<tr>
<td>Downstream to 3'</td>
<td>A35289G</td>
<td>Unknown</td>
<td>MTX efficacy in patients with psoriatic arthritis</td>
<td>(52)</td>
</tr>
<tr>
<td>UTR</td>
<td>A1171T</td>
<td>Unknown</td>
<td>MTX efficacy in patients with RA</td>
<td>(53)</td>
</tr>
</tbody>
</table>
Table summarizes positive associations of DHFR polymorphisms with related disorders; DHFR, dihydrofolate reductase; MTX, methotrexate; ALL, acute lymphoblastic leukemia; RA, rheumatoid arthritis; NTD, neural tube defects; del, deletion. *polymorphism in the DHFR minor and major promoter defining *1 and *1b haplotype, respectively associated with higher DHFR expression and higher risk of relapse in ALL patients. Position of the polymorphism is given relative to the transcription or translation initiation site, or refers to the position within indicated intron. ** rs SNP number from dbSNP database at National Center for Biotechnology Information (NCBI) is provided as long it is available.
Chapter IV
Article II

Thymidylate synthase polymorphism is associated with a resistance of leukemic blasts to methotrexate in an in vivo xenogeneic mouse model

Authors: Bahram Sharif Askari,*1,3 François Fontaine,*1 Daniel Sinnett,1,2 Caroline Laverdière,1,2 Maja Krajinovic,#1-3 and Elie Haddad#1,2,4
Affiliations:

¹Research Center, University Health Center Sainte-Justine, Montreal;
²Department of Pediatrics,
³Department of Pharmacology,
⁴Department of Microbiology, Infectiology and Immunology, University of Montreal, Montreal, QC
* Both authors contributed equally to this work
# Both authors share the senior authorship of this manuscript

Running title (50 characters including spaces): TS variants associated with MTX resistance in vivo

Primary Scientific Category: Lymphoid Neoplasia

Text word counts: 1192
Abstract word counts: 149
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Reference counts: 25

Key points:

- Thymidylate synthase 3R/3R genotype in cells from ALL patients is predictive of methotrexate response in vivo in a dose-dependent fashion.
- Increase in MTX dose may overcome MTX resistance associated with this genotype.
AUTHORS’ CONTRIBUTION

In this paper, my contribution is:

- Design of the study;
- Genotyping of bone marrow samples of ALL patients to define presence or absence of TS 3R/3R genotype.
- Performing the experiments with F.F
- Treatment the randomized mice with MTX at either 0.25; 1 or 5 mg/kg/day or PBS
- Writing the manuscript

Contributions of other authors are: F.F. M.K. and E.H. contributed to design the experiments and writing the manuscript; F.F. collected and analyzed the data; D.S. and C.L. contributed biological and clinical information and critically revised results and manuscript.

Conflict-of-interest disclosure: The authors declare no conflict of interest.

Correspondence: Elie Haddad, Department of Pediatrics, Research Center, University Health Center Sainte-Justine, Montreal, Canada
Abstract

Pharmacogenetics study conducted in children with acute lymphoblastic leukemia (ALL) showed that $3R/3R$ genotype of tandem repeat polymorphism in thymidylate synthase ($TS$) gene confers higher risk of relapse. Using xenogeneic mouse model, we investigated whether this genotype is associated with resistance to methotrexate (MTX). Response was estimated in mice injected with leukemia cells from two $3R/3R$ patients and two patients negative for this genotype. The response was dose-dependent: mice injected with patients’ cells with $3R/3R$ genotype were resistant to 0.25 and 1 mg/kg MTX doses, relative to mice injected with patients’ cells without this genotype; dose escalation to 5mg/kg efficiently bypassed genotype-associated MTX resistance. Obtained results provide functional explanation for the significantly higher risk of relapse seen in $3R/3R$ ALL patients and suggest that these patients might benefit from an increase in MTX dose. Furthermore, xenogeneic mice model can serve as a preclinical tool to explore individualized treatment options.
Introduction

The treatment of paediatric acute lymphoblastic leukemia (ALL) has greatly improved in the past four decades due to the introduction of effective combination risk-adapted therapies.\textsuperscript{1} Therapy resistance nevertheless remains a major obstacle to successful treatment\textsuperscript{2} due to unfavourable pharmacokinetics and pharmacodynamics or particular disease features.\textsuperscript{3} Several pharmacogenetics markers have been shown to contribute to this variability and to correlate with the risk of relapse in ALL (reviewed by Pui\textsuperscript{1} and Ansari\textsuperscript{4}), including tandem repeat polymorphism in thymidylate synthase (TS) gene. ALL patients who are homozygous for high expression triple repeat (3R) allele\textsuperscript{5} had significantly higher risk of relapse,\textsuperscript{6-11} whereas low expression double repeat (2R) allele was associated with higher frequency of treatment-related toxicity.\textsuperscript{12-14} TS is inhibited by methotrexate (MTX) polyglutamates\textsuperscript{15} and the presence of TS 3R/3R may suggest a need for different doses and/or schedules in order to improve the treatment efficacy. However, the poly-chemotherapeutic combinations commonly used for ALL treatment do not allow the clear demonstration of a direct relationship between TS genotype and a resistance to MTX only. To address genotype-based treatment response we used xenogeneic mice models that are well suited for \textit{in vivo} studies of human ALL\textsuperscript{16,17} since they retain the fundamental biological characteristics of the original disease such as leukemogeneicity, clonality and chemotherapy response.\textsuperscript{18-20} We investigated the effect of various MTX doses on leukemia development in mice injected with \textit{in vivo} expanded pre-B ALL patient’s blasts harboring or not the relapse-predisposing \textit{TS} 3R/3R genotype.
Methods

Bone marrow aspirates were obtained from newly diagnosed paediatric ALL patients treated at the CHU Sainte-Justine in Montréal, Canada. DNA was extracted from bone marrow samples of ALL patients and genotyping was performed to define presence or absence of $TS\ 3R/3R$ genotype. Genotyping was performed as described previously.\textsuperscript{5,6} Bone marrow mononuclear cells (BMMC) samples were evaluated for phenotypic analysis of blasts content by flow cytometry. Patient’s blasts were injected intravenously into NSG mice (NOD/LtSz-scid IL-2Rgamma$^{null}$). Mice were randomized to receive MTX at either 0.25; 1 or 5 mg/kg/day or PBS as described.\textsuperscript{18,21} Leukemia development was monitored by FACS staining in the peripheral blood. Details are provided in supplemental Methods.

The study has been approved by the Institutional Ethical Review Board in Research of the CHU Sainte-Justine and an informed consent was obtained from the patients and/or their parents. All animal experimental procedures were approved by our Institutional Committee for Good Animal Practices in Research.
Results and discussion

We analyzed two ALL patients with the TS 3R/3R genotype (TS positive, TS+) and two consecutive ALL patients without this genotype (TS negative, TS-), identified by genotyping as 2R/3R. None of the patients had the fusion gene transcripts that might have interfere with MTX efficacy,22 as shown by routine testing of the major chromosomal translocations. In all cases, the hCD45+ cells were confirmed to be leukemic blast because they harboured the same B-cell phenotype as in the primary leukemic cells from which they were expanded (Supplemental Table S1). Likewise, TS genotype remained the same throughout the experiments (Figure S1).

We evaluated the efficacy of 3 MTX doses (0.25, 1.0 and 5.0 mg/kg) between mice injected with TS+ and TS- patients cells. The time-to-leukemia, the kinetics of development and the number of patient blasts found in mice peripheral blood, either expressed in percentage of human cells (Figure 1A) or in absolute counts/μL of leukemic cells (Figure 1B), differed from one patient to another in untreated mice (Figure 1). Despite these differences, the response pattern to MTX could be clearly delineated between TS+ and TS- patients with a resistance to MTX observed in the NSG mice injected with TS+ leukemia cells relative to mice injected with TS- cells (Figure 1). The results were concordant for patients with same genotype. Lower efficacy of MTX in mice injected with TS+ cells was observed for both 0.25 and 1 mg/kg MTX doses, with the differential effect of genotype being particularly apparent for the 1mg/kg dose. However, in all cases, high MTX dose (5 mg/kg) was efficient to maintain very low levels of circulating blasts.
In order to normalize the response to MTX for all patients regardless the kinetics of blasts development in mice, we assessed the relative efficacy of MTX treatment for each dose compared to the untreated (PBS) group by assuming that the mean levels of blasts in the PBS group corresponded to 0% of treatment efficacy, and that a complete absence of circulating blasts corresponded to a 100% efficacy (Figure 2). MTX efficacy at 0.25 and 1 mg/kg increased with time for the mice injected with blasts from TS- patients, whereas the MTX efficacy decreased with time for the mice injected with blasts from TS+ patients (P ranging from .01 to < .0005 by week +1 post MTX), strongly suggesting an absence of efficacy of MTX at these doses against TS+ cells. For the 5 mg/kg MTX dose, the MTX efficacy was similar in mice injected with patients’ TS+ and TS- cells with somewhat lower efficacy in mice with TS+ cells, particularly from week+3 to week+5. Importantly, in mice injected with TS+ cells, an obvious increase in MTX efficacy was seen when compared to lower doses (68% vs. 1-9%, P < .0001), illustrating that dose escalation can maintain low levels of leukemia in this genotype condition.

Engraftment of human ALL cells in NSG mice has been extensively reported, however there is no evidence about the relevance of this xenogeneic model to investigate therapeutic implication of patient genotype and drug dose adjustment. Here, we demonstrate the relationship between the TS 3R/3R genotype and the resistance to MTX treatment in dose-dependent manner in this model. Dose-response curves and normalization of effect by calculating the relative efficacy of MTX as compared to PBS were useful to assess the efficacy of MTX among various specimens who may not have the same kinetics of proliferation, and to define the dose ranges where differential genotype effect is most apparent. Mice injected with blasts from TS+ ALL
patients were resistant to MTX dose of 0.25 and 1 mg/kg MTX and the resistance status was overcome by an increase in the dose. These results are in accordance with the association studies strongly supporting the possibility that ALL patients with higher risk of relapse due to $TS\ 3R/3R$ genotype develop MTX resistance and might need higher doses for effective target inhibition.

We also addressed how much the circulating murine cells could be affected by the chemotherapy and could therefore influence the MTX concentration caused by their metabolic uptake. The enumeration of murine CD45+ cells by flow cytometry showed no difference of the murine leukocytes of NSG mice in the blood (data not shown), suggesting that in this xenogeneic model of ALL, MTX at the doses that we used has an effect only on human cells and that the murine system does not interfere with the MTX effect.

In conclusion, our data provide a functional demonstration that the blasts from patients with $3R/3R$ genotype are more resistant to MTX suggesting that the association observed in clinical setting was indeed due to genotype-driven resistance to MTX. Chemotherapy response in dose-dependent manner and through data normalization showed that this model could serve as a preclinical tool to explore individualized treatment options.
Acknowledgements

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References


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Figures legends

Figure 1. Response to MTX in NSG mice injected with patients’ leukemia cells without and with TS 3R/3R genotype. (A) Percentage of human blasts among total lymphocytes in NSG mice injected with $5 \times 10^6$ pre-B ALL patient cells, treated with MTX or PBS. (B) Absolute counts of human blasts/μL of blood in NSG mice. Results are shown as mean ±SEM. n = 4-5 mice in each group. TS+ 1 and TS+ 2: TS positive patients with 3R3R genotype; TS- 1 and TS- 2: TS negative patients, without TS 3R3R genotype

Figure 2. Efficacy of MTX treatment per dose. Efficacy of MTX (in %) is normalized as compared to the not treated groups (PBS), 0% efficacy corresponding to a level of blasts identical to the one observed in the corresponding group of PBS-treated mice and 100% efficacy corresponding to undetectable blasts. TS- (full line) represent all mice injected with blasts of the 2 TS- ALL patients (n = 4-10 mice/week). TS+ (dotted line) represent all mice injected with blasts of the 2 TS+ patients (n = 3-10 mice/week). Results are showed as means ±SEM. Difference in efficacy is calculated by Mann-Whitney two-tailed test at each time point. ns: not significant, *: P < .05; **: P < .005; ***: P < .0005. Week 0 corresponds to the last value obtained prior to MTX treatment.
Figure 1.
Figure 2.

MTX 0.25 mg/kg

MTX 1 mg/kg

MTX 5 mg/kg

MTX efficiency (%)
SUPPLEMENTAL MATERIAL TO:

Thymidylate synthase polymorphism is associated with a resistance of leukemic blasts to methotrexate in an in vivo xenogeneic mouse model

Bahram Sharif Askari,*1,3 François Fontaine,*1 Daniel Sinnett,1,2 Caroline Laverdière,1,2 Maja Krajnović,1,3 and Elie Haddad1#1,2,4

¹Research Center, University Health Center Sainte-Justine, Montreal;
²Department of Pediatrics,
³Department of Pharmacology,
⁴Department of Microbiology, Infectiology and Immunology, University of Montreal, Montreal, QC

* Both authors contributed equally to this work

# Both authors share the senior authorship of this manuscript
Supplemental Methods:

Patient samples

Bone marrow aspirates were obtained from newly diagnosed paediatric ALL patients treated at the CHU Sainte-Justine (Montréal, Canada). The patient’s samples were collected on heparinized tubes and bone marrow mononuclear cells (BMMC) were obtained after Ficoll-Hypaque (GE Healthcare) density gradient centrifugation. BMMC were cryopreserved in DMSO 10% for later use. The study has been approved by the Institutional Ethical Review Board in Research of the CHU Sainte-Justine and an informed consent was obtained from the patients and/or their parents.

Genotyping

DNA was extracted from bone marrow samples of ALL patients and genotyping was performed as described previously.¹, ² In brief, the presence or absence of TS triple repeat, 3R/3R genetic polymorphism was resolved by polymerase chain reaction (PCR) amplification. The primer sequences used for TS 3R/3R amplification were 5’-GTG GCT CCT GCG TTT CCC CC-3’ and 5’-CCA AGC TTG GCT CCG AGC CGG CCA CAG GCA TGG CGC GG-3’, for the forward and reverse primers, respectively. The PCR was performed for 3 min at 95°C followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, completed by a final extension step at 72°C for 7 min. PCR amplification was followed on 3% agarose gel electrophoresis at 85V for 1h30.

Xenograft mouse model of human ALL and MTX chemotherapy

NSG mice (NOD/LtSz-scid IL-2Rγc null, Jackson Laboratory) were bred and maintained under specific pathogen-free conditions in micro-isolator cages and provided with autoclaved food and water in the research animal facility of the CHU Sainte-Justine.
BMMCs samples were thawed and evaluated for phenotypic analysis of blasts content by flow cytometry (see Methods below). Patient’s blasts were expanded in a primary round of in vivo expansion by injecting between 1 to 5 x10^6 thawed ALL patient blasts in the lateral tail vein of one to three non-irradiated NSG mice. In order to deplete the T lymphocytes present in the sample that may have caused a xeno-Graft-versus-Host Disease (GvHD) in the primary injection, mice received anti-CD3 antibody as previously described. Mice were evaluated for leukemia development by detection of circulating human blasts in the blood by flow cytometry (see Methods below). The mice were sacrificed when human blasts were ranging from 30-90% of total CD45 and/or CD19 and/or CD10-positive lymphocytes. The spleen was then crushed on a 70μm strainer using PBS. Human blasts were phenotyped and enumerated by flow cytometry. For the assessment of MTX sensitivity experiments, 7- to 10-week-old NSG mice were injected intravenously with 5 x10^6 in vivo expanded pre-B patient’s leukemic blasts with or without TS 3R/3R genotype. When the percentage of human ALL reached 1-5% of CD45+ and/or CD19+ and/or CD10+ cells in peripheral blood, mice were randomized to receive MTX (Accord Healthcare) intra-peritonealy in groups of 5 individuals at either 0.25; 1 or 5 mg/kg/day or PBS (negative control) on 4 consecutive days, every 14 days for a maximum of 8 weeks when possible. Peripheral blood was weekly collected from the saphenous vein on Microvette-200 heparinized tubes (Sarstedt). Red blood cells were lysed using ammonium chloride 0.3 M solution and cells were then analyzed by flow cytometry analysis. Mice were appropriately sacrificed at the end of MTX treatment or when they reached established limit points (hunch-back, weight loss and ruffled fur, assessed in blinded fashion as to assigned treatment). All animal experimental procedures were approved by our Institutional Committee for Good Animal Practices in Research.
Flow cytometry analysis

Flow cytometry analyses of xenograft leukemia cells in peripheral blood and spleen were performed using monoclonal antibodies specific for murine CD45 conjugated with fluorescein isothiocyanate (FITC) (Ref. 553080), anti-human CD45 conjugated with allophycocyanin (APC) (Ref. 555485), anti-human CD19 conjugated with phycoerythrin (PE) (Ref. 555413), anti-human CD3 conjugated with BD Horizon V500 (Ref. 561416) (from BD Biosciences) and anti-human CD10 conjugated with phycoerythrin-cyanine7 (PE-Cy7) (Ref. 312214) (from BioLegend) were used for enumeration in absolute counts or in proportions of human blasts in patient’s bone marrow and in mice peripheral blood and spleen. Dead cells were excluded by 7-amino actinomycin D (7-AAD) staining (BD Biosciences). Count Bright Beads (Life Technologies) were added in each tube for absolute counts determination. Flow cytometry analysis was performed using an LSRFortessa flow cytometer (BD Biosciences). The development of leukemia in mice was determined by enumeration of the proportion of human cells among total lymphocytes (human + murine) as previously described \(^4\) and by the enumeration of absolute counts of blast cells. The results were analyzed using FCS Express 4 for Flow Cytometry RUO Edition (De Novo Software).

Statistical analysis

Results were analyzed using GraphPad Prism 4.0 (GraphPad Softwares Inc., La Jolla, USA) and are shown as mean ±SEM. Elsewhere, two-tailed Mann-Whitney tests were used with significance set at \(P \leq .05\).
References


Supplemental Table

Table S1: Patient samples characteristics. Bone marrow aspirates were obtained from pediatric pre-B ALL patients. Phenotype of pre-B ALL blasts were determined by flow cytometry using monoclonal antibodies and DNA was extracted for evaluation of the presence or absence of the TS 3R/3R genetic variant. Primary blasts were also frozen and thawed for \textit{in vivo} expansion in NSG mice. BM: bone marrow, M: male.

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**Supplemental Figures**

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**Figure S1: TS genotypes before and after leukemic cell engraftment in NSG mice.**

TS-1 and TS-2: Samples from 2 ALL patients without TS 3R/3R genotype; TS+1 and TS+2: Samples from 2 ALL patients with 3R/3R genotype. Samples from each patient are obtained at 3 points, prior to engraftment (P) and following first (1) and second (2) engraftment in mice. Ctrl: Genotype controls for 2R/2R, 2R/3R and 3R/3R.
Chapter V
Article III

Polymorphisms of asparaginase pathway and asparaginase-related complications in children with acute lymphoblastic leukemia

Running title: Asparagine synthetase gene and asparaginase toxicity in ALL

Mohsen Ben Tanfous,¹ Bahram Sharif-Askari,¹ Francesco Ceppi,¹ Haithem Laaribi¹ Vincent Gagné,¹ Julie Rousseau¹, Malgorzata Labuda,¹ Lewis B. Silverman,⁴,⁵ Stephen E. Sallan,⁴,⁵ Donna Neuberg,⁶ Jeffery L. Kutok,⁷ Daniel Sinnett,¹ Caroline Laverdière and Maja Krajinovic¹-³
\textsuperscript{1}Research Center, CHU Sainte-Justine, Montreal, QC, Canada; \textsuperscript{2}Department of Pediatrics, University of Montreal, QC, Canada; \textsuperscript{3}Department of Pharmacology, University of Montreal, QC, Canada; \textsuperscript{4}Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA; \textsuperscript{5}Division of Hematology/Oncology, Children’s Hospital, Boston, MA; \textsuperscript{6}Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA; \textsuperscript{7}Department of Pathology, Brigham and Women’s Hospital, Boston, MA

\textbf{Correspondence}: Maja Krajinovic, Centre de recherche, CHU Sainte-Justine, 3175 chemin de la Côte-Ste-Catherine, Montréal, QC, H3T 1C5 Canada

\textbf{Running title}: \textit{ASNS} gene and asparaginase-related toxicities

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**Statement of translational relevance**

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy of childhood. The treatment of pediatric ALL has greatly improved in the past four decades due to the introduction of effective combination risk-adapted therapies. However, therapy resistance in a significant number of children is still a major obstacle to successful treatment. Intensive treatment has also significant short-term side effects and long-term consequences. Identification of genetic component underlying this variability, would allow traditional treatment to be complemented by genotype-based drug dose adjustment.

Asparaginase is a critical component of ALL treatment. We recently analyzed genes of asparaginase action pathway in relation to the risk of relapse in ALL. Here we investigated whether the same genes may affect asparaginase-related toxicities and found that particular polymorphisms and haplotypes in asparagine synthetase gene may affect these adverse reactions in ALL patients. The study provides a new insight into the pharmacogenetics of asparaginase-related treatment complications in ALL.
Abstract

Purpose. Asparaginase is a standard and critical component in the therapy of childhood acute lymphoblastic leukemia (ALL), but it is also associated with several toxicities.

Experimental design. We recently reported the results of an association study between asparaginase pathway genes and event free survival (EFS) in childhood ALL patients. The same polymorphisms were interrogated here in relation to allergies, pancreatitis and thrombotic events following treatment with E.coli asparaginase.

Results. Among patients of discovery group, allergies and pancreatitis were more frequent in individuals who are homozygous for the triple repeat allele (3R) of asparagine synthetase (ASNS) gene, resulting in remarkably higher risk of these toxicities associated with 3R3R genotype (OR for allergies =14.6, 95% CI= 3.6-58.7, p<0.0005 and OR for pancreatitis = 8.6, 95% CI= 2.0-37.3, p=0.01). In contrast, the ASNS haplotype *1 harbouring double repeat (2R) allele had protective effect against these adverse reactions (p≤0.01). The same haplotype was previously reported to confer reduction in EFS. The risk effect of 3R3R genotype was not replicated in validation cohort, whereas the protective effect of haplotype *1 against allergies was maintained (p≤0.002). Analysis with additional polymorphisms in ASNS locus in CEU cell lines showed that haplotype *1 is diversified in several subtypes of which one was associated with reduced in vitro sensitivity to asparaginase (rs10486009, p=0.01) possibly explaining an association seen in clinical setting.

Conclusions. This finding might have implication for treatment individualization in ALL and other cancers employing asparagine depletion strategies.
Keywords: asparaginase, polymorphisms, pharmacogenetics, childhood leukemia, treatment, adverse reactions
Introduction

Over the past four decades, treatment of childhood acute lymphoblastic leukemia (ALL) has improved importantly such that ~ 80-85% of patients are cured with current therapy regimen. Up to 20% of patients experience treatment failure whereas treatment-related toxicities are often life-threatening and are the primary cause of interruption or discontinuation of chemotherapy. Asparaginase (ASNase) is a standard component in the childhood ALL treatment (1). It is required by all cells for survival and is normally produced by the enzyme asparagine synthetase (ASNS). Malignant lymphoblasts are thought to have low ASNS levels and thus depend on extracellular sources of asparagine for their rapid growth. Depletion of asparagine by ASNase selectively kills leukemia cells by decreasing protein biosynthesis (2). Associations between success of ALL treatment and ASNase dose intensity or formulation have been reported in several clinical studies (3-5). E.coli derived enzymes are more potent, are associated with higher efficacy, but also with higher toxicity (5-7). Side effects related to ASNase treatment include allergic reactions that occur in 20–40% patients and require change of drug formulation. Two most serious and most frequent dose-limiting asparaginase-related toxicities are pancreatitis and thrombotic events reported in up to 18% and 5% of ALL patients, respectively (8, 9). Pancreatitis usually develops after the first few doses of asparaginase suggesting that it may occur as a result of an underlying predisposition rather than as a cumulative drug effect (9).

We recently analyzed relationship between event free survival (EFS) in childhood ALL patients and genes in ASNase pathway (10), which were differentially expressed
between asparaginase resistant and sensitive cells (11-13). We showed that promoter variant of transcriptional factor \textit{ATF5} involved in \textit{ASNS} regulation, is associated with higher promoter activity and confers higher risk of ALL relapse in patients who received E.coli ASNase (10). Association with lower EFS has been also found with tandem repeat (14) in \textit{ASNS} gene and with resulting haplotype (arbitrarily named haplotype*1) (10). Here we report the analysis of the same set of polymorphisms in \textit{ASNS}, \textit{ATF5} and \textit{ASS1} (arginosuccinate synthase 1) in relation to ASNase-related acute complications (allergies, pancreatitis and thrombotic events) in two independent childhood ALL cohorts.

**Patients and methods**

**Study population and endpoints in the analysis**

The study population consisted of 285 Caucasian children (98% of French-Canadian origin) diagnosed with ALL at the Hospital Sainte-Justine, (HSJ, Montreal, Quebec, Qc, Canada) between January 1989 and July 2005 (QcALL cohort or test group) who received E.coli asparaginase as a part of Dana-Farber Cancer Institute ALL Consortium protocols DFCI 87-01, 91-01, 95-01 or 00-01 (Table 1) (5, 6, 10, 15). Details of asparaginase administration across these treatment protocols are described elsewhere (10, 16). The information on asparaginase-related toxicity was assessed by retrospective chart review. Pancreatitis was defined as an elevation in the serum amylase level >3 times normal associated with clinical signs and symptoms consistent with the diagnosis (9). Pancreatitis cases were classified by duration of symptoms as severe or mild/moderate (16). Hypersensitivity reactions to asparaginase were characterized by local manifestations at the injection site as well as systemic manifestations (erythema,
swelling, urticaria, rash, pruritus, tachypnea, and wheezing) (17). Thrombosis was identified by clinical symptoms and confirmed by radiological imaging based on institutional guidelines (18).

Previously obtained genotypes in asparaginase pathway genes were used for the analysis, as described in Rousseau et al (10), including 8, 2 and 4 SNPs in \textit{ATF5}, \textit{ASNS} and \textit{ASS1} genes, respectively (Supplemental Table 1). The estimates of linkage disequilibrium (LD), and haplotype phase was obtained by PHASE software, version 2.0 (19). Association of genotypes/haplotypes with presence of each ASNase related toxicity was assessed by chi-square test. Adjustment for multiple testing (including all polymorphisms and all toxicities analyzed) was estimated by false discovery rate (FDR) (10). For significant associations, genotypes/haplotypes were grouped in two categories and the genotype-associated risk was expressed as odds ratio (OR) with 95% confidence interval (CI). Analyses of haplotypes within significantly associated gene were not further corrected.

A validation set of Caucasian patients called the Dana-Farber Cancer Institute (DFCI) group was composed of a 248 patients who received E.coli ASNase within DFCI 95-01 and 00-01 ALL treatment protocol in remaining (without HSJ) consortium institutions (5, 6, 16). Baseline characteristics did not significantly differ from discovery group (Table 1).

\textit{Cellular proliferation assay}

In vitro sensitivity to asparaginase was assessed in lymphoblastoid cell lines (LCLs) from 89 individuals of Northern and Western Europe (CEU), as described by Chen et al. (17) The drug concentration resulting in 50% inhibition of cell growth (IC50) during 48h
incubations time was estimated using several E.coli asparaginase concentrations ranging from 0.01-10 IU and the GraphPad software by fitting sigmoid dose-response curves. Obtained values were correlated to genotypes using Mann-Whitney or Kruskal-Wallis test.

Informed consents were obtained from parents or guardians before enrolment into the study. The study was approved by institution ethics committees.

Results
Allergies, pancreatitis and thrombotic events occurred in discovery group (QcALL) with the frequency of 15.8%, 5.6% and 3.5%, respectively. Pancreatitis was in most cases severe (in 13 out of 16 cases) and systemic allergies also occurred more frequently (in 37 out of 45 subjects with allergic reactions). Analysis between these toxicities and SNPs in \textit{ASNS}, \textit{ATF5} and \textit{ASS1} genes revealed an association of tandem repeat polymorphism (rs3832526) in \textit{ASNS} gene with both pancreatitis and allergies (p=0.008 and p<0.0005, respectively, Figure 1A). These complications were more frequent among patients that were homozygous for the triple repeat allele (3R) resulting in 8-14 fold risk elevation (OR for allergies =14.6, 95% CI= 3.6-58.7, and OR for pancreatitis = 8.6, 95% CI= 2.0-37.3). The association with allergy remained significant with FDR of lower than 1%, whereas association with pancreatitis remained significant only with FDR of 16%. We further analyzed \textit{ASNS} haplotypes composed of the tandem repeat polymorphism and promoter \textit{C-181T} substitution (rs3757676). Two haplotypes were associated with allergies and pancreatitis; Homozygosity for haplotype *2, uniquely tagged by 3R allele conferred higher risk of these toxicities (high sensitivity haplotype), whereas haplotype *1 defined by \textit{C-181} and 2R alleles had protective effect (low-sensitivity haplotype, OR
for allergies = 0.4, 95% CI = 0.2-0.8, and OR for pancreatitis = 0.2, 95% CI = 0.07-0.7, p ≤ 0.01, Figure 1B). In our previous analysis the low-sensitivity haplotype *1 conferred reduction in EFS in QcALL cohort (10). We further performed analyses of ASNS gene in replication (DFCI) cohort. Pancreatitis and allergies occurred in this group with the frequency of 8.5% and 23%, similar to the frequencies reported for the 00-01 clinical trial (16). Distribution of severe/moderate pancreatitis and systemic/local allergies differed from discovery group, 33.3% pancreatic cases had moderate form and among patients with allergies, 50.1% had local manifestation. The risk effect of 3R3R genotype (or haplotype *2) was not seen, whereas protective effect of haplotype *1 against allergies was maintained, particularly against local allergic manifestation and in patients assigned to high risk group (p = 0.002 and p < 0.0005, respectively, Figure 1B).

To further understand the variability of ASNS gene, we retrieved an information on all SNPs in coding and regulatory regions with minor allele frequency higher than 5%, that might have been identified (20) since our first analysis of this gene (10). We also included SNPs within 5kb upstream and downstream from gene boundaries. Sixteen additional tag SNPs were analyzed, which were all except one (Val210Glu) located beyond the coding region (Figure 2A). Given high number of resulting haplotypes and low frequency of adverse events, detailed stratified analysis would have limited power in patients. We analyzed instead whether polymorphisms defining particular haplotype subtypes might have functional role as estimated by in vitro sensitivity assay in lymphoblastoid CEU cell lines. The protective *1 haplotype was diversified in 5 subtypes (defined by SNPs at 5 different positions, Figure 2B). Two polymorphisms (rs10486009 and rs6971012, positions 4 and 5 in Figure 2B) correlated with in vitro sensitivity to
ASNase, (p=0.01 and p=0.002, respectively, Figure 2C). The TT genotype of rs6971012 had lower sensitivity, which was further reduced by the G allele of rs10486009 (Figure 2C, p=0.001) suggesting that it may contribute to protective effect of haplotype *I observed in clinical setting. In contrast, only one haplotype defined by 3R allele remained after addition of other ASNS polymorphisms and it did not affect in vitro sensitivity to ASNase.

Discussion

Differences in susceptibility to asparaginase have been attributed to variable levels of ASNS expression in number of studies: ASNS levels in leukemia cell lines, patients lymphoblasts and surrounding mesenchyme cells suggested that elevated ASNS levels may counteract the ASNase effect and underlie the resistance to treatment (11, 21, 22). Lower ASNS expression might then be expected to mediate higher sensitivity to treatment and possibly higher frequency of ASNase-related complications. We found that the 3R3R genotype of tandem repeat polymorphism correlated more frequently with pancreatitis and allergies in discovery group. Tandem repeat polymorphism is located in intron 1, but upstream from translation initiation site and was reported to act as an enhancer element (14). The 3R allele increased ASNS promoter activity in embryonic kidney cell line (14). We did not observe relationship between 3R3R genotype with mRNA level (10) or in this study with in vitro sensitivity to ASNase. The effect of 3R allele on therapeutic responses to ASNase is also ambiguous. Recent study reported that 3R allele can affect early response to ALL treatment, as defined by the number of leukemic blasts following one ASNase dose (23). We did not find an association of 3R3R
genotype with reduced EFS (10) and in this study we did not replicate an association of 3R3R genotype with adverse reactions of ASNase. Other polymorphisms/haplotypes in ASNS gene cannot explain this discrepancy; there was only one haplotype defined by 3R allele when additional polymorphisms were included in the analysis. The differences can nevertheless be due to low frequency of 3R3R genotype, different distribution of severe/moderate and systemic/local allergies, or different geographical origin of discovery and replication group. Other genes beyond those studied here, as well as disease and treatment characteristics might also play a role. For example, it has been shown that leukemic cells carrying TEL/AML1 fusion gene are more sensitive to treatment with ASNase compared to other subtypes of ALL (24); several polymorphisms of aspartate metabolic pathway have been associated with ASNase sensitivity in vitro using ALL cells and LCL cell lines (25); top ranking SNPs for ASNase-related allergies have been identified in the gene coding for glutamate receptor in genome-wide association study (17). Distribution of treatment protocols differed between discovery and replication group (Table 1) and patients might have received different ASNase doses, however association with pancreatitis and allergies with 3R3R genotype was maintained in the discovery group when analysis was limited to patients treated with DFCI 95-01 and 00-01 protocols (p ≤ 0.02, not shown). Further and larger studies are needed to confirm the role of 3R3R genotype and to explore whether this finding is applicable to other populations.

The results obtained for haplotype *I, harbouring 2R allele, seems more consistent and correlated with lower frequency of allergies in both discovery and replication patient cohort. This is in agreement with previously reported association of
the same haplotype with reduced EFS (10), suggesting its lower sensitivity in response to ASNase treatment. Analysis of additional polymorphisms in *ASNS* locus revealed diversification or haplotype *I* in several subtypes; one of them (defined by minor allele of *rs10486009*) seems particularly interesting because it was associated with reduced sensitivity to ASNase in vitro, possibly explaining lower sensitivity of haplotype *I* seen in clinical setting. Our finding might be as well of interest for the treatment of other cancers since asparagine depletion strategies using ASNS inhibitors and asparaginase have been suggested in pancreatic and ovarian malignancies (26, 27). Tumor specific up-regulation of ASNS was also reported in castration-resistant prostate cancer and correlated with the progression to a therapy-resistant disease state (28).

In conclusion, we reported an association of 3R3R genotype of *ASNS* gene with a higher frequency of ASNase–related adverse reactions. The association was not seen in the replication group suggesting limited study power or possible modulating role of other genes and/or disease and treatment characteristics or different patient origin. Haplotype harboring 2R allele seems to have protective role against ASNase allergies in both discovery and replication patient set. Extension of the analysis to additional polymorphisms and cellular proliferation assay in response to ASNase treatment, identified variants possibly explaining lower sensitivity of this haplotype observed in clinical setting.
Acknowledgement

We thank all patients and their parents who consented to participate in genetics studies related to leukemia. Canadian Institutes of Health Research, Leukemia Lymphoma, Society of Canada, Charles Bruneau Foundation, and Centre d'excellence en Oncologie pédiatrique et en soins palliatifs supported this study. Dana-Farber Cancer Institute ALL treatment protocols are supported by the National Cancer Institute/NIH grant P01 CA 68484.

Authorship and conflict of interest statement

Contribution: B.SA, V.G., J.R., M.L performed experiments; M.BT, H.L. D.S., F.C., C.L., S.E.S., L.B.S., D.N. and J.L.K. contributed to sample and clinical data collection and processing; V.G., B.SA. and M.K. performed the data analysis; M.K. designed the research and drafted the article; All authors contributed to the interpretation or data and revised the manuscript.

The authors declare no competing financial interests.
References


Table 1. Characteristic of ALL patients in the test (QcALL) and validation (DFCI) cohort

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Protocol distribution is different between QcALL and DFCI group (p=0.001), whereas the remaining characteristics do not differ significantly. WBC, white blood cell count.
Figure Legend

Figure 1. Asparaginase-related acute complications in childhood ALL in relation to tandem repeat polymorphism and resulting haplotypes in asparaginase synthase (ASNS) gene

A. Tandem repeat polymorphism in relation to allergies and pancreatitis in ALL patients of discovery cohort. The frequency of individuals with genotypes of tandem repeat polymorphism in patients with (+, dark gray bars) and without (−, light gray bars) allergies and pancreatitis (left and right side panel, respectively). Numbers of individuals represented by each bar, p value and risk associated with 3R3R genotype (odds ratio, OR, with 95% confidence interval, CI) are indicated on each plot.

B. Risk of allergies and pancreatitis in relation to ASNS haplotypes. Linear display of risk (OR with 95% CI) associated with haplotype *1 and *2 in discovery (QcALL) and replication group (DFCI) based on C-189T substitution (rs3757676) and tandem repeat polymorphism (rs3832526). Protective effect of haplotype *1 in DFCI group was more apparent against local allergies (LA) and in high risk (HR) group. Sequence and frequency of 3 major haplotypes are indicated in the box on the right-bottom side.

Figure 2. ASNS polymorphisms, haplotype *1 sub-classification and cellular proliferation assay

A. Linear display of SNPs in ASNS locus. Linear display refers to all SNPs with minor allele frequency (MAF) > 5% located in coding regions and within upstream and downstream gene boundaries of ~5kb. Exonic and coding sequences are represented by
open and gray boxes, respectively, and SNPs are represented by gray dots. Rs number and MAF are indicated next to each polymorphism. The black and gray arrows indicate the transcription and translation start site, respectively, estimated according to reference sequence NM_183356.3. Polymorphisms analyzed in patients are indicated in bold character. Arrows next to the polymorphisms indicate those depicted in Figure 2B.

B. **Haplotype *1 subtypes.** Diversification of ASNS haplotype *1 on several subtypes, based on the identified polymorphic positions (positions 1 to 5 indicated by respective rs numbers). Haplotype resolution is performed using all SNPs presented in Figure 2A; only those that are relevant for haplotype *1 diversification are presented.

C. **In vitro sensitivity to asparaginase in relation to rs6971012 and rs10486009.** Box plots representing ASNase IC50 in CEU lymphoblastoid cell lines with and without C allele of rs6971012, with and without G allele of rs10486009 and with combined rs6971012 and rs10486009 genotypes (G+, C+ or none of these alleles, (CG)-) are presented in left, right and middle panel, respectively. The number of individuals represented by each bar and the p value obtained by Mann-Whitney or Kruskal-Wallis test is indicated on the plot.
Figure 1.

A

Allergies

Pancreatitis

B

*1

Allergies QcALL

Pancreatitis QcALL

Allergies DFCI (LA)

Allergies DFCI (HR)

*2

Allergies QcALL

Pancreatitis QcALL

OR (95%CI)

56.7% C 2

12.4% C 3

30.8% T 2
Figure 2.
Supplemental Table 1. Polymorphisms in *ATF5*, *ASNS* and *ASS1* genes analyzed in the relation to ASNase related complications

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>dbSNP</th>
<th>position</th>
<th>variation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATF5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3826777</td>
<td>-1072</td>
<td>G/A</td>
<td></td>
</tr>
<tr>
<td>rs1273686</td>
<td>-670</td>
<td>A/C</td>
<td></td>
</tr>
<tr>
<td>rs1152232</td>
<td>-34</td>
<td>A/G</td>
<td></td>
</tr>
<tr>
<td>rs2241702</td>
<td>321</td>
<td>A/T</td>
<td></td>
</tr>
<tr>
<td>rs11554772</td>
<td>1562</td>
<td>C/T</td>
<td></td>
</tr>
<tr>
<td>rs283525</td>
<td>3288</td>
<td>C/T (Leu83)</td>
<td></td>
</tr>
<tr>
<td>rs283526</td>
<td>3403</td>
<td>T/C (Pro121Leu)</td>
<td></td>
</tr>
<tr>
<td>rs8667</td>
<td>3911</td>
<td>G/A</td>
<td></td>
</tr>
<tr>
<td><strong>ASNS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3757676</td>
<td>-181</td>
<td>T/C (-strand)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2R/3R ou 4R (-strand)</td>
<td></td>
</tr>
<tr>
<td>rs3832526</td>
<td>232</td>
<td>R :</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCTGCGCCCCG(C/T)GC</td>
<td></td>
</tr>
<tr>
<td><strong>ASS1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs928518</td>
<td>-1343</td>
<td>G/T</td>
<td></td>
</tr>
<tr>
<td>rs11244367</td>
<td>-621</td>
<td>G/T</td>
<td></td>
</tr>
<tr>
<td>rs2071367</td>
<td>34</td>
<td>G/T</td>
<td></td>
</tr>
<tr>
<td>rs10901072</td>
<td>26133</td>
<td>C/T (His167)</td>
<td></td>
</tr>
</tbody>
</table>

Ancestral allele is given in bold and minor allele is underlined. The polymorphisms is presented as a change from ancestral to derived allele, unless ancestral allele is not known, when the change is given from major to minor allele. SNPs in coding region leading or not to amino-acid substitutions are indicated. SNP position is given respective to transcription initiation site which is estimated for *ATF5*, *ASNS* and *ASS1* based on respective mRNA sequences NM_012068, NM_001673 and NM_00005.
## Supplemental Table 2. Identity of polymorphisms genotyped only in controls

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>gene</th>
<th>dbSNP</th>
<th>position</th>
<th>variation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATF5</strong></td>
<td>rs36220131</td>
<td>-1919</td>
<td>G/T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs36220132</td>
<td>-1867</td>
<td>T/G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs4402677</td>
<td>-1150</td>
<td>T/G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs36220128</td>
<td>-1112</td>
<td>G/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs36220125</td>
<td>-731</td>
<td>G/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs36220126</td>
<td>-705</td>
<td>C/T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs4802639</td>
<td>-581</td>
<td>T/C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs4802640</td>
<td>-514</td>
<td>C/T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs35018314</td>
<td>-270</td>
<td>C/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs11083991</td>
<td>-162</td>
<td>G/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs1152231</td>
<td>-9</td>
<td>A/G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs892027</td>
<td>1276</td>
<td>G/T</td>
<td></td>
</tr>
<tr>
<td><strong>ASNS</strong></td>
<td>rs11554430</td>
<td>144</td>
<td>T/C (-strand)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs1803163</td>
<td>20238</td>
<td>G/T (Pro547His) (-strand)</td>
<td></td>
</tr>
<tr>
<td><strong>ASS1</strong></td>
<td>rs7043097</td>
<td>-1790</td>
<td>A/C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs4740430</td>
<td>-1636</td>
<td>C/T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs11244364</td>
<td>-1357</td>
<td>G/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs12352216</td>
<td>-706</td>
<td>A/G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs7854616</td>
<td>-456</td>
<td>A/T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs35269064</td>
<td>13843</td>
<td>G/T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs10121464</td>
<td>56450</td>
<td>T/C</td>
<td></td>
</tr>
</tbody>
</table>

SNPs genotyped only in controls and excluded from the analysis in ALL patients either because of low frequency or LD with other SNPs. dbSNP number is provided. Ancestral allele is given in bold and minor allele is underlined. The polymorphisms is presented as a change from ancestral to derived allele, unless ancestral allele is not known, when the change is given from major to minor allele. SNPs in coding region leading to amino-acid substitutions are indicated. SNP position is given respective to transcription initiation site which is estimated for **ATF5**, **ASNS** and **ASS1** based on respective mRNA sequences NM_012068, NM_001673 and NM_00005 and reference\(^{32}\) for **ATF5**.
Supplemental Table 3. In silico prediction of transcription factor binding sites affected by \textit{ATF5} polymorphisms

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Identity</th>
<th>dbSNP</th>
<th>Minor Allele</th>
<th>MA Gained TFBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1072A</td>
<td>rs3826777</td>
<td>A</td>
<td></td>
<td>SOX/SRY-sex/testis determining and related HMG box factors</td>
</tr>
<tr>
<td>A-670C</td>
<td>rs1273686</td>
<td>C</td>
<td></td>
<td>Fork head domain factors, Grainyhead-like transcription factors, Cellular and viral myb-like transcriptional regulators</td>
</tr>
<tr>
<td>C-514T</td>
<td>rs4802640</td>
<td>T</td>
<td></td>
<td>Twist subfamily of class B bHLH transcription factors, TALE homeodomain class recognizing TG motifs</td>
</tr>
<tr>
<td>C-270A</td>
<td>rs35018314</td>
<td>A</td>
<td></td>
<td>Cart-1 (cartilage homeoprotein 1), Lim homeodomain factors, GATA binding factors</td>
</tr>
<tr>
<td>A-34G</td>
<td>rs1152232</td>
<td>G</td>
<td></td>
<td>Vertebrate TATA binding protein factor</td>
</tr>
<tr>
<td>A321T</td>
<td>rs2241702</td>
<td>T</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>G1276T</td>
<td>rs892027</td>
<td>T</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>C1562T</td>
<td>rs11554772</td>
<td>T</td>
<td></td>
<td>MEF3 binding sites</td>
</tr>
</tbody>
</table>

The gain or loss of transcription factor binding sites (TFBS) induced by minor allele (MA) of SNPs (including genotyped polymorphisms and polymorphisms in correlation) defining 5 (*1-*5) \textit{ATF5} haplotypes, as obtained by Matinspector-Genomatix software, version 2.1 (http://www.genomatix.de). Ancestral allele is given in bold and minor allele is underlined.
CHAPTER VI
Unpublished results

A. *In vitro* approach

B. *In vivo* xenograft mouse model of human ALL

The results assessing *in vivo* approach using xenograft mouse model are presented in the article 2. Here are presented only the results of *in vitro* approach.

1. MTX targets genes, DHFR and TS:

   For *in vitro* assay 93 CEPH cell lines were tested. Results of cell viability test against MTX indicate that 42.6% of cell lines are sensitive, 23.4% are resistant to MTX treatment, whereas remaining had intermediate phenotype. In our experiment IC50 threshold for sensitive, intermediate and resistant status were <0.01, 0.01-0.1 and >0.1 µM respectively. Genotyping analysis showed that 9 (9.67%) carried *DHFR *1b haplotype, 26 (27.95%) had 3R3R TS genotype, 3 (3.22%) had combination of these genotypes and the remaining had no at risk genotypes. Association analysis between sensitivity status of LCLs and these genotypes revealed absence of association (Table 1). No association was seen if IC50 distribution was analyzed across genotype groups (*DHFR *1b haplotype, P= 0.6 and 3R3R TS genotype, P= 0.2).
Table 1: Association analysis between sensitivity status of LCLs and *DHFR* *1b* haplotype and *3R3R TS* genotype.

<table>
<thead>
<tr>
<th>Gene</th>
<th>genotype</th>
<th>Sensitivity status</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>1b haplotype</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHFR</td>
<td>0</td>
<td>Sensitive 36 (94.74%)</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Intermediate 29 (82.86%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant 19 (95%)</td>
<td></td>
</tr>
<tr>
<td>TS</td>
<td>3R/3R</td>
<td>Sensitive 26 (66.67%)</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Intermediate 25 (17.43%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Resistant 16 (84.21%)</td>
<td></td>
</tr>
</tbody>
</table>

2. ASNase pathway genes:

*In vitro* sensitivity test on 93 LCLs against E.coli ASNase indicates that 60.21% of cell lines were sensitive (IC50<0.3 IU), 13.98% were resistant (IC50>0.4 IU) to E.coli ASNase treatment and remaining cell lines had intermediate phenotype (Figure 1). The results assessing *in vitro* sensitivity to ASNase and ASNS polymorphisms are presented in the article 3. Here are presented only the results on *ATF5*. Genotyping analysis of *ATF5 T1562C* variation showed that 83 (93.25%) carried *CC*, 5 (5.61%) had *CT* and 1 cell line had *TT* genotype. Based on our experiment in lymphoblastoid cell lines in response to Ecoli ASNase, we did not find an association between *ATF5 T1562C* variation and sensitivity to ASNase, as determined by IC50 values (P= 0.2) (Table 2).
Figure 1: Frequency of sensitive, resistant and intermediate LCLs against E.coli ASNase.

Table 2: Results of association of IC50 distribution across *ATF5 T1562C* variation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sensitivity status</th>
<th></th>
<th></th>
<th></th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ATF5 T1562C</em></td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>CC</em></td>
<td>52 (92.86%)</td>
<td>23 (95.83%)</td>
<td>12 (92.30%)</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td><em>CT</em></td>
<td>3 (5.36%)</td>
<td>1 (4.17%)</td>
<td>1 (7.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>TT</em></td>
<td>1 (1.78%)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter VII

Discussion
Importance of pharmacogenomics approaches for childhood ALL leukemia:

The treatment of paediatric ALL has greatly improved due to the introduction of effective combination risk-adapted therapies. However, therapy resistance is still a major obstacle to successful treatment, whereas intensive treatment has also important short-term side effects and long-term consequences. Unfavourable clinical pharmacokinetics and pharmacodynamics as well as cellular drug resistance play a major role in ALL treatment failure [8, 9]. Candidate gene analysis and GWAS are two fundamental approaches that improved pharmacogenetic knowledge and gave an insight into the specific drug metabolism and signalling pathways in paediatric ALL [10]. Identification of polymorphisms modulating treatment response may lead to different treatment schedules, improving the efficacy of treatment and allowing for a reduction in drug side effects. Individualizing MTX and ASNase dose based on pharmacogenetic markers in childhood ALL patients, may lead to better cure rate compared with standard doses of these drugs [11]. Several steps are needed prior to the translation of pharmacogenetic marker to clinical practices. These include replication through an independent association studies, functional significance of identified polymorphisms, which is prerequisite for subsequent dose adjustment, and finally prospective clinical where the effect of genotype-based treatment adjustment is analyzed. The step of functional validation in ALL is complex, since childhood ALL treatment protocols include several chemotherapeutic agents. Findings in clinical setting reflect usually an association of a given polymorphisms with overall response to treatment, therefore an evaluation in the context of individual drug of these protocols is crucial for understanding the impact of pharmacogenetics markers [12].
**ALL as a model of cancer disease:**

ALL is the most frequent malignancy of childhood and a principal cause of cancer-related mortality in children due to a persistent group of patients that does not respond to standard treatment [8, 13, 14]. Pharmacogenetics approaches can help understanding genetic basis of relapse in ALL. Childhood ALL treatment protocols include chemotherapeutics which are generally given at doses near those that lead to severe toxicity. Thus, individualized therapy may reduce toxic episodes in susceptible patients [10]. Well known clinical symptoms and biological profile of ALL facilitate identification of genetic component influencing therapeutic responses [11]. Polymorphism in drug metabolising enzymes and targets, have been shown to affect drug pharmacokinetic and pharmacodynamic pathway in childhood ALL [11, 12]. However, despite increasing information obtained through pharmacogenetic studies, only polymorphisms in thiopurine methyltransferase (TPMT), an enzyme that catalyses S-methylation of mercaptopurine and thioguanine, have been introduced in clinical practice [15, 16] suggesting that much work still remains to facilitate transition to clinical implementation.
Lymphoblastoid cell lines (LCLs) and their utility:

The LCLs are Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines derived from B-lymphocytes. LCLs are promising model system to study the genetics of drug response and are useful tool for identifying polymorphisms related to drug sensitivity, including the sensitivity to anticancer agents. Importantly many data are publicly available for hundreds of established LCLs such as genome-wide genotype and gene expression data as well as data derived from next-generation sequencing (DNA and RNA-Seq) [17, 18]. Centre d'Étude du Polymorphism Humain (CEPH) families, International HapMap Project and Human Variation Panel are three main collections of LCLs established from healthy individuals of different origin. As a complement to clinical studies, LCLs can be used to investigate associations between genetic variation and chemotherapeutic susceptibility. Several cellular phenotypes can be measured to determine cellular sensitivity to a drug, including cytotoxicity, apoptosis, gene expression changes and intracellular concentration of the drug or metabolite. In several pharmacogenomics studies LCL model have been used to identify genes and variants that predict drug response and toxicity [18]. Genome-wide analysis has been successfully used to identify novel genetic variants predicting sensitivity to a variety of chemotherapeutics, including etoposide, cisplatin, carboplatin, daunorubicin, cytarabine and ASNase [19][93, 94]. Because of adverse side effects of anti-cancer drugs, there are no healthy volunteers for genetic studies. Likewise, due to narrow therapeutic index of many anticancer drugs, small change of dosage can be associated with extensive toxic response and in some cases could be fatal. Thus, challenge to study drug toxicity is one of
advantages of using LCLs in pharmacogenomics studies [18]. In addition, availability of different data sets allows overlaying them on the top of each other investigating at the same time genetic influences on gene expression and cellular phenotypes. LCLs offer the opportunity to test the finding via experimental manipulation and therefore begin to get at the underlying biology. LCLs are an unlimited resource and allow for the evaluation of toxic drugs in a controlled testing system [94]. There is nevertheless several disadvantages of working with LCLs for pharmacogenomics discovery. They are representing one cell type and the phenotype observed from in vitro experiments may not be recapitulated in vivo. Experiments with LCLs are also subject to in vitro confounders, such as Epstein-Barr virus (EBV) that can infects B-lymphocytes in vitro and affects the gene expression patterns of LCLs [21], growth rate differences between cell lines, and thaw effects. A disadvantage that is especially important to take into consideration for pharmacogenomic studies is that most LCLs lack expression of many CYP450 enzymes and several transporters; therefore, they are most useful for identifying the contribution of pharmacodynamics genes [94]. In spite of these limitations, LCL-based systems have been so far proven useful in advancing the field of pharmacogenomics.

In this project we used 93 CEU LCLs to access the role of particular variation in DHFR, TS, ASNS and ATF5 genes with in vitro sensitivity to ANSase and MTX.
Xenograft mouse model of ALL:

The xenograft mouse model gradually provides a stable, reproducible and clinically relevant model, used to study different malignancies and diseases [22, 23], including bone marrow mesenchymal stem cells transplantation and ALL [24, 25]. Two extremely immunodeficient mouse lines, the nonobese diabetic severe combined immune deficient (NOD/SCID) strain and NOD/LtSz-scid IL-2Rγc null (NSG) mouse have been developed, which can be humanized very effectively [25-27]. Lack of T, B, and NK cells in NSG mice due to a deficient common cytokine receptor g-chain, result in defective cytokine signalling of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [23, 28]. NSG mice bearing additional mutation on IL-2 receptor gamma chain (IL2ry) gene, lead to severe defect in natural killer (NK) cells [29, 30]. In a case of engraftment of leukemia cells in mice, the immune system of NSG mice can not reject injected cells and their immune system will have no influence on the outcome of leukemia, because of lack- of mature T, B and NK cells [23, 31, 32]. The recipient NSG mice will show the same clinicopathologic pattern and type of leukemia as seen in donor patients. Accordingly mice retain the fundamental biological characteristics of the original disease [22, 31-33]. Although leukemia cells developed in both NOD/SCID and NSG mice strains, NSG mice demonstrate superior engraftment of human cord blood, adult CD34+ hematopoietic stem cells and multilineage differentiation when compared with NOD/SCID [23, 25, 29]. Diamanti et al compared the engraftment of childhood T-ALL and B-cell precursor ALL cells in NSG and NOD/SCID strains as well as the phenotype and functional ability of cells capable of engrafting these mice. They concluded that engraftment of unsorted leukemia cells was 1.2 to 7.7-fold higher in NSG mice than in NOD/SCID mice,
regardless of subtype or risk stratification group [25]. Likewise, Agliano et al (2008) also demonstrated that human acute leukemia cells engraftment in NSG mice was accompanied with faster development of leukemia related symptoms and higher percentage of leukemia cells in blood, marrow and spleen of NSG mice [34]. The NSG strain is a more permissive host for engraftment of paediatric leukemia [25]. NSG mice showed higher engraftment levels, shorter length of time to engraftment, earlier presence of leukemia cells from blood and bone marrow compared to NOD/SCID strain [25, 28, 29]. Xenograft mouse model has been used for engraftment of patient’s leukemia cells and expansion of leukemia cells in NSG mice, has been shown by several groups [25, 27, 34-36]. Importantly, the recent study by Woiterski provided an evidence for a validity of such model to constitute a surrogate marker for clinical parameters. They performed an analysis in primary and serial engraftment using 54 primary paediatric B-ALL, myeloid leukemia (AML) and T-ALL samples. They characterized the leukemogenic profile and correlated engraftment kinetics with clinical outcome. PCR-based minimal residual disease marker expression and fluorescence in situ hybridization confirmed the presence of patient-specific genetic aberrations in mice. Analysis of genes known to be important in the leukemogenesis of all three diseases revealed that well-known tumor-regulating genes were expressed to a comparable extent in mice and men. Overall survival of NSG mice highly correlated with the individual prognosis of B-ALL, AML and T-ALL patients. These studies suggest that in vivo model provides a valuable preclinical tool to explore the heterogeneity of leukemic disease and exploit patient-tailored leukemia-targeting strategies [27]. This model has been also used to explore apoptosis in leukemia cells [37], cytotoxic effects of cord blood cytokine-induced killer (CB-CIK) cells or
interleukin (IL)-27 against human B-ALL [38]. Other haematological malignancies, such as AML were investigated with these models as well demonstrating disease recapitulation and self-renewal capacity [35].

**Importance of polymorphisms in DHFR gene:**

The antileukemia effect of methotrexate involves the inhibition of DHFR [2]. DHFR plays a critical role in the conversion of DHF to THF that are required for nucleic acid synthesis and methylation reaction [39]. The change in DHFR expression and activity caused by genetic polymorphisms may thus affect an individual predisposition to respond to the treatment in terms of efficacy and drug side effects. Recent pharmacogenetic studies in our lab showed an association of DHFR promoter polymorphisms with lower event-free survival and inferior ALL outcome. The polymorphisms might have affected the function of the non-coding transcript involved in DHFR expression regulation [2, 3]. Indeed, particular haplotype (named *1b, defined by particular allelic combination derived from six polymorphic site in the major and minor promoter of DHFR) increased mRNA levels possibly explaining an association with a higher risk of ALL relapse seen in the clinical setting [3]. In my research project I attempted to get further insight into the effect of DHFR polymorphism on MTX sensitivity using cellular proliferation assay. DHFR genotypes assessment carried on in 93 CEU LCLs identified DHFR *1b haplotype with similar frequency as obtained in ALL patients; 9 (9.67%) cell lines carried this haplotype. Analysis with an in vitro sensitivity to MTX in LCLs did not reveal significant association. Several attempts have been made to establish the role of DHFR expression in MTX resistance in childhood ALL and other diseases such as rheumatoid or psoriatic
arthritides. Matherly and coworkers (1995), have been shown that, DHFR overexpression is associated with resistant to MTX in childhood ALL patients [40]. Likewise, Goker and coworkers (1995) demonstrated that DHFR gene overexpression may be a significant cause of acquired MTX resistance in ALL patients with p53 gene mutations, which may lead to defective cell cycle control [41]. Based on estimation in 40 newly diagnosed patients with ALL, there was a correlation between unfavorable EFS and DHFR expression [42]. Matheson et al (2007) also reported low level amplification of DHFR in ALL patients both at presentation and relapse [43]. One of the polymorphisms studied by several groups is insertion/deletion (indel) of 19pb sequence in the first intron of DHFR gene. Ongaro et al, 2009 demonstrated that, deletion allele was associated with an increased risk of hepatotoxicity in adult ALL [44], whereas association of homozygosity for the insertion allele with thrombocytopenia in childhood ALL was reported by Salazar et al, 2012 [45]. Ongaro et al, (2009) and Vagace et al, (2011) reported that homozygosity for DHFR 19 bp deleted allele has been associated with increased hepatotoxicity in leukemia patients treated with MTX [44, 46]. In contrast, Dulucq et al, (2008) did not find such an association [2]. They identified linkage disequilibrium (LD) of 19bp indel with the promoter polymorphisms; several haplotypes carried either 19-bp ins or 19-bp del allele [2], which might explain some discrepancy observed among studies. Polymorphisms in DHFR gene have been investigated in other diseases treated with MTX. Some studies found an association of the polymorphism located several Kb downstream from the 3’UTR in patients with psoriatic and rheumatoid arthritis [39, 47, 48].
**Importance of polymorphisms in TS gene:**

TS plays a critical role in maintaining a balanced supply of deoxynucleotides required for DNA synthesis. Due to its major role in cell proliferation TS is a target of several chemotherapeutics including MTX. Change in TS expression in ALL patients has been reported [49, 50]. Our group conducted an association studies in ALL patients treated with MTX, and demonstrated that patients who are homozygotes for high activity TS 3R genotype had a reduced event-free survival (EFS) [51]. The similar observations were made by others. Rocha et al (2005) reported that TS 3R3R genotype was associated with a higher risk of hematologic relapse [52]. Similarly, Sepe et al (2012) found an association of TS 3R4R genotype and poor prognosis in ALL [53]. In contrast, the low activity 2R allele correlated with increased toxicity in ALL patients treated with MTX including more frequent osteonecrosis and stomatitis [54, 55]. TS polymorphism seems also to influence susceptibility to ALL. High expression 3R allele could limit DNA damage in rapidly dividing tissues that have greater requirement for DNA. Indeed, several studies showed as association of 3R allele with a reduced risk of leukemia suggesting potential protective role against childhood ALL development [56, 57].

We addressed the effect of TS polymorphisms on MTX sensitivity using *in vitro* and *in vivo* approach. Analysis of cellular proliferation assay in relationship to 3R3R genotype did not reveal significant association, whereas experiments conducted in mice seem to recapitulate well observation seen in clinical setting. Although engraftment of human ALL cells in NSG mice has been shown by us and others [25, 34-36], there was no evidence about the relevance of this model to investigate therapeutic implication of patient genotype and drug dose adjustment. We showed the relationship between the TS
3R/3R genotype and the resistance to MTX treatment in dose-dependent manner. Mice injected with blasts from TS+ ALL patients (with 3R3R genotype) were resistant to MTX dose of 0.25 and 1 mg/kg MTX, relative to mice injected with patients’ cells without this genotype (TS-) that responded well to the dose of 1mg/kg. Importantly, genotype-related resistance was bypassed by an increase in the MTX dose. Our results are in accordance with association studies in patients with childhood ALL showing relationship between 3R3R TS genotype and higher risk of relapse [51] strongly supporting the possibility that higher risk of relapse in TS 3R/3R patients is due to MTX resistance. These patients might need higher MTX doses for effective target inhibition. In order to define minimal increase in MTX dose that can overcome resistance related to TS 3R/3R genotype, we performed additional experiments in mice injected with blasts from TS+ ALL patients using MTX doses of 1, 1.5 and 2 mg/kg. Based on these results, the suppression of leukemia cell proliferation was initiated in NSG mice that were treated with dose of 2 mg/kg (Figure 1, unpublished results). The resistance status can be thus reversed with the doubling MTX dose, which might be of importance for intensification and maintenance phase of treatment of 3R3R positive ALL patients. Given that our association study did not detect a correlation between 3R3R genotype and higher risk of toxicity [58], increase in MTX dose might be justified, and prospective study with the dose adjustment according to the genotype could be considered as well. Some factors might influence our in vivo experiments in mice, such as environment and characteristics of mice. The environment within a mouse model is different from that in human leukemia; therefore, populations that form leukemia in mice may differ from those that contribute to disease progression in the patients [25]. Similarly, characteristics of mice, like for example
different sex of NSG, might influence the engraftment [27, 59, 60]. Our experiments included analysis in several patients and compared relative difference in MTX response between several mice injected with leukemia cells with and without TS 3R3R genotype (five mice each), minimizing the impact of mice environment. The analysis carried at different point of experiments revealed that leukemia cells expanded in mice retain the same B cell phenotype and same TS genotype as in patients they originated from. To further address whether the significant differences observed, are MTX specific, we plan to test response of mice with the same TS genetic background to other drugs used in ALL treatment, like for example vincristine, which does not employ same action pathway.

There are several reasons for the discrepancy seen between in vitro and in vivo model. There is a significant difference in MTX exposure between these models. In in vitro approach, cell lines are continuously exposed to MTX, whereas in in vivo models, the MTX is undergoing pharmacokinetic and dynamic changes, which more similarly reflect changes in patients [22]. In regard to other TS polymorphisms a G to C substitution was described in the second repeat of 3R allele that may further modulate TS expression [1, 61]. C allele seems to abolish the binding site for upstream regulatory factor resulting in different pattern of TS expression that has been proposed based on this additional polymorphism: high (3RG3RG), median (3RG3RC and 2R3RG) and low TS expression profile (2R2R, 2R3RC and 3RC3RC) [61, 62]. Our group has studied the effect of substitution in 3RG allele on ALL outcome [1]. Although the 3RG3RG individuals had lowest EFS, this reduction was not significantly different compared to other 3R3R subgroups [1]. And finally, an association of TS 28bp repeat polymorphisms with other malignancies and diseases was reported in several studies. Increased risk of colorectal
cancer among TS 3R/3R individuals [63]; and association of 3R3R genotype with poor prognosis in breast and gastric cancer was reported. Colorectal cancer patients with TS 3R3R genotype who were treated with 5-FU, presented a poor prognosis [64-67]. Similar was noted in rheumatoid patients (RA) treated with MTX. Kumagai et al (2003), demonstrated that RA patients who are homozygous for the 3R allele require higher dose of MTX compared to those with 2R allele [68].
Figure 1: MTX increase dose to overcome resistance in NSG mice injected with blasts from TS+ ALL patients: Additional experiments with different doses (1, 1.5 and 2 mg/kg). The suppression of leukemia cell proliferation starts in NSG mice injected with blasts from TS+ ALL patients, treated with 2 mg/kg MTX.
**ASNase-related polymorphisms in childhood ALL and personalized ASNase therapy:**

ASNase is a critical component of ALL treatment whose anti-leukaemia effect is related to asparagine depletion. Differences in susceptibility to asparaginase correlated to variable levels of *ASNS* expression in different studies. Increase of *ASNS* level in leukemia cell lines, patient’s lymphoblasts and surrounding mesenchyme cells have been reported [69-72]. The increase in *ASNS* levels can counteract the ASNase effect leading to subsequent resistance to ALL treatment, whereas lower *ASNS* expression can mediate higher sensitivity to treatment leading to higher frequency of ASNase-related complications [5]. Some studies conducted in clinical setting reported that an elevation of *ASNS* expression was predictive of inferior disease outcome [4]. Stams et al found that ASNase-resistant, *TEL-AML1*-negative B cells which are usually associated with inferior prognosis, had significantly higher *ASNS* expression compared with *TEL-AML1*-positive B-lineage ALL [73]. There are nevertheless other studies which did not find an association between an expression of *ASNS* and response to ASNase [74]. Interesting observation has been made by Iwamoto et al who have shown that BM-derived mesenchymal cells express much higher levels of ASNS compared with leukemic lymphoblasts and may mediate resistance to ASNase [69]. Wing, et al contradicted this finding by reporting absence of ASNS association with an increase of asparagine level in bone marrow of patients [70]. Our lab focused on the polymorphisms in ASNase action pathway, particularly on the genes that were shown by microarray profiling as differentially expressed between ASNase sensitive and resistant cells [71, 72, 75-78]. Association of these polymorphisms with event free survival (EFS) in childhood ALL
patients revealed event predisposing role of several genetic variations, particularly tandem-repeat ASNS polymorphism and T1562C variation in ATF5 gene, transcription ASNS regulator [4]. Based on these results, the T1562 variation was associated with reduced EFS in ALL in both the test and the replication cohorts, and was limited to patients who received Escherichia coli asparaginase which might be related to higher asparaginase activity. Indeed, E coli asparaginase treatment has been shown to have higher efficacy and toxicity compared with Erwinia-ASNase [4]. ATF5, as a transcriptional activator of ASNS, binds to the nutrient-sensing response unit of the ASNS gene and activate ASNS transcription after nutrition deprivation [79]. ATF5 C1562T is located in the regulatory 5′UTR region and may affect promoter activity and mRNA levels or translation ATF5 efficiency, which is regulated by alternatively spliced 5′UTR α and β [80]. Our analysis indicated that ATF5 T1562 allele confers higher ATF5 promoter activity. Mechanism behind this association could have been proposed: more important asparagine depletion caused by E.coli ASNase leads to higher ATF5 up-regulation which is further potentiated in individuals with high-activity allele, such as T1562, leading to resistance to ASNase [4]. Our experiment in lymphoblastoid cell lines in response to ASNase treatment meant to establish this mechanistic relationship. However no association between ATF5 T1562C variation and sensitivity to ASNase, as determined by IC50 values, was found which might be due to the number of reasons: discrepancy between in vitro and in vivo finding, concomitant role of other medications administered or contribution of surrounding mesenchymal cells in clinical setting.
Considering *ASNS* gene variations, tandem repeat polymorphism with most frequent double (2R) and triple repeat (3R) alleles was also associated with lower EFS. Particularly, haplotype harbouring 2R allele (arbitrarily named haplotype *1*) was associated with reduction in EFS [4]. Our analysis of polymorphisms in ASNase pathway with ASNase-related complications revealed also the implication of tandem repeat polymorphism. Patients who were homozygous for 3R allele was more susceptible to pancreatitis and allergies [5], whereas carriers of haplotype *1* had lower frequency of these complications. This is in accordance with results of Rousseau et al (2011) which demonstrated the association of the same haplotype with reduced EFS, suggesting all together lower sensitivity of this haplotype in response to ASNase treatment [4, 5].

Further analysis of additional polymorphisms in *ASNS* gene revealed diversification of haplotype *1* in 5 subtypes; two polymorphisms (rs10486009 and rs6971012,) defining particular haplotype subtypes correlated with *in vitro* sensitivity to ASNase. ASNase sensitivity *in vitro* seems to be driven by minor allele of rs10486009 possibly providing mechanistic explanation for lower sensitivity of haplotype *1*, observed in clinical setting. In contrast, only one haplotype defined by 3R allele remained after addition of other *ASNS* polymorphisms and it did not affect *in vitro* sensitivity to ASNase. Akagi et al (2009) demonstrated that the tandem repeat sequences in *ASNS* gene functions as a transcriptional element. They have shown by luciferase reporter assay performed in embryonic kidney cell lines, that the number of tandem repeats increase promoter activity [81]. In accordance with this, Pastorrczak et al (2014) showed worse response in 3R3R ALL patients, particularly in early phases of treatment protocol following one ASNase dose [82]. Our finding does not support these observations, since 3R3R genotype was
rather associated with higher sensitivity to treatment. We did not notice an association of this genotype with mRNA levels [4] or in vitro sensitivity to ASNase [5]. It is possible that the DNA copy number alteration is not causing ASNS up-regulation in all tumours, and it might be that other mechanisms are involved in ASNS overexpression as well [83]. For example, it has been shown that high ASNS levels in TEL/AML1 [+ ] patients correlate with better prognosis compared to other subtypes of ALL [84]. Mechanism of mutant p53-mediated oncogenesis has been demonstrated by several studies [83, 85]. P53 mutants with the gain of function properties, can bind to the ASNS promoter and induce ASNS expression [83, 85]. Increased prevalence of mutated p53 in advanced castration-resistant prostate cancer (CRPC) and its association with ASNS overexpression was reported by Sircar and coworkers (2012) [83]. It would be possible that this mechanism plays a role in childhood ALL leukemia patients as well. Another mechanism of ASNS overexpression could be associated with the relative glucose and asparagine amino acid deficiency which exists in metabolically active advanced tumours. The glucose and asparagine amino acid deficiency in advanced tumours such as CRPC might as well lead to ASNS up-regulation [83]. To overcome ASNS overexpression due to glucose and asparagine amino acid deficiency, using ASNS inhibitors paired with ASNase, would be potent strategy in many tumours. In another study related to asparaginase hypersensitivity, Chen et al (2010) identified through genome-wide interrogation an association of genetic variations in the GRIA1 (glutamate receptor, ionotropic, AMPA 1) on chromosome 5q33 with risk of asparaginase allergy in ALL patients [86].
**Perspective of personalized therapy in ALL:**

MTX is an antifolate drug that acts through inhibition of DHFR and TS interrupting folate homeostasis. MTX was introduced as antileukemic drug about 60 years ago and still remains an important component of ALL treatment protocols. In spite of a number of studies that evidenced the influence of genetic polymorphisms of folate metabolizing enzymes on MTX response [2, 3, 51], pharmacogenetic tests to predict the outcome of MTX treatment are not yet available. There are several reasons for the lack of personalized MTX treatment:

1. The large number of observations was limited to single center cohorts, without replication sets.
2. Small numbers of patients due to disease prevalence and long duration of ALL treatment protocol may reduce power of study increasing possibility of false positive associations.
3. Considering that MTX exerts its action via folate pathway, these effects could be changed by patient’s folate level. Accordingly there are no evidences considering patient’s folate level through pharmacogenetic study on ALL.
4. ALL treatment protocols are composed of multiple drugs with overlapping toxicities leading to difficulties in assigning these complications to MTX (or to one particular drug).
5. Different populational background of patients enrolled in studies could increase chance of bias and make difficult comparison of results between studies.
6. Different dose of MTX and administration schedule among studies due to different ALL treatment protocol could modulate an association between polymorphisms and treatment outcome limiting the reproducibility of studies.

7. Given complex pathway of MTX action few SNPs are not sufficient to explain MTX-related resistance and adverse effects. Other variants in the same gene and epistatic interactions with other genes should be considered and analyzed.

8. The association between polymorphism of folate metabolizing enzymes and MTX responses may be affected by other genes and their variations such as those involved in DNA repair or apoptosis. Most of pharmacogenetic studies focused only on polymorphisms of the folate metabolizing enzymes, which may increase false positive results [87]. Similar conclusion can be made for ASNase pharmacogenomics. Several other strategies may assist further pharmacogenetic studies such as i) understanding the mechanism of drug resistance through molecular profiles of leukemia cells in order to identify genes which affect sensitivity to ALL drugs [10, 87]. ii) Increase a number of studies that analyze an association with the MRD status, which was shown by many studies to help identifying patients who are at increased risk of relapse or death. iii) Pharmacogenomics studies with large sample sizes and replication cohorts are necessary to create stability and consistency in quantifying association between genotype and drug response [87-89]. iv) Pharmacogenetic models should include both genetic and clinical factors which can together better predict the disease outcome. v) Improve our understanding on the functionality of genetics variants. vi) Conduct studies which will monitor drug efficacy/toxicity in relation to polymorphisms in prospective manner. vii)
Genome-wide association studies approach may offer an alternative and unbiased approach conditional on the availability of large cohorts [87].

**Impact of ALL pharmacogenomics:**

In spite of extensive investigation regarding pharmacogenomics biomarkers, the potential and utility of pharmacogenomics in clinics, especially in case of ALL has be lowered than expected [90, 91]. Most of predictions may come true, but many years later than expected. Among all market chemotherapeutics, only 1-2% has pharmacogenomic-based recommendations. Among drugs used in childhood ALL protocol treatment only 6-MP, has a recommendation for TPMT genetic test which can help individualized therapy adapting initial doses based on TPMT genotypes [90-92]. For the other components of ALL treatment protocols, the data are not sufficiently consistent or, are only emerging to allow specific recommendations [91]. Many of them await replication, including applicability to different treatment protocols and different populations [10].

In order to identify genetic component of treatment response combination of mutations in several genes, is more helpful than single gene approach, especially in case of drugs with complex intracellular pathway [46, 91]. The scenario of ALL pharmacogenetics is still complex and requires further research to identify whole array of genetic factors involved in drug efficacy and toxicity in childhood ALL patients. This will ultimately lead to precise risk stratification and personalized medicine by translating and integrating molecular tools into clinical practice. Some other important factors such as identification of leukemia specific genetic profile (somatic mutations), cytogenetics of leukemia cells, gene expression studies and minimal residual disease investigation can shed a light on the road of ALL pharmacogenomics research.
Limitations:

The results of this thesis need to be considered in the context of several potential limitations. First, there was limited number of patients whose leukemic cells were available for engraftment in mice, for which reason we could not investigate by in vivo model the effect of DHFR and combined TS and DHFR genotypes. Although xenotransplantation model following engraftment of acute lymphoblastic leukemia cells with thymidylate synthase gene polymorphisms in NSG mice was successful, there are nevertheless several limitations. For example due to limited access for NSG mice of different sex, we used both female and male mice in the same experiment. Based on recent evidence, sex associated factors may play a role in growth and proliferation of engrafted leukemic cells in mouse model [27, 59, 60], although the data are not consistent. Some studies showed that engraftments is faster in male recipient compared to female mouse model [27, 59] whereas others showed that, female mouse model has instead faster engraftment [60]. Considering LCLs, in spite of advantages of their use in pharmacogenomics studies, there are also drawbacks, these include difference in drug-induced effects due to cell type or the influence of non-genetic factors such as baseline growth rates, EBV copy numbers and ATP levels which may affects drug-induced phenotypes in LCLs [17, 18]. Other cells models also used in pharmacogenetic studies are fibroblasts and peripheral blood mononuclear cells; nevertheless they have no extensive genetic information available like LCLs [18]. Therefore illustrating the complexity of drug effects in the human body requires more than a single-model system.
**Conclusions:** In conclusion, this project provided an explanation for the mechanism behind association of *TS* and *ASNS* genotypes with therapeutic response in ALL. Although both *in vitro* and *in vivo* approach used in this thesis have their own inherent limitations, they have been shown useful to i) understand how variability in *ASNS* gene modulate sensitivity to ASNase treatment and ii) to confirm through *in vivo* model the *TS* genotype-driven resistance to MTX. This may have an implication for genotype-based drug dose adjustment in order to decrease risk of relapse and drug-related toxicity in patients undergoing ALL treatment. Further work is needed to get an insight into the role of *DFHR* and *ATF5* variations. Similar mouse model as one described for *TS* should be evaluated to define how these polymorphisms modulate sensitivity to MTX and ASNase. Chemotherapy response in dose-dependent manner and through data normalization showed by our NSG mice experiments suggested that this model can serve as a preclinical tool to explore individualized treatment options. The knowledge acquired through this work, can be useful for optimal treatment selection or tailoring therapy based on individual genetics in childhood ALL, but is also important for other haematological malignancies, using MTX and ASNase for treatment. Likewise, this model will allow validation of the variants in the genes controlling the action of other drugs used in ALL treatment that are or will be detected through pharmacogenetics studies of ALL. Finally, by anticipating that pharmacogenomics approaches and technology continue to improve, molecular genetic profiles associated with drug sensitivity in both ALL patients and their leukemia cells, will ultimately lead to better risk stratification of patients and individualized therapies, resulting in better health outcomes for children with ALL.
References:


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