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Predictors of Treatment Toxicity and Prognosis in Pediatric Acute Lymphoblastic Leukemia in Belgian and Vietnamese Populations

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List of Abbreviations

Abbreviations were used in this thesis:

5-MeTHF: 5-methyltetrahydrofolate

5-MeTHFp: 5-MeTHF polyglutamates

6-MP: 6-mercaptopurine

6-TGDP: 6-thioguanine diphosphate

6-TGTP: 6-thioguanine triphosphate

6-TXMP: 6-thioxanthosine monophosphate

6-MMPR: 6- methylmercaptopurine ribonucleotides

ABC-transporters: ATP-binding cassette transporters

ABCB1: ATP-binding cassette sub-family B member 1

AICAR-Tase: aminoimidazolecarboxamide ribotide transformylase

ALL: Acute lymphoblastic leukemia

AO: Aldehyde oxidase

ATP: Adenosine triphosphate

AZA: Azathiopurine

BFM: Berlin-Frankfurk-Münster

BM: Bone marrow

CCND1: Cyclin D1

CCR5: chemokine receptor

CDKs: Cyclin-dependent kinases

cDNA: Complementary deoxyribonucleic acid

CKIs: CDK inhibitors

CNS: Central nervous system

CSF: Cerebrospinal fluid

CTMA: Centre de Technologies Moléculaires Appliquées

CYP2C9: Cytochrome P450 2C9

DHFp: Dihydrofolate polyglutamate

DHFR: Dihydrofolate reductase

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

DPK : Diphosphate kinase

dTMP: Deoxythymidine monophosphate

dTTP: Deoxythymidine triphosphate

dUMP: Deoxyuridine monophosphate

EM: Extramedullary

eNOS: Endothelial nitric oxide synthase

FAB classification: French- American-British classification

FDA: Food and Drug Administration

FPGS: Folylpolyglutamate synthetase

FRALLE: French acute lymphoblastic leukemia

GAR-Tase: Glycinamide ribonucleotide transformylase

HD-MTX: High dose methotrexate

HGPRT: Hypoxanthine guanine phosphoribosyltransferase

HR : Hazard ratio

IMP: Inosine monophosphate

IMPDH : Inosine monophosphate dehydrogenase

INK4: Inhibitors of kinase 4

IT: Intrathecal

ITP: Inosine triphosphate

ITPA: Inosine triphosphatase

IV: Intravenous

IVC: Intravenous continuous

GD : Guanine deaminase

GGH: ɣ-glutamyl hydrolase

LD-MTX: Low dose methotrexate

LLA : La leucémie lymphoblastique aiguë

MDR: Multi-drug-resistance protein

MGRS: Multilocus genetic risk score

MP: Mercaptopurine

MPK: Monophosphate kinase

MRD: Minimal residual disease

mRNA: Ribonucleic acid messenger

MTHFR: Methylenetetrahydrofolate reductase

MTX: Methotrexate

MTXPGs: Methotrexate polyglutamates

PCR: Polymerase chain reaction

pRb: Retinoblastoma protein

RBC: Red blood cell

RFC: Reduced folate carrier

RFS: Relapse-free survival

RNA: Ribonucleic acid

SC: Subcutaneous

SNC: Système nerveux centrale

SLC19A1: Solute Carrier Family 19, member 1

SNP: Single base polymorphism

TIDP: Thioinosine diphosphate

TIMP : Thioinosine monophosphate

TITP: Thioinosine triphosphate

TG: Thioguanine

TGMP : Thioguanine monophosphate

TGNs: Thioguanine nucleotides

TPMT: Thiopurine methyltransferase

TS: Thymidylate synthase

TSER: TS promoter enhancer region

TXMP: Thioxanthylic acid

TYMS : Thymidylate synthase

UCL: Université catholique de Louvain

ULB : Université libre de Bruxelles

UPNT : University of Medecine Pham Ngoc Thach

WBC: White Blood Cell

XO : Xanthine oxidase

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Predictors of Treatment Toxicity and Prognosis in Pediatric Acute Lymphoblastic Leukemia in Belgian and Vietnamese Populations

Summary

Acute lymphoblastic leukemia (ALL) is the most common cancer diagnosed in children, representing nearly one-third of all pediatric cancers. Although current treatment protocols can cure approximately 80% of pediatric ALL patients, a number of children will relapse, with a relapse rate shown to vary within a group of patients with similar risk even though their leukemia blast carry the same characteristics. Indeed, genetic variations involved in drug metabolism could, at least partially, be responsible for heterogeneous responses to standardized leukemia treatments, hence requiring more personalized therapy. Pharmacogenetics investigates genetic variations that affect pharmacokinetics and pharmacodynamics of drugs by changing the structure of proteins involved in drug metabolism and cellular transport, and their influence on drug response phenotypes.

The methotrexate (MTX) and 6-mercaptopurine (6-MP) are an essential part of the multi-drug regimens used for successful treatment of childhood ALL. Although MTX and thiopurines have been in use for over sixty years, there is significant uncertainty associated with their use, which is mainly caused by inter-individual differences in the bioavailability and metabolism of these extensively metabolized drugs.

Study I:

We compared the relapse free survival (RFS) in Vietnamese (n=141) and Caucasian (n=94) children with ALL living in Vietnam and Belgium, respectively, and treated by the same FRALLE 2000 protocol.

RFS was significantly worse in Vietnamese children (hazards ratio=4.48; 95% confidence interval [CI], 2.16-9.3; P<0.01). The 5-year RFS was 83.8% (95% CI, 76.3%-92.0%) and 47.8% (95% CI, 35.6%-64.2%) for Caucasian and Vietnamese children, respectively. In Vietnamese group, relapses occurred in bone marrow (BM) and cerebrospinal fluid (CSF) at a much earlier stage.

Several factors may contribute to the poor RFS in Vietnamese children, which include the time interval before the first intrathecal therapy and differences in the management of drugrelated toxicity. However, additional contribution of socioeconomic factors and/or variations in pharmacogenetic polymorphisms in Vietnamese patients cannot currently be ruled out.

Study II:

The aims of this study were to (a) to determine the prevalence of seven common genetic polymorphisms (cyclin D1 (CCND1) G870A, ɣ-glutamyl hydrolase (GGH) C452T, inosine triphosphatase (ITPA) C94A, methylenetetrahydrofolate reductase (MTHFR) A1298C, MTHFR C677T, thymidylate synthase (TSER), and thiopurine methyltransferase (TPMT)) including those that affect the folate and thiopurine metabolic pathways between two clinical series and (b) to assess the impact of seven genetic polymorphisms on RFS between Vietnamese clinical series (n= 141) and Caucasian (n=94) clinical series.

The prevalence of *MTHFR*-677TT genotype was significantly higher in Caucasians (P=0.008), in contrast to the prevalence of *TYMS-TSER*3R/3R* and *ITPA*-94AA/AC genotypes which were significantly higher in Vietnamese (P<0.001 and P=0.02, respectively). Compared with children with a low MGRS (≤3), those with a high MGRS (≥4) were 2.06 (95% CI = 1.01, 4.22; P = 0.04) times more likely to relapse. Adding MGRS into a multivariate Cox regression model with race/ethnicity and four clinical variables improved the predictive accuracy of the model (AUC from 0.682 to 0.709 at 24 months).

Including multilocus genetic risk score (MGRS) into a clinical model improved the predictive accuracy of short and medium term prognosis, hence confirming the association between well determined pharmacogenotypes and outcome of paediatric ALL. Whether variants on other genes associated with folate metabolism can substantially improve the predictive value of current MGRS is not known but deserves further evaluation.

Résumé

La leucémie lymphoblastique aiguë (LLA) est le cancer le plus fréquent chez l'enfant et représente environ un tiers d'entre eux. Même si de nos jours, les protocoles de traitement parviennent à guérir près de 80% des enfants atteints de LLA, un certain nombre d'enfants rechutera, avec un taux de rechute qui varie, alors que nous sommes en présence d'un groupe de patients homogène au niveau des facteurs de risque et avec des caractéristiques identiques au niveau des blastes leucémiques. Des variations au niveau des gènes impliqués dans le métabolisme des médicaments pourraient en partie être responsables de cette hétérogénéité de réponse à un traitement standard, nous encourageant à évoluer vers des traitements plus personnalisés. La pharmacogénétique étudie les variations génétiques qui influencent la pharmacocinétique et la pharmacodynamique des drogues, en modifiant la structure des protéines impliquées dans le métabolisme médicamenteux et leur transport cellulaire ainsi que leur influence sur la réponse thérapeutique.

Le MTX et 6-MP sont essentiels dans les conditionnements impliquant des multiples chimiothérapies, nécessaires pour l'obtention de succès thérapeutiques dans la LLA. Même si le MTX et les thiopurines ont été utilisés depuis plus de soixante ans, il existe une certaine incertitude liée à leur utilisation, essentiellement due aux différences entre individus au niveau de la biodisponibilité et du métabolisme de ces drogues.

Premier article :

Dans notre première étude, nous avons comparé la survie sans rechute d'enfants Vietnamiens (n=141) et d'enfants Caucasiens (n=94) atteints de LLA, vivant au Vietnam et en Belgique et traités selon le même protocole FRALLE 2000.

La survie sans rechute était significativement plus mauvaise chez les enfants Vietnamiens (hazards ratio=4.48; 95% confidence interval [CI], 2.16-9.3; P<0.01). La survie sans rechute à 5 ans est de 83.8% (95% CI, 76.3%-92.0%) et 47.8% (95% CI, 35.6%-64.2%) pour les enfants de race blanche et Vietnamiens respectivement. Dans le groupe d'enfants Vietnamiens, la rechute au niveau médullaire et du système nerveux centrale survient de façon plus précoce.

Plusieurs facteurs peuvent intervenir dont le temps écoulé entre le diagnostic et la première injection intrathécale ainsi que des différences d'adaptation de traitement au niveau de la

toxicité médicamenteuse. De plus des facteurs socio-économiques et/ou des différences de polymorphismes pharmacogénétiques ne peuvent être exclus et ont mené à l'étude suivante.

Deuxième article :

La deuxième étude vise (a) à analyser la prévalence de sept polymorphismes génétiques (CCND1 G870A, GGH C452T, ITPA C94A, MTHFR A1298C, MTHFR C677T, TSER, and TPMT) y compris ceux qui influencent le métabolisme des folates et thiopurines entre les deux groupes et (b) à évaluer l'impact de ces polymorphismes sur la survie sans rechute des enfants Vietnamiens et Caucasiens.

La prévalence du génotype *MTHFR*-677TT était significativement plus élevée chez les Caucasiens (P= 0.008) par rapport aux génotypes *TYMS-TSER*3R/3R* et *ITPA*-94AA/AC qui étaient significativement plus fréquents chez les Vietnamiens (P<0.001 et P=0.02). Comparés aux enfants avec un MGRS bas (≤3), ceux qui ont un MGRS élevé (≥4) ont 2.06 fois plus de risque de rechute (95% CI = 1.01, 4.22; P = 0.04).

Le fait d'ajouter MGRS dans un modèle de régression multivariée de Cox au facteur race/groupe ethnique et à quatre variables cliniques augmente la précision du modèle (AUC de 0.682 à 0.709 au 24 mois) au niveau du pronostic à court et moyen terme, confirmant l'association entre certains pharmacogénotypes et le pronostic de la LLA chez l'enfant. Reste à déterminer si l'adjonction de l'analyse d'autres gènes associés au métabolisme des folates peut encore améliorer la valeur prédictive des MGRS telle que présentée ici.

1. Introduction

1. Introduction

Acute lymphoblastic leukemia

ALL is a fast-growing cancer of a type of white blood cells (WBC) called lymphocytes. These cells are found in the BM and other parts of the body. ALL occurs when the body produces a large number of immature lymphocytes. The cancer cells grow quickly and replace normal cells in the BM. BM is the soft tissue in the centre of bones that helps form all blood cells. Leukemic cells accumulate in the BM, replace normal blood cells and spread to other organs including liver, spleen, lymph nodes, central nervous system (CNS), kidneys and gonads. ALL prevents healthy blood cells from being made. Life-threatening symptoms can occur.

Cause, incidence and risk factors

Most of the time, no clear cause can be found for ALL. But the following may play a role in the development of leukemia in general:

- Chromosomal abnormalities
- Exposure to radiation, including x-rays before birth
- Past treatment with chemotherapy drugs
- Receiving a BM transplant
- Toxins such as benzene

The following increase the risk of ALL:

- Down syndrome or other genetic disorders
- A brother or sister with leukemia

This type of leukemia usually affects children aged 3 to 7. ALL is the most common childhood cancer, but it can also occur in adults.

Acute leukemias represent a clonal expansion and arrest at a specific stage of normal myeloid or lymphoid hematopoiesis (Hoffbrand and Mehta 2005). The leukemogenic event may occur in committed lymphoid cells of B- or T-cell lineages or in early precursors, which gives rise to the different subtypes of ALL based on the stage of lymphoid differentiation of the cell in which the event occurred (Reaman 2002). About 80% of all cases of ALL express cell-surface markers indicative of a precursor B-cell lineage. Only 1% to 2% of cases express a phenotype typical of a mature B cell. T-cell ALL accounts for about 15% to 20% of cases and is commonly associated with features at diagnosis, such as older age, male predominance,

high WBC and extramedullary (EM) disease, all of which indicate the need for increased intensity of chemotherapy (Pizzo, Poplack et al. 2006).

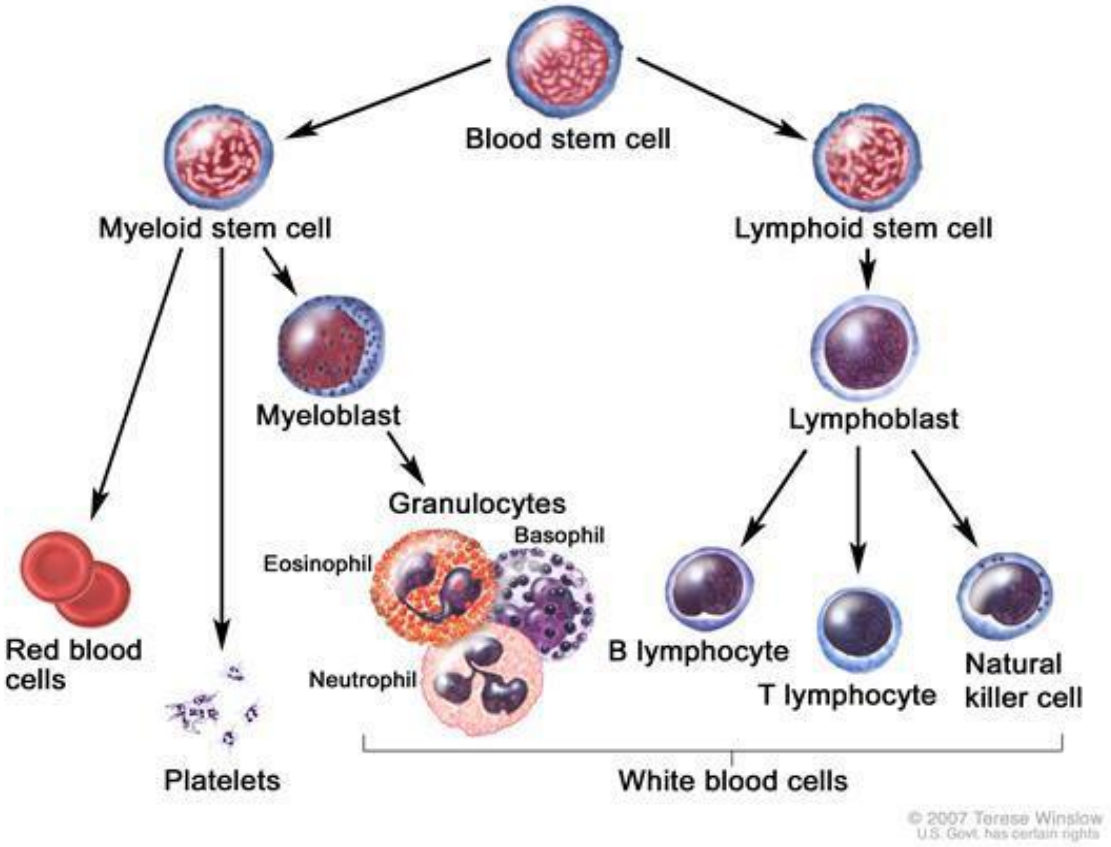


Figure 1: Blood cell development. A blood stem cell goes through several steps to become a red blood cell, platelet, or white blood cell (National Cancer Institute USA., 2010).

Bone marrow is found in the center of most bones and has many blood vessels. There are two types of bone marrow: red and yellow. Red marrow contains blood stem cells that can become red blood cells, white blood cells, or platelets. Yellow marrow is made mostly of fat. A myeloid stem cell becomes one of three types of mature blood cells: red blood cells, white blood cells and platelets. A lymphoid stem cell becomes a lymphoblast cell and then one of three types of lymphocytes (white blood cells): B lymphocytes, T lymphocytes and natural killers cells.

Cure rates of B-cell–progenitor ALL among children are very high, but they are not as high among adults. Approximately one fifth of children with B-cell progenitor ALL have recurring disease, which is difficult to treat. Thus, this form of ALL varies with respect to the potential for relapse and resistance to chemotherapy. It is generally accepted that B-cell–progenitor ALL originates in B-cell restricted progenitors that have accumulated critical genetic lesions.

Cytogenetic studies of B-cell–progenitor ALL have revealed aberrations in chromosomal sites of regulatory molecules that are implicated in signaling and transcriptional regulation (Greaves and Wiemels 2003).

Multiple genetic hits to deoxyribbonucleic acid (DNA) are necessary to cause cancer and when cancer strikes in childhood it is probably due to only a few severe genetic hits. Thus in childhood ALL we see the same genetic alterations in more than half of the children (hyperdiploidy 35% and t(12;21) translocation 25%). However these alterations alone are not enough to cause cancer; 0.5% of adults harbour the t(12;21) translocation and have never had childhood ALL (Olsen, Madsen et al. 2006).

We do not know the cause of the initiating genetic alteration. Suggestions include chemicals, ionising radiation, food, smoking, past chemotherapy, and delayed infection. The hypothesis of delayed infections is based on the theory that early exposures to common infectious agents are required for the proper maturation of the immune system, lack of these exposures results in aberrant responses when children are finally in contact with the agent. ALL develops in the biological context of an aberrant immune response due to delayed infections, and thus, the infectious agents are only an indirect trigger of the leukemogenic process. The latter hypothesis (Hayashi, Fujimaki et al. 2007) is supported by a study showing that children in day-care have reduced risk of childhood cancer compared to children with less contact to other and thus less exposure to infections (Urayama, Buffler et al. 2010). In the industrialized parts of the world, we see more cases of childhood ALL; this could also support Greaves's hypotheses as children in the Western world have fewer infections due, for example, to vaccinations programs.

ALL in children is a rare disease, but devastating when it occurs. It offers a unique treatment challenge to the pediatrician. Not long ago this diagnosis was tantamount to a death sentence, but today the disease is curable in the majority of patients treated with effective chemotherapy, in combination with intensive supportive care. As survival has improved, it has become increasingly possible to define both inherent and treatment-related adverse prognostic features. The treatment has advanced from use of single agents to combination therapy, and today it is complex and multidisciplinary.

The prognosis has improved considerably in the last two decades (Schrappe, Camitta et al. 2000). In the Nordic countries the overall event-free survival has risen from 57 to 75%. Children without unfavourable features have benefited most strikingly from the

intensification of therapy (event-free survival is today 80-85%). As they constitute almost 70% of all patients, the improvement has made a great impact on the overall result. However, in children with high-risk ALL, the progress has only been modest. The relapse rate has of course decreased in parallel with the improving results, but the prognosis after relapse has not improved during the above mentioned period. Only about 25-30% of children who relapse will reach and remain in a second remission. Cytotoxic treatment in FRALLE 2000 protocol for childhood ALL is divided into four essential phases: induction, consolidation, intensification and maintenance phase.

Both molecular and immunological criteria are widely used in contemporary treatment protocols.

The FRALLE 2000 criteria for standard and high-risk ALL form a good basis for comparing the treatment results of different protocols, but they are not sufficiently specific for modern stratification of treatment. Stratification of treatment aims at finding those children who need intensive treatment, but on the other hand, also those whose treatment could be less intensive and thus lead to less adverse effects.

So, general aims of the study were to find new possible predictive factors in childhood ALL, and in the context of contemporary intensive treatment, to assess the value of established predictive factors for adverse effects of MTX and 6-MP in treatment.

Epidemiology

Until the 1940's there were no effective treatments for leukemia in children. However after recognising that folic acid seemed to accelerate the disease, Sidney Farber introduced the folate antagonist, aminopterin, into the treatment in 1948 and was the first to induce remission in children with ALL (Miller 2006). Today we individualize treatment in childhood leukemia based on pharmacology, cytomorphology, immunology, cytogenetics and molecular biology (minimal residual disease (MRD)) (Whitehead, Vuchich et al. 1992, Barredo, Synold et al. 1994, Synold, Relling et al. 1994, Evans, Relling et al. 1998, Coustan-Smith, Sancho et al. 2000) and pharmacogenetics as mentioned earlier with the example with TPMT and 6-MP.

ALL is the most common cancer in children, accounting for 75-80% of all acute leukemias in children. Approximately half to two-third of ALL cases occur in children. An incident peak is seen between 2 and 5 years among children living in the Western world (Greaves 2006).

Every year approximately 180-200 children are diagnosed with ALL in the Nordic countries (Denmark, Finland, Iceland, Norway and Sweden) (Gustafsson, Schmiegelow et al. 2000); and 110-125 children are diagnosed with ALL in Pakistan (2000-2004) (Yasmeen and Ashraf 2009).

Compared to the total cancer burden in Belgium, childhood cancer comprises less than 1%. Every year, about 320 children (0-14 years) and 175 adolescents (15-19 years) are diagnosed with cancer (Belgian-Cancer-Registry 2010). Between 2004 and 2009 a total number of 625 new diagnoses of leukemia are registered in children (N = 500) and adolescents (N = 125) in Belgium. The most frequent subtype, acute lymphoid leukaemia, represents 74% of the total number of leukaemia cases. In this age group (1-9 years) ALL represents 80% or more of all leukaemia diagnoses. From the age of 10 years onwards, the incidence rates for ALL remain fairly stable (Belgian-Cancer-Registry 2010).

Until recently, there has been very little information on the pattern of occurrence of childhood cancer in Vietnam. Incidence rates of childhood cancer for the Cancer Centre Hospital at Ho Chi Minh city are presented for the first time for 3 years period 1995-1997 in Paediatric and Perinatal Epidemiology 2000. A total of 302 cancer cases were registered in children under 15 years of age, with a male to female ratio of 1:1. Leukaemia (principally ALL), brain tumours and lymphomas were the most common childhood neoplasms. Among 121 cases of leukemia, ALL was the most common subcategory, accounting for 66%. It occurred most commonly 41% in the age 3-5 rears group. In 1995, the incidence of ALL observed in Cancer Centre Hospital at Ho Chi Minh city and elsewhere in south-east Asia is lower than in the white Caucasian population of Australia, Europe and North America (Quoc, Hung et al. 2000).

FRALLE 2000 protocol

FRALLE 2000 protocol is shown in Figure 2. Drugs used in treatment FRALLE 2000 protocol are shown in Table 1. Patients were assigned on sub-groups A1/A2/A3, B1/B2 or T1/T2 according to the results of flow cytometry, myelogram on day 21 and/or MRD evaluated at day 35.

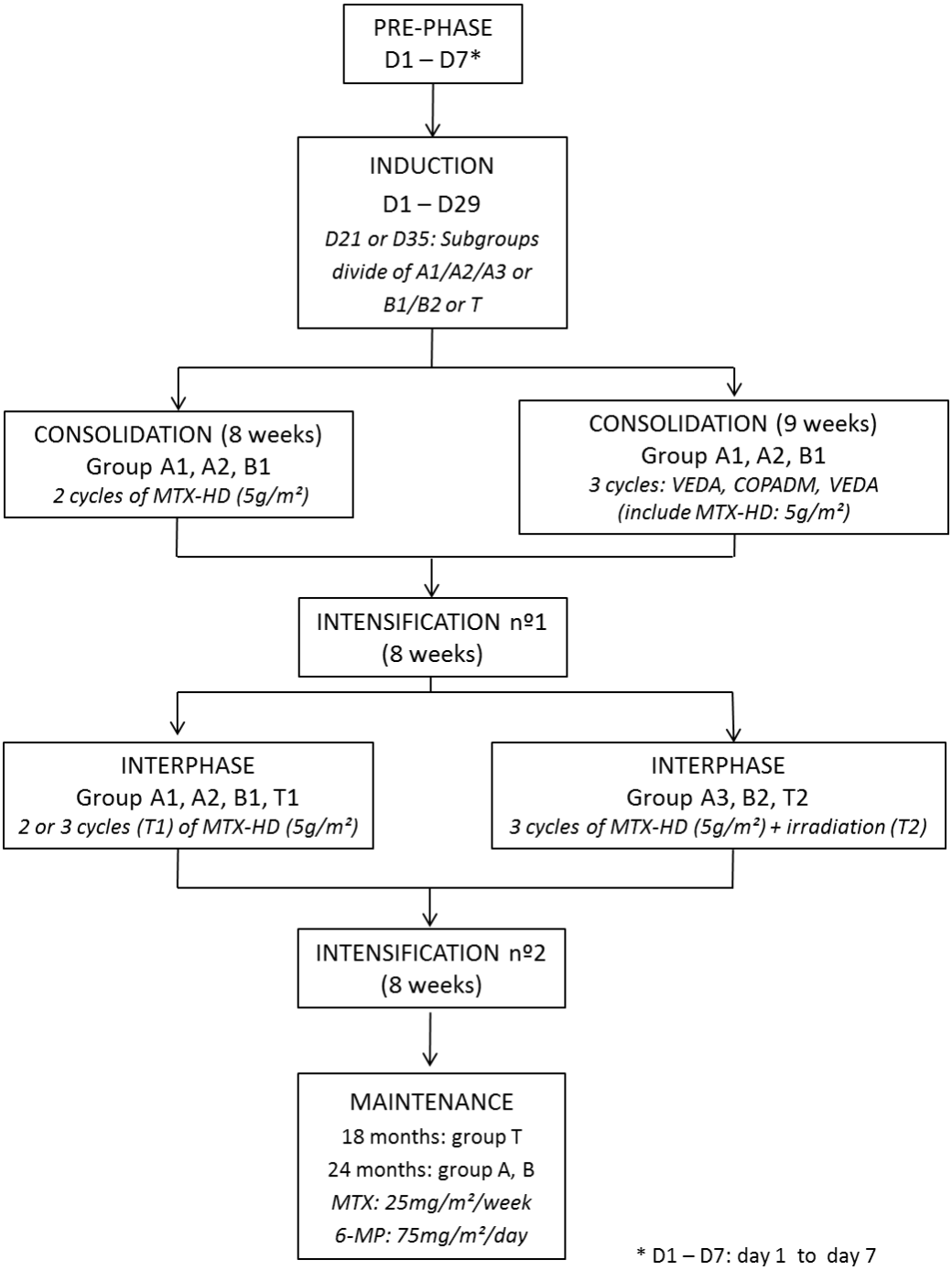


Figure 2: Diagram of Phases in FRALLE 2000 protocol.

Table 1: Treatment with FRALLE 2000 protocol details

Phase/Drugs	Daily Dose	Days of Application per Element	Note of toxicity/dose
Pre-phase			
- Prednisone (oral or IV)	60mg/m^2/d	D1 – D7 continuous	
- MTX (IT)	8-15 mg	D1	
Induction			
- Dexamethasone (oral or IV)	6mg/m^2/d	D8 – D28 continuous, reduce dose - off D35	Group A
- Prednisone (oral or IV)	40mg/m^2/d	D8 – D28 continuous, reduce dose - off D35	Group B/T
- Vincristine (IV) *	1,5mg/m^2/injection (2mg max)	D8-15-22-29	
- Daunorubicine (IVC in 1h)	40mg/m^2/injection	D22-29 or D8-15-22-23(+/-)-29	Group A3 or B
		D8-9-10-15	Group T
- L-Asparaginase (IVC in 1h)	6.000 UI/m^2/injection	D10-12-14-16-18-20-22-24-26	Group A/B
		D8-10-12-15-17-19-22-24-26	Group T
- Endoxan (IVC in 1h)	1.000mg/m^2/injection	D22 or D8	Group B2 or T
- MTX (IT)	8-15 mg	D8-15	Single in A
- Aracytine (IT)	15-30 mg	D8-15	Triple in B/T
- Depomedrol (IT)	20 mg	D8-15	Triple in B/T
Consolidation			
Cycle 1			B1 or T1
- Lanvis (oral)	60mg/m^2/d	D1 – D21 continuous	B1/T1
- Vepeside (IVC in 1h)	150mg/m^2/injection	D1-8-15	B1
- Aracytine (SC)	30mg/m^2/injection	D1-2/ D8-9/ D15-16	B1/T1
- Endoxan (IVC in 1h)	1.000mg/m^2/injection	D1-15	T1
- MTX (IT)	8-15 mg	D1- 30	B1
- Aracytine (IT)	15-30 mg	D1- 30	B1
- Depomedrol (IT)	20 mg	D1- 30	B1
Cycle 2			A1/A2/B1/T1
- Dexamethasone (oral or IV)	6mg/m^2/d	D1-D5/ D29-D33/D57-D61 continuous	A1/A2
- Prednisone (oral or IV)	40mg/m^2/d	D29 - D35 continuous	B1/T1
- Mercaptopurine (oral)	75mg/m^2/d	D1 - D77 continuous	A1/A2
	50mg/m^2/d	D29 - D35 continuous	B1/T1
- Vincristine (IV) *	1,5mg/m^2/injection	D1-8-29-36-57-64 or D29-43	A1/A2 or B1/T1
- MTX-HD (IVC in 24h)	5.000mg/m^2/d	D29- 43	B1/T1 (higher toxicity)
- MTX (oral)	25mg/m^2/	D36 or D8-15-22-36-43-50-64-71-78	B1/T1 or A1/A2

Phase/Drugs	Daily Dose	Days of Application per Element	Note of toxicity/dose
- MTX (IT)	8-15 mg	D2-30-58	Single in A1/A2
	8-15 mg	D1-30-44 or D1-15-30-44	B1 or T1
- Aracytine (IT)	15-30 mg	D1-30-44 or D1-15-30-44	B1 or T1
- Depomedrol (IT)	20 mg	D1-30-44 or D1-15-30-44	B1 or T1
Or VEDA1/COPADM/VEDA2			
VEDA			A3 or B2
- Dexamethasone (oral or IV)	20mg/m^2/d	D1-D5 continuous	
- Vincristine (IV) *	1,5mg/m^2/injection	D1	
- Aracytine (IVL in 1h) **	2.000mg/m^2/injection (8g max)	D1-2	
- Vepeside (IVL in 1h)	150mg/m^2/d	D3-4-5	
- MTX (IT)	8-15 mg	D5	Single in A3
- Aracytine (IT)	15-30 mg	D5	Triple in B2
- Depomedrol (IT)	20 mg	D5	Triple in B2
COPADM			
- Prednisone (oral or IV)	60mg/m^2/d	D1 – D5 continuous	
- Vincristine (IV) *	1,5mg/m^2/injection	D1	
- Adriamycine (IVL in 1h)	40mg/m^2/d	D2	
- Endoxan (IVC in 1h)	1.000mg/m^2/injection	D2-3	
- MTX-HD (IVC in 24h)	5.000mg/m^2/d	D1	Higher toxicity
- MTX (IT)	8-15 mg	D2	Single in A3
- Aracytine (IT)	15-30 mg	D2	Triple in B2
- Depomedrol (IT)	20 mg	D2	Triple in B2
Intensification I			
Cycle 1			
- Dexamethasone (oral or IV)***	10mg/m^2/d (10mg max)	D1 – D14 continuous, reduce dose - off D21	
- Vindesine (IV) ****	3mg/m^2/injection (4mg max)	D1-8-15	
- Adramycine (IVC in 1h)	25mg/m^2/injection	D1-8-15	
- L-Asparaginase (IVC in 1h)	6.000mg/m^2/injection	D4-6-8-10-12-15	
Cycle 2			
- Lanvis (oral or IV)	60mg/m^2/d	D29 – D49 continuous	
- Vepeside (IVC in 1h)	150mg/m^2/injection	D29-36-43	
- Aracytine (SC)	30mg/m^2/injection	D29-30/ D36-37/ D43-44	
- MTX (IT)	8-15 mg	D3-30	Single A
	8-15 mg	D1-29	Triple B/T
- Aracytine (IT)	15-30 mg	D1-29	Triple B/T
- Depomedrol (IT)	20 mg	D1-29	Triple B/T

Phase/Drugs	Daily Dose	Days of Application per Element	Note of toxicity/dose
Interphase			
- Dexamethasone (oral or IV)	6mg/m^2/d	D1-D5/ D29-D33 continuous	A
- Prednisone (oral or IV)	40mg/m^2/d	D1-D5/ D29-D35 continuous	B/T
- Mercaptopurine (oral)	50mg/m^2/d	D1 – D49 continuous	A3/B/T
	75mg/m^2/d	D1 – D49 continuous	A1/A2
- Vincristine (IV) *	1,5mg/m^2/injection	D15 or D1-15-29-43(+/-)	A3 or B/T
- MTX-HD (IVC in 24h)	5.000mg/m^2/d	D1-29	B1
		D1-15-29-43(+/-)	A3/B2/T
- MTX (oral)	25mg/m^2/d	D8-15-22-36-43-50	A1/A2
		D8-22-36 or D8-22-36-43-50	B/T or A3
- MTX (IT)	8-15 mg	D2-16-30 or D2-30	Single A3 or A1/A2
	8-15 mg	D2-30 or D2-16-30-44	B or T
- Aracytine (IT)	15-30 mg	D2-30 or D2-16-30-44	B or T
- Depomedrol (IT)	20 mg	D2-30 or D2-16-30-44	B or T
Intensification II			
Cycle 1			
- Prednisone (oral or IV)	40mg/m^2/d	D1 – D14 continuous, reduce dose - off D21	A3/B/T
- Vincristine (IV) *	1,5mg/m^2/injection (2mg max)	D1-10-20-30	A1/A2
		D1-8-15	A3/B/T
- Daunorubicine (IVC in 1h)	40mg/m^2/injection	D1-8-15	A3/B/T
- L-Asparaginase (IVC in 1h)	20.000mg/m^2/injection	D2-11-21-31	A1/A2
	6.000mg/m^2/injection	D3-5-8-10-12-15	A3/B/T
- MTX- DI (IVC)	100mg/m^2/injection	D1-10-20-30	A1/A2
- MTX (IT)	8-15 mg	D2 or D3-30	Single A1/A2 or Single A3
	8-15 mg	D1-29	Triple B1/T1
- Aracytine (IT)	15-30 mg	D1-29	Triple B1/T1
- Depomedrol (IT)	20 mg	D1-29	Triple B1/T1
Cycle 2			
- Lanvis (oral or IV)	60mg/m^2/d	D29 – D49 continuous	
- Endoxan (IVC in 1h)	1.000mg/m^2/injection	D29	
- Aracytine (SC)	30mg/m^2/injection	D29-30/ D36-37/ D43-44	
Maintenance		Month 1-24	
Re-induction		Month 1-12	
- Dexamethasone (oral or IV)	6mg/m^2/d	D1-D5 continuous	A
- Prednisone (oral or IV)	40mg/m^2/d	D1-D7 continuous	B/T
- Vincristine (IV) *	1,5mg/m^2/injection (2mg max)	D1	

Phase/Drugs	Daily Dose	Days of Application per Element	Note of toxicity/dose
Maintenance			
- MTX (oral)	25mg/m^2/week	3 weeks/month (M1-12)	
		4weeks/month (M13-24)	Higher toxicity
- Mercaptopurine (oral)	75mg/m^2/d	M1-24	Single in A
- MTX (IT)	8-15 mg	D1/M1-3	Triple in B/T
- Aracytine (IT)	15-30 mg	D1/M1-3	Triple in B/T
Depomedrol (IT)	20 mg	D1/M1-3	
Dose of MTX and Aracytine in IT depend on age. Subgroups (A1/A2/A3 or B1/B2 or T1/T2) divide at D21 or D35 of induction phase.			

The assignment to risk group in the FRALLE 2000 protocol with specified treatment according to age, WBC count at diagnosis, French- American-British (FAB) classification (L1/L2/L3) , and result of fow cytometry. Patients were stratified into three treatment groups:

- Standard risk (SR) group: FRALLE 2000-A (A1/A2/A3)
    - Patient with ALL, B-cell line, L1 or L2
    - Age > 1year or < 10years
    - WBC at diagnosis < 50.000/mm^3
    - Trisomy 21
- Hight risk (HR) group: FRALLE 2000-B (B1/B2)
    - Patient with ALL, B-cell line, L1 or L2
    - Age > 10 years
    - WBC at diagnosis ≥ 50.000/mm^3
    - Detectable CNS involvement
    - Present of t(9;22) or t(4;11) or hypodiploide ≤ 44 chromosomes
    - Present of BCR-ABL or MLL arrangement

- Very high risk (VHR) group: FRALLE 2000-T (T1/T2)
  Patient with ALL, T-cell line, L1 or L2

FAB classification of ALL and features of blasts:

- Type L1: Small cells with scant cytoplasm; nucleoli indistinct and not visible
- Type L2: Large, heterogeneous cells with moderately abundant cytoplasm; clefting and indentation of nucleus; large and prominent nucleoli.
- Type L3: Large cells with moderately abundant cytoplasm; regular, oval-to-round nucleus; prominent nucleoli; prominent cytoplasmic basophilia and cytoplasmic vacuoles.

Methotrexate

MTX (4-amino-10-methylpteroyl-L-glutamic acid) is an analogue to the folic acid antagonist aminopterin (Fig.3), the first drug to induce remissions in children with ALL (Schornagel and McVie 1983). Today MTX is the most widely used drug in chemotherapy and is used throughout the 2-2½ years of therapy in childhood ALL. Although the optimal dosage is still under active investigation, high dose methotrexate (HD-MTX) is commonly given as consolidation therapy, and low-dose oral MTX (LD-MTX) is given in continuation therapy in most childhood ALL treatment protocols. MTX has increased affinity for its target enzymes when MTX is polyglutamated intracellularly and the polyglutamated MTX is intracellularly retained far longer than the administered monoglutamated MTX. The antileukemic effect of MTX is well documented (Evans, Relling et al. 1998, Burke, Estlin et al. 1999) and it has been shown that intracellular and extracellular concentrations of MTX and its active polyglutamated metabolites are significant for the antileukemic effect and cure rate (Masson, Relling et al. 1996). Treatment efficiency is assessed by the number of lymphoblasts in BM (MRD) and outcome (van Dongen, Seriu et al. 1998, Coustan-Smith, Sancho et al. 2000, Schmiegelow, Nyvold et al. 2001).

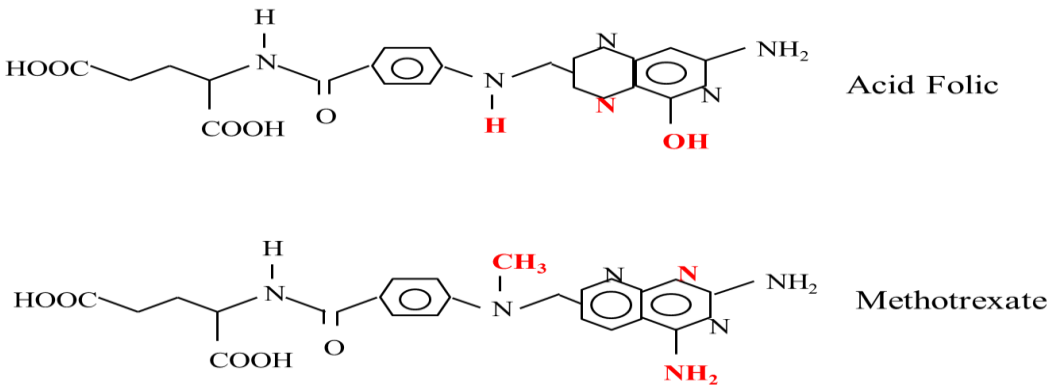


Figure 3: Folic acid and methotrexate only differ in an amino and a methyl-group.

Tetrahydrofolic acid itself is synthesized in the cell from folic acid with the help of an enzyme folic acid reductase. Methotrexate looks a lot like folic acid. So methotrexate binds to it quite strongly and inhibits folic acid reductase which is responsible for the conversion of folic acid to tetrahydrofolic acid.

Methotrexate metabolism pathway

Uptake of MTX into ALL cells is primarily mediated by reduced folate carrier (RFC) (Solute Carrier Family 19, member 1 (SLC19A1)), whereas efflux of MTX is mediated by ATP-binding cassette transporters (ABC-transporters) (Hooijberg, Broxterman et al. 1999, Zeng, Chen et al. 2001, Chen, Lee et al. 2002). Once inside the cell, MTX is quickly converted into poly (γ-glutamate) forms methotrexate polyglutamates (MTXPGs), a reaction catalyzed by folylpolyglutamate synthetase (FPGS) (McGuire, Hsieh et al. 1980). Compared to the parental drug, MTXPGs (especially long-chain MTXPGs) are retained longer in cells because they are not readily effluxed by ABC-transporters (Chabner, Allegra et al. 1985, Banerjee, Mayer-Kuckuk et al. 2002). The ability of ALL cells to form and accumulate MTXPGs has been shown to influence treatment response and outcome in childhood ALL (Whitehead, Rosenblatt et al. 1990, Barredo, Synold et al. 1994, Masson, Relling et al. 1996).

MTX and MTXPGs exert their effect by inhibiting enzymes essential for thymidylate synthesis and de novo purine synthesis, which will affect deoxyribonucleic acid (DNA) synthesis and cellular proliferation by primarily inhibiting the enzyme dihydrofolate reductase (DHFR), leading to depletion of 5-10 methylene tetrahydrofolic acid and N-10 formyl tetrahydrofolic acid. As these tetrahydrofolates function as co-factors to one-carbon transfer reactions, various configurations and synthetic reactions, which are dependent on one-carbon addition, are inhibited. Both the purine de novo synthesis and the thymidylate synthesis are dependent on one-carbon addition. For example, the thymidylate synthesis receives a one-carbon group from 5,10-methylene tetrahydrofolate to deoxyuridine monophosphate (dUMP) for the conversion to Deoxythymidine monophosphate (dTMP), one of the precursors essential for DNA-synthesis. In this process, the 5,10-methylene tetrahydrofolate is oxidized to dihydrofolate and DHFR would, if not inhibited by MTX and MTXPGs, reduce it to tetrahydrofolate again. The inhibition of DHFR also prevents DHFR from reducing MTXPGs to MTX. As MTXPGs is retained longer inside the cell, the accumulation of MTXPGs will extend the toxic effect to the cell. MTXPGs inhibits MTHFR, TS, glycinamide ribonucleotide transformylase (GAR-Tase) and aminoimidazole carboxamide ribonucleotide transformylase (AICAR-Tase), all essential

for either the thymidylate synthesis or the de novo purine synthesis (Schornagel and McVie, 1983; Schroder, 1990). It is also likely that MTX has inhibitory effects on the intracellular transport of glucose. MTX kills cells during the S phase of the cell cycle and has the highest efficacy on rapidly dividing cells. In high-dose MTX treatment, the cells are depleted and the patient will die, if leucovorin rescue is not given.

Leucovorin (folinic acid), which is also known as 5-formyl tetrahydrofolate, is an active form of one of vitamins known as folates. The folate coenzymes (including Folinic Acid and L-5-MTHF) are responsible for the following important metabolic functions and benefits: Formation of purines and pyrimidines, formation of the amino acid methionine from homocysteine (Vitamin B12 as methylcobalamin), formation of heme, and formation and maturation of red and white blood cells… Leucovorin is a faster acting and stronger form of folic acid (also known as vitamin B9). Leucovorin bypasses the metabolic block caused by MTX, thereby permitting normal cells to synthesize thymidylate and other compounds. It should be noted that failure to administer leucovorin in the right dose at the right time can be fatal.

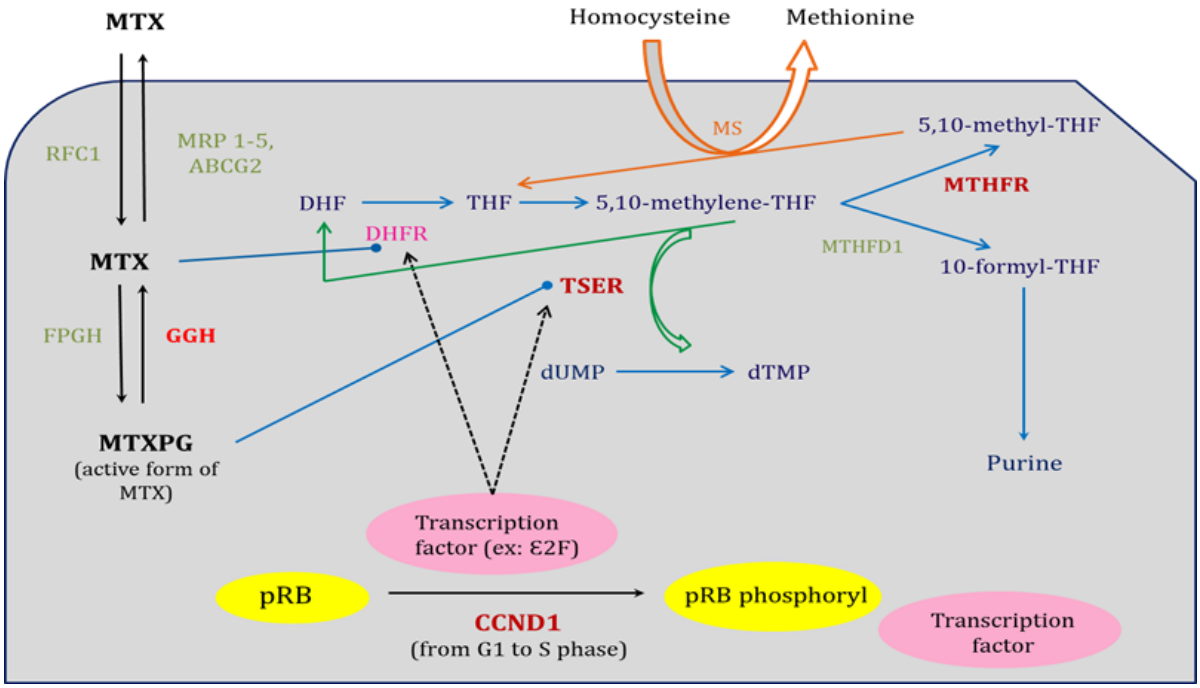


Figure 4: Mechanism of action of intracellular methotrexate (based on source Medecine/Sciences-vol 23- 11/2007).

ABCG2= ATP-binding cassette (ABC) family especially G2; CCND1= cyclin D1; DHF= dihydrofolate; DHFR= dihydrofolate reductase; dTMP= deoxythymidine monophospate; dUMP= deoxyuridine monophospate; FPGH= folylpolyglutamate synthetase; GGH= γ-

glutamyl hydrolase; pRB= Retinoblastoma protein; MRP 1-5= multidrug resistance proteins 1-5; MTHFD1= methylenetetrahydrofolate dehydrogenase; MTHFR= methylenetetrahydrofolate reductase; MTX= methotrexate; MTXPG= methotrexate polyglutamates; MS= methionine synthase; RFC1= reduced folate carrier; THF= tetrahydrofolate; TSER= thymidylate synthase.

MTX enters cells through the RFC1 or other transport systems. After entering the cell, MTX is transformed to MTXPG by the enzyme FPGH. FPGH increases the efficacy of methotrexate. Glutamates can be removed by GGH and MTX monoglutamate is rapidly effluxed from the cell via membrane transporters of the ATP-binding cassette (ABC) family, especially ABCC1-4 and ABCG2. The main intracellular target of MTX is DHFR, inhibition of which results in accumulation of DHF and depletion of cellular folates. The addition of glutamate residues to methotrexate also increases its affinity for other target enzymes (TSER and DHFR). Other enzymes that are indirectly affected by methotrexate are MTHFR, MTHFD1, CCND1.

Individualisation of therapy

Traditionally, the same kind of drug is given to patients with the same disease, and the dose is based on the patients' weight or body surface area (m^2). However, response to treatment can vary greatly, even though they all are treated with the same drug and the same dose per square meter (Eichelbaum, Ingelman-Sundberg et al. 2006). This variability in individual response can be caused by non-genetic factors, such as age affecting kidney function, liver function, environment, food, and concomitant diseases. However genetics is responsible for typically 15 to 30%, and in rare cases up to 95%, of individual differences in drug metabolism and treatment effect. These genetic differences can affect both pharmacokinetics (e.g drugmetabolising enzymes) and pharmacodynamics (e.g. drug targets or target related proteins) and thus affect both efficacy and toxicity (Nebert, Zhang et al. 2008). Patients with the same diagnosis may respond in different ways to the same drug. In the best case the prescribed drug is not toxic and beneficial, but in some cases it can be also not beneficial. In other cases the treatment can be toxic, but beneficial. The worse occurrence is when the drug is toxic and also not beneficial (Figure 5).

Through the use of pharmacogenetics, physicians will be able to profile variations between individual's DNA to predict responses to a particular medicine. Pharmacogenetics will have impact on medical care and medical aid at multiple levels (Roses 2000). Moreover, pharmacogenetic testing may provide the first example of a mechanism whereby DNA based testing can be applied to populations (Wolf, Smith et al. 2000). The intersection of

pharmacogenomics and medicine has the potential to yield a new set of molecular diagnostic tools that can be used to individualize and optimize drug therapy (Evans and Relling 2004).

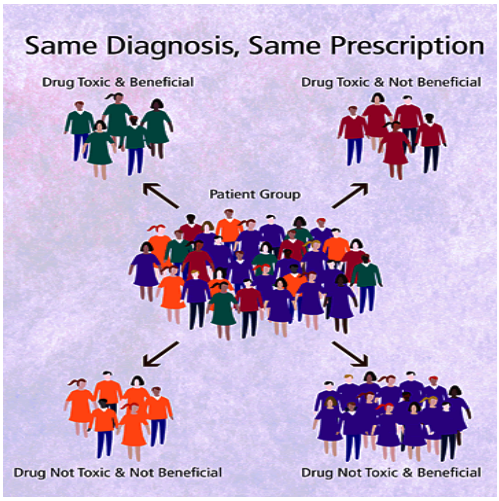


Figure 5: Individualized therapy means the use of pharmacogenomics to select the right drug for the right person prior to medication prescription (base on www.dana-farber.org)

Personalized medicine recognizes that individual patients may react in very different ways to the same treatment given for the same problem. The goal is to tailor therapies based on a patient's DNA profile. Personalized tumor molecular profiles, tumor disease site and other patient characteristics are then potentially used for determining optimum individualized therapy options.

Pharmacogenetics and pharmacogenetic terms

Individual variation in response to drugs is a substantial clinical problem. Individuality in drug response can be inherited. The genetically determined variability in drug response defines the research area known as pharmacogenetics. Pharmacogenetics has evolved in the past 50 years to a major driving force of clinical pharmacology. The first concept of pharmacogenetics was originated from Motulsky in 1957 (Motulsky 1957). The term pharmacogenetics was suggested by Vogel in 1959 (Vogel 1959), and the first book on the subject was written by Kalow (Tang and Endrenyi 1998). The first molecular defect was identified by Frank Gonzalez in the late 1980s (Gonzalez, Skodat et al. 1988). The term pharmacogenetics is used to cover both germline and somatic predictors of drug response (Goldstein, Tate et al. 2003). One of the main directions in development of pharmacogenetics is identifying genes and allelic variants of genes that affect our response to drugs (Wolf, Smith et al. 2000). To date, polymorphisms of genes encoding drug metabolism enzymes, drug transporters, and drug receptors, which are involved in drug responses, have been reported and in some of them the association between

pharmacodynamics and drug efficacy has been clarified (Evans and Relling 1999, Goldstein, Tate et al. 2003).

Pharmacogenetics is the study of variability in drug response due to heredity. The commonest form of genetic variation is the single nucleotide polymorphism (SNP) where a single nucleotide base is altered or deleted or an additional nucleotide base inserted. It is estimated that such a change (or polymorphism) occurs every 500–1000 bases in the human gene (Pirmohamed 2001). SNPs may occur within the coding or non-coding region of the gene. SNPs within the coding region may lead to changes in the amino acid produced (known as a non-synonymous polymorphism). SNPs within the non-coding region may still affect gene transcription, gene splicing or RNA stability. Given the rapid advances in high-throughput genotyping it is now possible to screen large numbers of these polymorphisms throughout the human genome that may influence drug response. This review will highlight the possible pharmacogenetic targets for MTX by considering its metabolism and mechanisms of action.

There are differences between pharmacogenetics and pharmacogenomics. Accordingly, most of scientists use pharmacogenetics to depict the study of single genes and their effects on interindividual differences in drug metabolizing enzymes, and pharmacogenomics usually used in broader context, to depict the study of not just single genes, but the functions and interactions of all genes in the genome in the overall variability of drug response, whether this is caused by pharmacokinetics, pharmacodynamics or both. Some have distinguished between pharmacogenetics and pharmacogenomics on the basis of either the type of genomic information or the quantity (Goldstein, Tate et al. 2003). The differences between the pharmacogenetics and pharmacogenomics are the initial approach of the science. Pharmacogenetics is based on an unexpected drug response result and looks for a genetic cause, while pharmacogenomics looks for genetic differences within a population that explain certain observed responses to a drug or susceptibility to a health problem (www.genetics.edu.au).

To summarize, classical pharmacogenetics has searched the genes for an abnormal drug response of proven clinical value, whereas pharmacogenomics presently and in the future will search the bearing if any of known genes, SNPs or haplotypes.

The Role of Ethnicity in Pharmacogenetics

Genetic differences between people contribute to interindividual differences in the response to many drugs. Findings of the Human Genome Project cleared that 99.9 % of the information in the estimated 20.000 human genes is identical from one person to the next. The small

differences in the remaining 0.1% of genes are the key to each individual (www.genetics.edu.au). Very often the variability in DNA consists of only one nucleotide base change, and if this occurs in more than 100 subjects at a given position in the DNA, it is referred to as a single nucleotide polymorphism. SNPs make up about 90% of all human genetic variation. In 2008, about 12 million SNPs and a large amount of other types of genetic variation were known in the human genome (Brockmöller and Tzvetkov 2008). The remainder of the variation is caused by insertions, deletions, tandem repeats and microsatellites (Marsh and McLeod 2006).

It is known, that many polymorphisms that influence drug response and which probably contribute significantly to phenotypic variation in drug response have significant allele frequency differences among racial or ethnic groups (Evans and Relling 1999, Tate and Goldstein 2004, Engen, Marsh et al. 2006). Recent studies report differences between African and non-African population groups in the structure of sequence variation in the human genome (Tishkoff and Verrelli 2003).

Geographic patterns of genetic variation, including variation at drug metabolizing enzyme loci and drug targets, indicate that geographic structuring of inter-individual variation in drug response may occur frequently. This geographic distribution of certain variants has highlighted the possible importance of average differences in drug response across populations (Wilson, Weale et al. 2001). For this reason we need to take into account not just differences between the genotypes of individuals, but the differences in genotypes between population groups of different origin and the substantial variation between the five main racial groups, which are based on continental ancestry (Daar and Singer 2005).

In conclusion, understanding of human genomic variation and its application in pharmacogenetics might shift our focus away from interindividual differences towards interpopulation differences (Daar and Singer 2005). Thus race or ethnicity will usually be a far inferior guide to response than direct determination of the underlying genetic variant (Need, Motulsky et al. 2005).

Drugs interacting with MTX

Delayed renal excretion can be caused by concurrent use of drugs in which reduce renal blood flow, are nephrotoxic or are weak organic acids (e.g. nonsteroidal anti-inflammatory agents, cisplatin and aspirin/piperacillin, respectively) and can lead to severe myelosuppression (Thyss, Kubar et al. 1986). 6-MP is used in ALL therapy together with MTX and gives a

synergistic in-vivo effect on both toxicity and efficacy (Bökkerink, Bakker et al. 1988, Schmiegelow, Schrøder et al. 1994, Schmiegelow, Schrøder et al. 1995, Schmiegelow and Bretton-Meyer 2001, Nygaard and Schmiegelow 2003).

Thiopurines

Both azathiopurine (AZA) and 6-MP need to undergo extensive metabolic transformations (Figure 6) before they can exert their immunosuppressive activity, as both substances have no intrinsic activity. Once absorbed, approximately 90% of the absorbed AZA undergoes a rapid non-enzymatic conversion in the liver, yielding 6-MP and methyl-4-nitroimidazol. The remaining 10% of AZA, which is also cleaved non-enzymatically, yields amongst others hypoxanthine and methyl-4-nitro-5-thioimidazol. The absorption of AZA is incomplete and (interindividually and intraindividually) variable resulting in a wide range of bioavailability from 16-72% (Schwab and Klotz 2001). Subsequently, 6-MP is metabolized by three competing enzyme systems, xanthine oxydase (XO), TPMT and hypoxanthine guanine phosphoribosyltransferase (HPRT). The enzyme XO catalyses the reaction of 6-MP to the pharmacologically inactive metabolite 6-Thiouric-acid, whereas the enzyme TPMT methylates 6-MP into 6-methyl MP. The HPRT enzyme is responsible for the conversion into 6-thioinosine monophosphate (6-TIMP). Subsequently, 6-TIMP can be further transformed by inosine monophosphate dehydrogenase (IMPD) into 6-thioxanthylic acid (6-TXMP), which subsequently is converted via guanosine monophosphate synthetase to 6-thioguanylic acid (6-TGMP). The 6-thioguanine nucleotides (6-TGN) have a half-life of approximately 5 days with a wide range of 3-13 days (Schwab and Klotz 2001). The different 6-TGN may all be methylated by TPMT. The metabolite 6-TIMP can be methylated via TPMT to form 6-methyl TIMP, 6-methyl thioinosine diphosphate (6-methyl TIDP) and 6-methyl thioinosine triphosphate (6-methyl TITP). The latter three metabolites are the so-called 6-MMPR. 6-Thioinosine-monophosphate can also be phosphorylated via monophosphate kinase (MPK) to 6- TIDP, and subsequently, via diphosphate kinase (DPK) to 6-TITP, and ultimately back to 6-TIMP following an enzymatic reaction with inosine triphosphatase (ITP) pyrophosphatase. 6-TGN are considered as the pharmacologically active end-metabolites. 6-TGN are considered as the pharmacologically active end-metabolites. In the classical immunosuppressive theory, the mode of action is specifically ascribed to antimetabolic (cytotoxic) pathways. The 6-TGN, as a result of their structural similarity to the endogenous purine bases, are incorporated into DNA or Ribonucleic acid (RNA) as fraudulent bases, resulting in strand breakage and

interference with de novo synthesis of proteins and nucleic acids (Fairchild, Maybaum et al. 1986, Marinaki, Ansari et al. 2004).

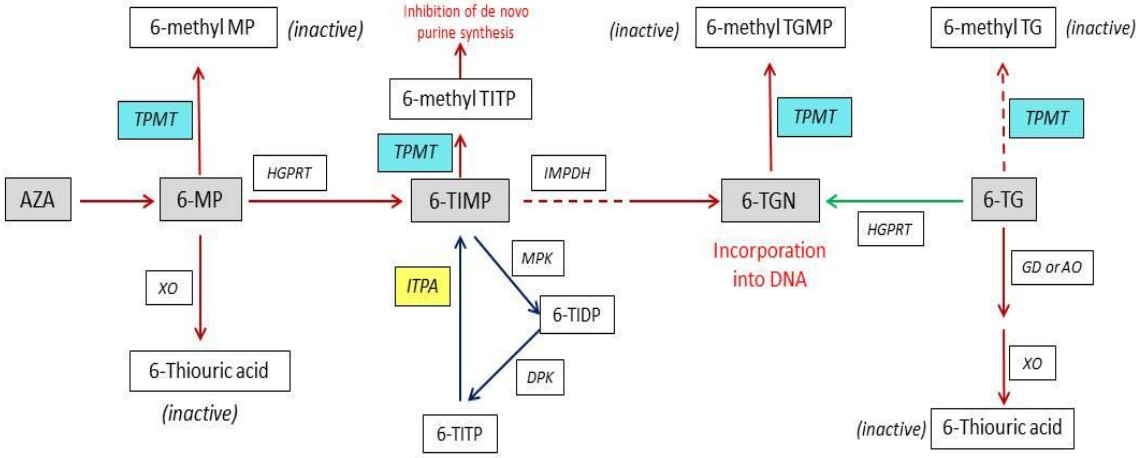


Figure 6: Metabolization of thiopurine based drugs Azathiopurine (base on Article "Assessment of Thiopurine S-Methyltransferase Activity in Patients Prescribeb Thiopurines: A Systematic Review". Ann Intern Med.2011).

AO = aldehyde oxidase; AZA = azathiopurine; DPK= diphosphate kinase; GD = guanine deaminase; HGPRT = hypoxanthine guanine phosphoribosyltransferase; IMPDH = inosine monophosphate dehydrogenase; ITPA= inosine triphosphatase; MP = mercaptopurine; MPK= monophosphate kinase; TG = thioguanine; TGMP = thioguanine monophosphate; TGN = thioguanine nucleotides; TIDP= thioinosine diphosphate; TIMP = thioinosine monophosphate; TITP= thioinosine triphosphate;TMPT = thiopurine S-methyltransferase; TXMP = thioxanthylic acid; XO = xanthine oxidase.

AZA is degraded to 6-MP and nitromethyl-imidazole, via a non-enzymatic step. Three competing enzymes metabolize 6-MP. XO inactivates 6-MP to 6-Thiouric acid. TPMT methylates 6-MP into 6-methyl MP, this metabolite is associated with hepatotoxicity. By HPRT, 6-MP is catalyzed to 6-TIMP, then via IMPDH to 6-TXMP, ultimately leading to the pharmacologically active 6- TGN (consisting of 6-TGMP, 6-TGDP and 6-TGTP) via the enzyme guanosine monophosphate synthetase. 6-TIMP can also be methylated to 6-methyl TIMP, 6- methyl TIDP and 6-methyl TITP. In a normal useless cycle, 6-TIMP is phosphorylated by MPK to 6-TIDP, subsequently by DPK to 6- TITP and ultimately back to 6-TIMP due to ITPA. When ITPase activity is impaired or lacking, 6-TITP accumulates. 6-TG is directly converted by HPRT to 6- TGN. TPMT metabolize 6-TG to 6-methylthioguanine (6-methyl TG). Guannine deminase (GD) or aldehyde oxidase (AO) metabolize 6-TG to 6- Thiouric acid. ITPA has no known role in the metabolism of 6-TG.

The thiopurine antimetabolites MP and TG, analogues of the purine nucleosides hypoxanthine and guanine, are key components in childhood ALL treatment protocols. MP or TG are used

during induction consolidation treatment as well as in maintenance therapy. After uptake via nucleoside transporters, these medications are metabolized into active cytotoxic TGNs and cytotoxicity occur mainly by incorporation of TGNs into DNA or RNA resulting in cell cycle arrest and apoptosis (Relling, Hancock et al. 1999). TPMT and ITPA are involved in the metabolism of 6-MP. Polymorphisms in the ITPA gene have been associated with neutropenia and thrombocytopenia during thiopurine therapy (Stocco, Cheok et al. 2009).

Linkage disequilibration

Linkage disequilibration is the phenomenon where two or more genetic variant combinations are inherited together, resulting in fewer haplotypes than expected. In humans this is often seen in genetic variants with a small physical distance between loci, resulting in fewer meiotic recombinations between the genetic variants.

Polymorphism

Polymorphism (Greek: multiple forms) is the phenomenon of alternative DNA sequences between individuals at a locus in the genome. There are different types of polymorphisms: single base polymorphism, deletions, or insertions of a section of DNA, the latter includes repeated sequences. The majority of human sequence variations are due to substitutions that have occurred once in the history of mankind at individual base pairs. SNP is the most common form of polymorphisms, found in approximately 1 of every 600 base pairs (Patil, Berno et al. 2001). In polymorphisms where a sudden variant causes diseases, the variant is often referred to as a mutation. The most frequent variant, which obviously does not cause disease, is called wild type. In pharmacogenetics a variant can be most frequent in one ethnic population but not in another ethnic population, hence the term wild type is avoided in the thesis. Instead, the specific nucleotides from each allele are mentioned (e.g. 3435CC, 3435TT or 3435CT, if the variant is heterozygous).

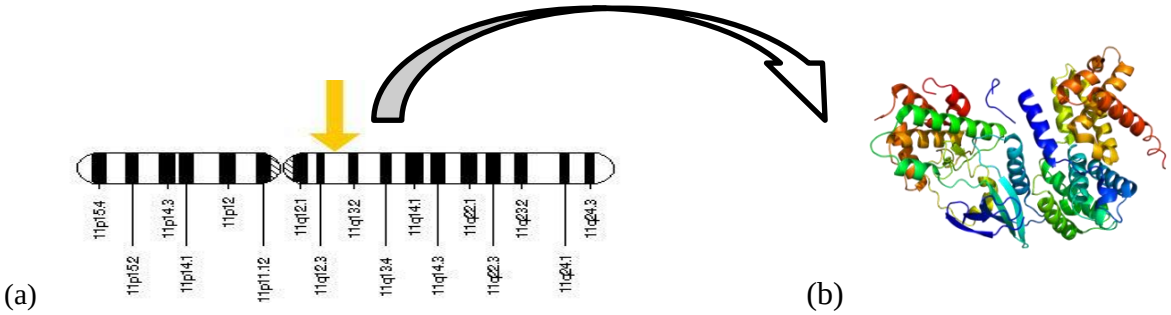
CCND1 G870A polymorphism

The CCND1 gene (located on chromosome 11 q13) (Figure 7) codes for CCND1, which is a key protein for the cellular division, as it regulates the transition from the G1 to the S phase of the cell cycle. Uncontrolled cell proliferation is one of the hallmarks of cancer. The cell cycle is the machinery that governs proliferation and growth of cells, thus, alteration in genes regulating the cell cycle could predispose to cancer. CCND1 has a critical role in control of cell cycle G1 to S phase transition. CCND1 coding gene CCND1 has a commonly occurring G to A

polymorphism (G870A, Pro241Pro) at position 6962 (rs 603965) in exon 4. Overexpression and deregulation of this gene may lead to abnormalities in the cell cycle resulting in carcinogenesis (Knudsen, Diehl et al. 2006). It has been postulated that the A allele results in a higher level of mRNA (transcript-b), encoding for a protein with an altered C-terminal domain, with enhanced cell transformation activity and possible prolonged half-life (Sobti, Kaur et al. 2006). This polymorphism produces two alternatively spliced forms of a transcript with varying stability and enzymatic activity. The protein product of the CCND1 A allele has higher enzymatic activity and is hypothesized to be more stable than the product of the G allele (Betticher, Thatcher et al. 1995).

While CCND1 870 AA genotype has been reported to increase the risk for childhood ALL in a Chinese population (Hou, Wang et al. 2005), it has also been associated with an increased MTX resistance with EFS reduction (Costea, Moghrabi et al. 2003, Costea, Moghrabi et al. 2006). CCND1 is the major D Cyclin in most cell types. All 3 Cyclin D molecules act in late G phase, just before entry into S phase. Many tumors have high CCND1 levels without amplification or mutation of the CCND1 structural gene. In blasts from patients with acute lymphocytic leukemia, resistance mechanisms found are decreased uptake and increased DHFR activity. A major cause of intrinsic resistance to MTX in soft tissue sarcoma cells and in acute myelocytic leukemia appears to be a lack of drug retention, due mainly to low levels of polyglutamylation. A novel association between lack of the retinoblastoma protein and intrinsic MTX resistance has been found. This has been attributed to an increase in DHFR activity, due to an increased rate of transcription of this gene, stimulated by an increase in levels of free E2F, not sequestered by hypophosphorylated retinoblastoma protein. CCND1 A870G polymorphism modulates the ratio of CCND1 mRNA isoforms, and the transcript associated with the A allele results in the protein with a longer half-life. CCND1 AA870 were previously associated with worse ALL outcomes (Bertino, Göker et al. 1996).

The reported prevalence of 870AA homozygotes in Caucasian and in Asian ALL children ranges from 22.3 to 25.7% and 32.4 to 38%, respectively (Phaichitchinda 2005, Onay, Aaltonen et al. 2008, Pabalan, Bapat et al. 2008, Yang, Lin et al. 2010).



(a) (b)

Figure 7: CCND1 gene. (a) CCND1 gene – chromosome 11 (base on http//: www.ghr.nlm.nih.gov) (b) CCND1 protein (base on en.wikipedia.org)

CCND1 G870A polymorphism and cancer risk

The CCND1 G870A A allele has been reported to be associated with increased risk for several cancers, including colorectal, esophageal, cardiac, bladder, head and neck, lung, prostatic, and renal cell carcinomas as well as leukemia (Knudsen, Diehl et al. 2006).

The results have not been entirely consistent since some studies have shown no risk and increased risk for cancer has even been reported for the GG genotype. Thus, the effect of this polymorphism may vary in distinct tumor types, although differences in study designs can also explain some of the discrepancies. The highest risks have, however, been reported in most studies for the AA genotype. The relative risks have typically been modest, which can in part be expected for a common allele such as the A allele in CCND1 polymorphism. The cancer risk with this low-penetrance variant could be modified by polymorphisms in other genes or environmental factors (Buch, Zhu et al. 2005, Shu, Moore et al. 2005).

Cell cycle

The human cell cycle is a firmly controlled machinery that regulates cell entry into mitosis and DNA replication, and thus, cell division. Cell cycle consists of different phases: G1 phase (preparation for DNA synthesis), S phase (DNA synthesis and replication), G2 phase (the second gap), and M phase (mitosis) (Figure 8). G0 (quiescence) is a biochemically different resting phase, where cells are kept in the absence of proliferation stimuli, but from which cells upon growth factor stimulation can re-enter into the active cell cycle. As cells receive enough environmental proliferation stimuli, they move from G1 to S phase. This G1/S transition is considered irreversible (Sherr 1996). The G2 phase is important for DNA replication and for the correction of errors in replication.

Cyclin-dependent kinases (CDKs) regulate the cell cycle and changes between different phases. CDK levels remain relatively constant throughout the cell cycle, but they are activated and inactivated by periodic changes in levels of their binding proteins. Cyclins are cell cycle regulator proteins that activate cell cycle progression by binding to CDKs. Active cyclin-CDK complexes drive phosphorylation cascades throughout the cell cycle. CCND1-CDK4/6 and cyclin E-CDK2 complexes act predominantly on G1 phase, cyclin A-CDK2 on S and G2 phases, and cyclin B1-CDK1 on G2 phase and mitosis. CKIs consist of large amount of inhibiting proteins, e.g. INK4 group inhibitors CDKN2A (p16 and p14), CDKN2B (p15), CDKN2C (p18), and CDKN2D (p19), and the second group of CIP/KIP (cyclin-dependent kinase inhibitory protein/kinase inhibitor protein) inhibitors CDKN1A (p21) and CDKN1B (p27) (Sherr and Roberts 1999). Inhibitors of kinase 4 (INK4s) bind to CDK4 and CDK6 to prevent their association with CCND1. CIP/KIP inhibitors do not affect cyclin binding, but form complexes with the G1/S transition CDKs. CKIs allow the cell cycle to be controlled at multiple levels.

The most important target of cyclin-CDK phosphorylation is the pRb and other retinoblastoma family proteins such as p107 and p130. Active pRb represses the transcription factors essential for G1/S transition. Thus, pRb is a negative cell growth regulator. Upon stimuli of proliferation factors, pRb becomes inactivated and transcription factors needed for G1/S transition are activated. The pRb hyperphosphorylation disrupts its association with E2F, and activated E2F mediates transcriptional activation required for G1 progression. CCND1-CDK4/6 complex is upregulated early after mitogenic stimuli and initiates the phosphorylation and inactivation of pRb (Baldin, Lukas et al. 1993). Cyclin E-CDK2 complex becomes activated later and is necessary in pRb phosphorylation in late G1 phase as well as in events leading to DNA synthesis (Sheaff, Groudine et al. 1997, Caldon, Daly et al. 2006). Recent data suggests that also the cell cycle reentry from G0 to G1 phase is regulated by specific retinoblastoma family proteins in association with specific E2F transcription factors. Cyclin C/CDK3 complex controls the pRb suppression at this phase and seems to be critical in promoting cell exit from the quiescent G0 phase (Ren and Rollins 2004). The G0 to G1 transition, however, is incompletely understood.

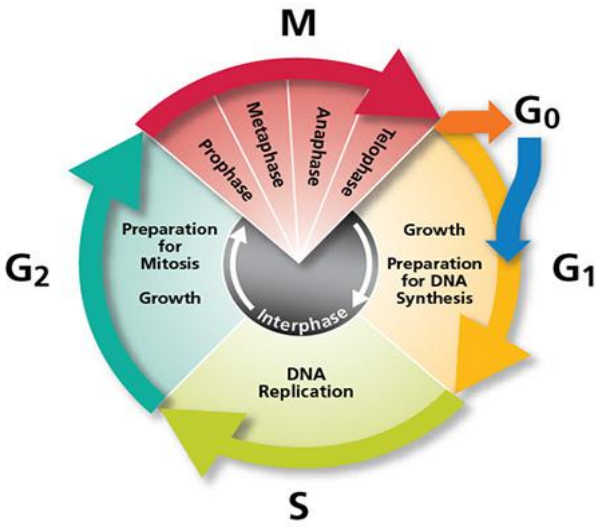


Figure 8: Cell cycle of cellular (based on www.bdbiosciences.com)

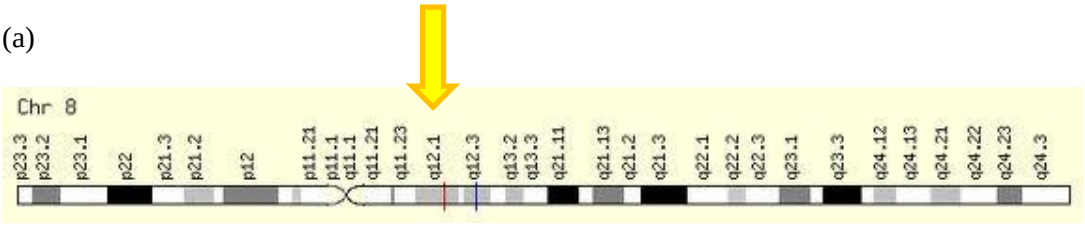
The cell cycle has two major phases: interphase, the phase between mitotic events, and the mitotic phase, where the mother cell divides into two genetically identical daughter cells. Interphase has three distinct, successive stages. During the first stage called G1, cells "monitor" their environment, and when the requisite signals are received, the cells synthesize RNA and proteins to induce growth. When conditions are right, cells enter the S stage of the cell cycle and "commit" to DNA synthesis and replicate their chromosomal DNA. Finally, in the G2 phase, cells continue to grow and prepare for mitosis.

GGH C452T polymorphism

GGH (located on chromosome 8p12.3) (Figure 9) is a lysosomal peptidase that catalyzes the removal of ɣ -linked polyglutamates of folates and anti-folates such as MTX, thus allowing folates to be exported from the cells or converting long-chain MTXPGs into short-chain MTXPGs and ultimately to MTX (Rhee, Lindau-Shepard et al. 1998, Panetta, Wall et al. 2002). Long-chain MTXPGs, which is the active form of MTX, directly blocks folate-dependent enzymes and leads to inhibition of de novo thymidine and purine synthesis, arresting DNA replication and causing cell death (Rhee, Lindau-Shepard et al. 1998, Cheng, Wu et al. 2004). MTXPGs cytotoxicity and efficacy are related to its cellular concentration and persistence (GALIVAN 1980, Koizumi, Curt et al. 1985). It has been previously shown that the T variant allele of GGH may result in decreased GGH activity, hence increasing intracellular MTXPGs. These observations suggest that GGH C452T polymorphism monitoring could be used to predict side effects and resistance to MTX (Hayashi, Fujimaki et al. 2007). Little is known however about MTX resistance in relapsed childhood ALL. Cells from relapsed precursor-B ALL have been shown to be threefold more resistant to MTX than those from

newly diagnosed ALL (Rots, Pieters et al. 2000). It can therefore be hypothesized that GGH C452T polymorphism could play a role in ALL relapse through its interaction with MTX metabolism. While the reported frequency of the GGH 452CT variant allele in Caucasian and in Asian ALL patients varies from 6 to 15.5% and 10 to 19%, respectively, it ranges between 0 and 4% or 0.4 and 1.5% for the GGH 452TT homozygote variant in both ALL populations, respectively (Hayashi, Fujimaki et al. 2007, Organista-Nava, Gómez-Gómez et al. 2010, Yang, Lin et al. 2010, Tiseo, Giovannetti et al. 2012).

(a)



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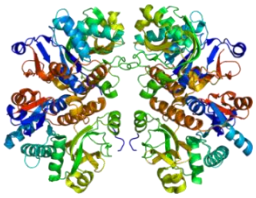


Figure 9: GGH gene. (a) GGH gene – chromosome 8 (base on en.wikipedia.org) (b) Gamma-glutamyl hydrolase protein (base on en.wikipedia.org)

ITPA A94C polymorphism

ITPA (located on chromosome 20p13) (Figure 10) catalyzes pyrophosphohydrolysis of ITP to IMP and deficiency of this enzyme leads to the abnormal accumulation of ITP (Kudo, Saito et al. 2008). It has been predicted that 6-MP is activated via a 6-TIMP intermediate and thus, toxic 6-TITP accumulates in ITPase-deficient patients on thiopurine drug treatment. In the ITPA gene, we detected a known nonsynonymous SNP, namely, 94C>A (Pro32Thr, rs1127354).

Interindividual differences in the therapeutic or toxic effects of 6-MP can be explained in part by variation in the formation of active metabolites. This variation is due to genetic polymorphisms in the genes encoding enzymes involved in thiopurine metabolism. Recent reports provide evidence for association between the ITPA 94CA or 94AA polymorphism and the occurrence of adverse drug reactions in patients treated with thiopurines (Sumi, Marinaki et al. 2002, Marinaki, Ansari et al. 2004). ITPA shows genetically determined polymorphic activity. The ITPA-C94A transversion reduces ITPA enzymatic activity to 25% and ~0% in

heterozygote and homozygote carriers, respectively (Stocco, Cheok et al. 2009). For patients without dose reduction of 6-MP according to TPMT genotype, those with ITPA-94AC/AA genotype were reported to have a higher probability of severe febrile neutropenia (Stocco, Cheok et al. 2009). Conversely, TPMT has been shown to have a higher impact than ITPA on 6-MP toxicity when the dose of 6-MP was not adjusted for TPMT phenotype (Stocco, Crews et al. 2010). In line with these data, it has been suggested that ITPA variants may have stronger impact in Asians than in Caucasians as the prevalence of TPMT*3A variant in Asians is lower than in Caucasians whereas the reverse is observed for the prevalence of ITPA variants (Marsh and Van Booven 2009, Kim, Kang et al. 2012). In contrast, Kim et al. showed that toxicity of 6-MP was related neither to TMPT nor to ITPA polymorphisms, but reported a lower event-free survival (EFS) rate in patients carrying the ITPA-94AC/AA genotypes (Kim, Kang et al. 2012). ITPA effect on RFS remains so far unclear. The prevalence of the ITPA-C94A transversion varies with ethnicity, ranging from 3.3 % in Africans, to 8.3 % in Caucasians, 11-19 % in Asians (Marsh and Van Booven 2009, Stocco, Cheok et al. 2009), and 3% in Chilean (Farfan, Salas et al. 2014).

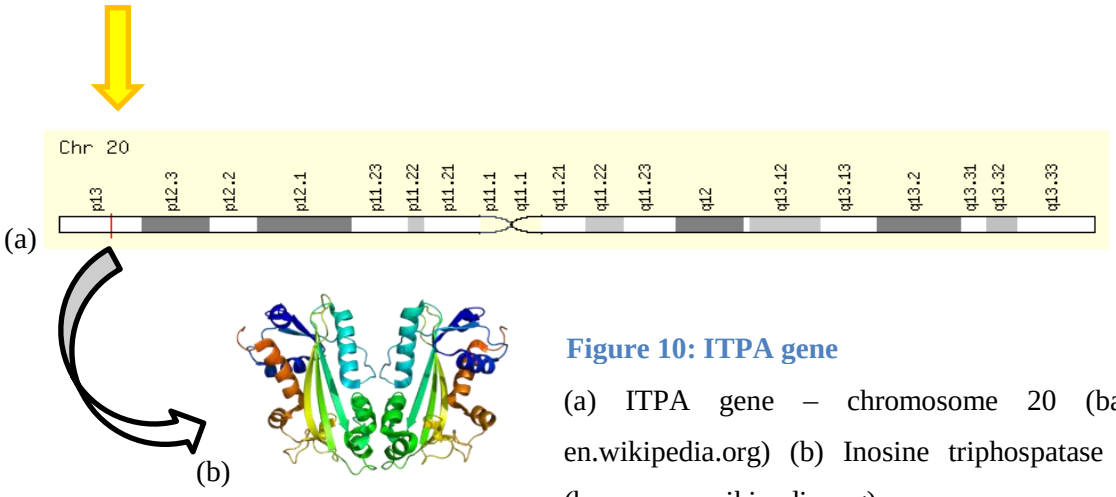


Figure 10: ITPA gene

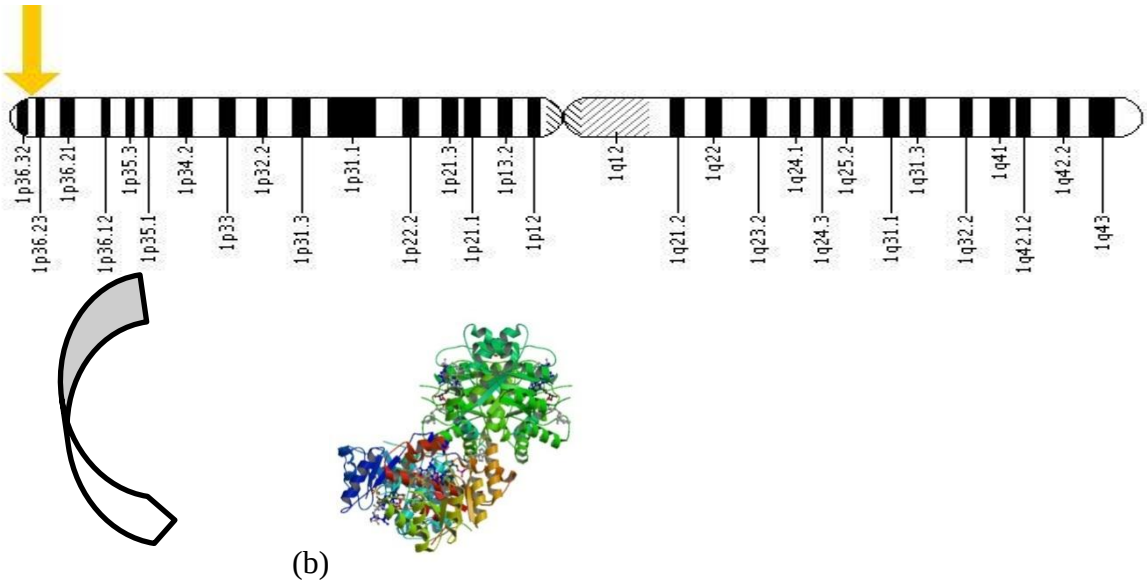
(a) ITPA gene – chromosome 20 (base on en.wikipedia.org) (b) Inosine triphospatase protein (base on en.wikipedia.org)

MTHFR polymorphism

MTHFR (located on chromosome 1p36.3) (Figure 11) catalyzes the reduction of 5,10-methylene-THF to 5,10-methyl-THF which is the predominant circulating form of folate and acts as the carbon donor for remethylation of homocysteine to methionine (Aplenc, Thompson et al. 2005). MTHFR plays a key role in determining the amount of folate available in the cell for DNA synthesis and methylation. The accumulation of 5,10-methyl-THF resulting from the MTHFR genetic polymorphism may play a role in MTX response (Giovannetti, Ugrasena et al. 2008). Specific MTHFR genotypes have allegedly been associated with MTX toxicity in

leukemia patients (Robien, Schubert et al. 2006), and with an increased risk of relapse in pediatric ALL patients (Krajinovic, Lemieux-Blanchard et al. 2004). The prevalence of MTHFR C677T and the A1298C variant alleles varies with ethnicity: the prevalence of 677TT ranges between 9 to 11% and 2.5 to 8.5% in Caucasian and Asian ALL children, respectively (de Jonge, Hooijberg et al. 2005, Giovannetti, Ugrasena et al. 2008, They-They, Hamzi et al. 2009), versus 9% to 16.5%, and 0% to 9%, respectively, for the prevalence of 1298CC (Krajinovic, Lemieux-Blanchard et al. 2004, de Jonge, Hooijberg et al. 2005, Sirachainan, Wongruangsri et al. 2008).

(a)



(b)

Figure 11: MTHFR gene.

(a) MTHFR gene – chromosome 1 (base on http//: www.ivfconnections.com) (b) MTHFR protein (base on http//: www.lovingourguts.com)

TPMT polymorphism

TPMT (located on chromosome 6p22.3) (Figure 12) catalyzes the S-methylation of thiopurine drugs, such as 6-MP, 6-TG and AZA, to inactive metabolites (Weinshilboum and Sladek 1980, McLeod, Krynetski et al. 1995, Krynetski, Tai et al. 1996). Thiopurines are part of the routine treatment for patients with malignant (ALL) and inflammatory diseases (rheumatoid arthritis), and are used as immunosuppressant to prevent rejection after organ transplantation. TPMT enzyme activity in human tissues is controlled by a spectrum of TPMT genetic polymorphisms (Weinshilboum and Sladek 1980, McLeod, Krynetski et al. 1995, Krynetski, Tai et al. 1996, Tai, Krynetski et al. 1997). Variations in TPMT activity impact on treatment toxicity and

efficacy. Patients with low or undetectable levels of TPMT activity have the highest risk of developing severe myelosuppression when treated with standard doses of thiopurines whereas patients with very high TPMT activity are more likely to have a reduced clinical response to these cytotoxic agents (Evans, Horner et al. 1991, Lennard, Gibson et al. 1993, Lilleyman and Lennard 1994). Four common TPMT alleles have been identified in the TPMT gene (i.e., TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C) which account for approximately 80% of Caucasians with low or intermediate TPMT activity. The TPMT*3A allele is mutated at both nucleotides G460A and A719G whereas TPMT*3B and TPMT*3C only have the G460A or A719G mutation, respectively (SZUMLANSKI, OTTERNESS et al. 1996, Otterness, Szumlanski et al. 1997, Yates, Krynetski et al. 1997).

The association between low TPMT activity and excessive hematological toxicity is now well recognized (McLeod, Lin et al. 1994, McLeod, Krynetski et al. 1995, Otterness, Szumlanski et al. 1997). Patients with low or undetectable levels of TPMT activity develop severe myelosuppression when treated with "standard" doses of thiopurines, while patients with very high TPMT levels are more likely to have a reduced clinical response to these agents (Evans, Horner et al. 1991, McLeod, Miller et al. 1993, Lilleyman and Lennard 1994). A 1999 study in 180 children identified an important role for TPMT genotype on tolerance to 6-MP therapy (Relling, Hancock et al. 1999). Two of the patients were TPMT-deficient and tolerated a full dose of 6-MP for only 7% of weeks of the planned therapy. Heterozygous and homozygous wild type patients tolerated full doses for 65% and 84% of weeks of therapy over 2.5 years of treatment, respectively. The percentage of weeks in which 6-MP dosage had to be decreased to prevent toxicity was 2%, 16%, and 76% in wild type, heterozygous, and homozygous variant individuals respectively (Relling, Hancock et al. 1999).

Interethnic variability in RBC, TPMT activity has been reported in several populations. RBC TPMT was 29% higher in Saami subjects in Northern Norway compared to white subjects from the same geographic region (Klemetsdal, Tollefsen et al. 1992). African American subjects have 17-33% lower RBC TPMT activity than American white subjects (Jones, Smart et al. 1993, Krynetski, Schuetz et al. 1995). The TPMT activity in African and white Americans was substantially lower than that reported in 119 Chinese subjects (Lee and Kalow 1993).

The frequency and pattern of variant alleles is different among various ethnic populations. For example, Southwest Asians (Indian, Pakistani) have a lower frequency of variant TPMT alleles, and all variant alleles identified to date are TPMT*3A (Collie-Duguid, Sludden et al.

1999). This is in contrast to Kenyans and Ghanaians where the frequency of variant alleles is similar to Caucasians but all variant alleles are TPMT*3C (Ameyaw, Collie-Duguid et al. 1999, McLeod, Pritchard et al. 1999). Conversely, in Caucasian populations TPMT*3A is the most common allele, but TPMT*2 and TPMT*3C are also found (Collie-Duguid, Sludden et al. 1999). Among African Americans TPMT*3C is most prevalent, but TPMT*2 and TPMT*3A are also found (Hon, Fessing et al. 1999). This highlights the genetic admixture in the African American population that has been observed by others. It also points to the desire to perform genotype analysis in individual patients, in order to optimize the safety and efficacy of specific medications.

The prevalence of TPMT*3A mutation ranges from 0.9 to 5.7% in Caucasians and 0% in Asians (Ameyaw, Collie-Duguid et al. 1999, Zhou 2006, Tumer, Ulusoy et al. 2007) whereas the prevalence of TPMT*3C mutation varies between 0.6 to 3% in Asians and 0.3 to 1.6% in Caucasians (Spire-Vayron de la Moureyre, Debuysere et al. 1998, Ameyaw, Collie-Duguid et al. 1999, Sasaki, Goto et al. 2006, Zhou 2006).

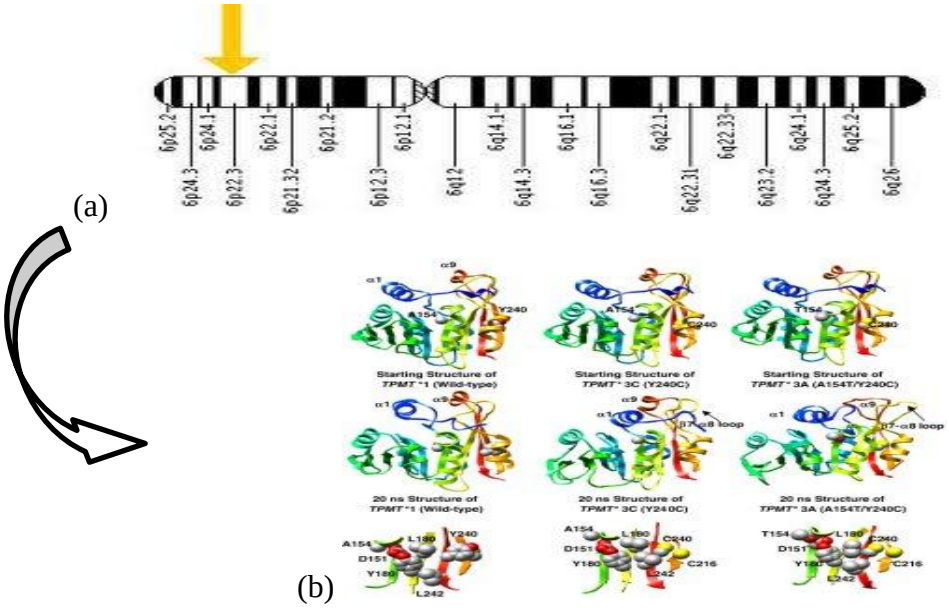


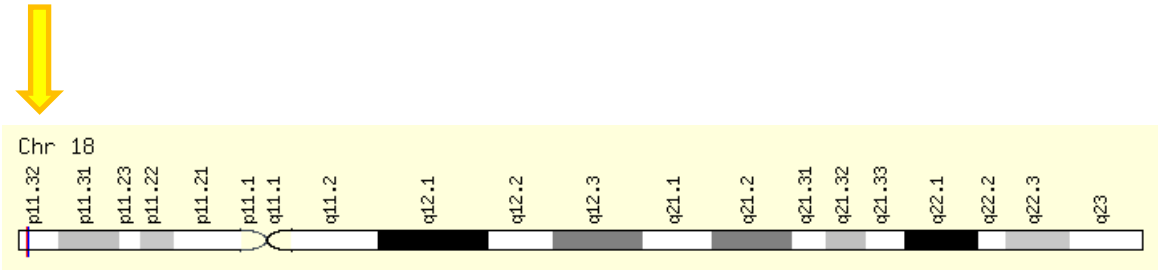
Figure 12: TPMT gene. (a) TPMT gene – chromosome 6 (base on http//: www.ghr.nlm.nih.gov) (b) TPMT protein (base on http//: www.depts.washington.edu)

TS polymorphism

TS (located on chromosome 18p11.32) (Figure 13) catalyzes the conversion of dUMP to dTMP which is required for DNA synthesis and repair (Radparvar, Houghton et al. 1988). The inhibition of this enzyme leads to deoxythymidine triphosphate (dTTP) depletion, chromosome

break and cell death (Welsh, Titley et al. 2000). A 28-bp polymorphic tandem repeat sequence in the 5' untranslated enhancer region of the human TS gene has been shown to influence TS expression. A polymorphism in the TSER has been shown to contain either two (TSER*2) or three (TSER*3) 28 bp tandem repeats. Compared to a double tandem repeat (TSER*2), the presence of a triple tandem repeat (TSER*3) increases TS expression in vitro, and is associated with higher in vivo tumor TS activity (Marsh 2005). Whereas homozygous TSER*3 correlates with increased TYMS expression in childhood ALL (Horie, Aiba et al. 1995), this variant may also be associated with MTX resistance and with a higher risk of relapse (Horie, Aiba et al. 1995, Giovannetti, Ugrasena et al. 2008). Accordingly, determining the TS genotype may help predict the patient response to cytotoxic treatment (Krajinovic, Costea et al. 2002). The prevalence of homozygous TSER*3 lies between 28 to 30% and 50 to 77% in Caucasian and Asian ALL children, respectively (Marsh, Ameyaw et al. 2000, de Jonge, Hooijberg et al. 2005, Giovannetti, Ugrasena et al. 2008).

(a)



(b)

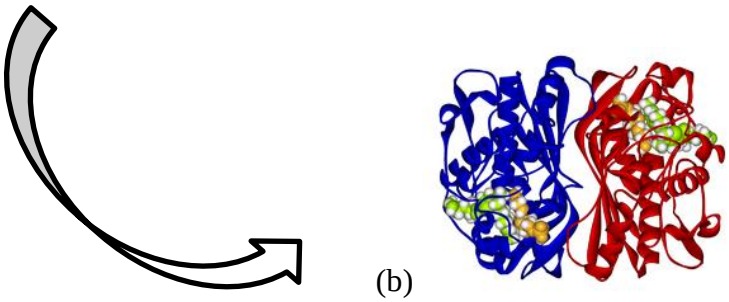


Figure 13: TS gene. (a) TS gene – chromosome 18 (base on en.wikipedia.org) (b) TS protein (base on http://www.ott.nih.gov)

2. Aims of study

2. Aims of study

General Aim:

Objectives of our study are to clarify roles of pharmacogenetics affecting transport and metabolism of chemotherapeutic drugs in the treatment of childhood ALL and will focus particularly on MTX. Thereby it might be possible to give more individualized treatment to improve therapeutic and reduce toxic effects. This study is based on the FRALLE 2000 protocol with extensive use of HD-MTX therapy. The overall aim is to find out new possible predictive factors in childhood ALL which are helpful to assess the value of established predictive factors for adverse effects of MTX and 6-MP in treatment.

Specific Aims:

1. To analyse therapeutic effects of protocol FRALLE 2000 on two groups of childhood ALL, Asian patients in Vietnam and Caucasian patients in Belgium:

1.1. To compare the characteristics of patients and the application of protocol FRALLE 2000 between these two groups.

1.2. To compare the side effects of HD-MTX and 6-MP, two main drugs in protocole FRALLE 2000, between these two group.

1.3. To analyse RFS of these two groups:

1.3.1. To compare RFS between these two groups.

1.3.2. To analyze which group's characteristics have an impact on the RFS.

1.4. To analyse the impact of relapse between these two group.

2. To analyse the roles of 7 important polymorphisms (CCND1 G870A, GGH C452T, MTHFR A1298C, MTHFR C677T, TS, and ITPA C94A, TPMT) influencing the transport and metabolism of HD-MTX and 6-MP.

2.1. To identify the frequency of those 7 polymorphisms in both series.

2.2. To analyse the correlations between 7 polymorphisms and RFS as well as ALL risk.

3. Materials and Methods

3. Materials and Methods

Patients and treatment

Caucasian and Vietnamese series of patients:

The Caucasian series included 94 ALL patients diagnosed under the age of 21 (mean age 6.66 ± 4.57 years with a median of 5.0 years, range: 1-20 years) between 2000 and 2011, and followed at the Cliniques Universitaires Saint-Luc (Université catholique de Louvain (UCL)), Brussels, Belgium. The Vietnamese series included 141 ALL patients diagnosed under the age of 16 (mean age 5.57 ± 3.52 years with a median of 5.0 years, range: 1-15 years) between 2005 and 2011, and followed at the Blood Transfusion and Hematology Hospital, University of Medicine Pham Ngoc Thach (UPNT) at Ho Chi Minh city, Vietnam. In both series, the patients were treated according to the FRALLE 2000 protocol. Only patients with therapy compliance were considered for this study.

Four and 25 patients were excluded from the Caucasian and Vietnamese series, respectively either because of a very young age (less than 1-year-old) or the presence of the Philadelphia chromosome. In these conditions, the treatment protocol differed between Belgium and Vietnam. Furthermore, the treatment protocol also differed between Belgium and Vietnam for relapsing patients. Accordingly, the current study compared the RFS between both series of ALL children.

Acute Lymphoblastic Leukemia features in both series

The diagnosis of ALL was based on BM and blood morphology according to the FAB classification, immunology, cytogenetic and molecular biology. Karyotype was performed in 50% of Vietnamese patients and 100% of Caucasian patients. Regarding cytogenetic studies, abnormalities were categorized in good and poor prognostic factors. Good prognostic factors include hyperdiploidy with more than 55 chromosomes, presence of t (12; 21), t (1; 19), and normal karyotype. Poor prognostic factors include: hypodiploidy with less than 45 chromosomes, MLL-rearrangement, or t (4; 11).

Treatment follow-up and dose intensity

To assess treatment response, BM examination and MRD evaluation were carried out at day 35 of the induction phase in both groups. CSF was studied at each IT injection. After completion of therapy, BM and CSF were examined on a regular basis during 3 years. At the end of this

time interval, children were considered to be cured. In contrast, treatment failure was classified as isolated CNS relapse, isolated BM relapse, combined BM relapse, and EM relapse.

As there was no reduction of dose in both series during the induction or consolidation phases, dose intensity of MTX and 6-MP were only calculated during the maintenance therapy. Indeed, it is worth noting that occurrence of toxicity was not handled the same way in Vietnam and in Belgium. In Belgium, the dose was temporarily reduced but drug regimens were only exceptionally stopped. In contrast, medications in Vietnam were stopped and resumed later so that each patient received ultimately 100% of the total dose required but not during the same time period. Accordingly, the occurrence of toxicity gave rise to longer treatment duration in Vietnam. The dose intensity was therefore computed for each patient of both series as the ratio between the total received dose and the treatment duration.

Toxicity recorded in the Caucasian and Vietnamese series

Toxicities of HD-MTX included mucositis, neutropenia, diarrhea, skin rash, liver failure, central neurotoxicity, and leukoencephalopathy. Only grade 3 or 4 were considered.

Laboratory technique

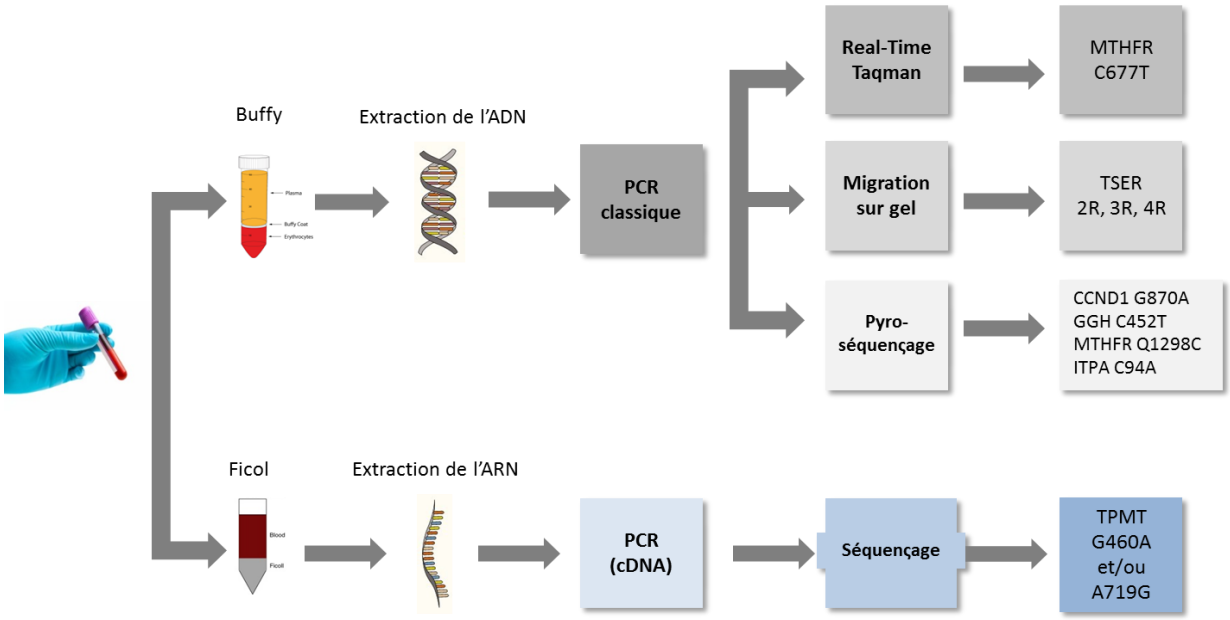


Figure 14 : Methods of laboratory

DNA and RNA isolation

For each subject, two venous blood samples (7.5 mL) were collected in EDTA tubes. The extraction of genomic DNA was performed from peripheral blood lymphocytes using the EZ1 DNA Blood kit and BioRobot EZ1 (Qiagen, Leusden, The Netherlands), according to the manufacturer's protocol (Figure 14).

Total RNA was isolated from whole blood over a Ficoll Hypaque and extracted with TRIzol® reagent (Invitrogen Life Sciences, Merelbeke, Belgium) according to the manufacturer's protocol (Figure 14).

Concentrations of extracted DNA and RNA were measured both using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and were stored at -20°C and -80°C until use, respectively.

Pyrosequencing

Identification of polymorphisms of the following genes was carried out: CCND1- (G870A), GGH- (C452T) and MTHFR- (A1298C) genes. Pyrosequencing was performed on a PSQ 96 MA (Qiagen, Vento, Belgium, Nertherlands). Primers used in polymerase chain reaction (PCR) and pyrosequencing (QIAGENE GmbH, Hilden, Germany), and size of corresponding amplicons are given in Table 2. The PCR reaction mixture was as follows: 5 µL buffer (10 mM Tris hydrochloride, and 50 mM potassium hydrochloride, pH 8.3), 3 mM $MgCl_2$, 1U Taq Gold polymerase, 200 µM of each dNTP, 10 pmol of each primer and 5 µL (~150-200 ng) of genomic DNA in a final volume of 50 µL. The cycling conditions were 95°C for 5 minutes, followed by 40 cycles at 95°C for 40 seconds, 60°C for 40 seconds, and 72°C for 80 seconds, with a final extension step at 72°C for 7 minutes.

For each target gene, a sequencing primer was further hybridized to the PCR product and incubated with reagents containing enzymes and substrates. One dNTP was added to the reaction mixture at a time and was incorporated into the DNA strand if it was complementary to the base in the template strand. The incorporation resulted in a release of pyrophosphate that was converted to ATP, which in turn drived conversion of the substrate luciferin to oxyluciferin, therefore generating light corresponding to a peak.

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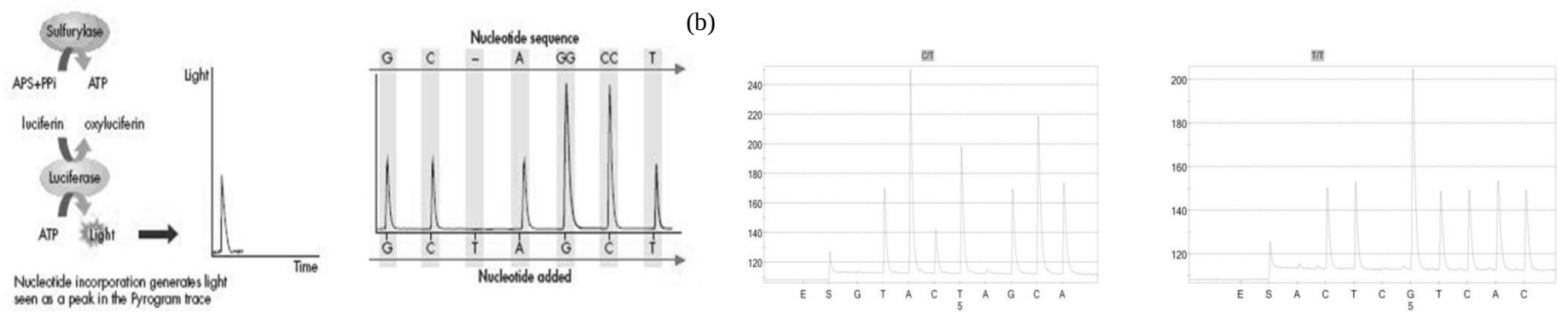
Figure 15: Pyrosequencing. (a) Pyrosequencing technical (jonhanneswilbertz.com) (b) Result of our study: GGH C452T (C/T) and CCND1 G870A (T/T)



(b)

(a) Pyrosequencing technical

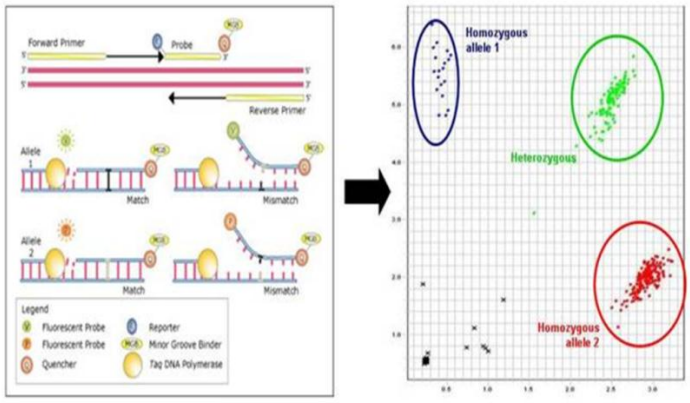
Table 2: Target genes and related single nucleotide polymorphisms: primers and probes used in the various pharmacogenetic assays

Target Gene and related polymorphism	Genbank (NCBI) Accession	Location	RS Number	Forward and reverse primers (5' – 3') used in polymerase chain reaction	Target size (bp)	Pyrosequencing primer [(1)], Taqman probes [(2)] (5' – 3')
CCND1 (G870A)	NG_007375.1	11q13	rs603965	F: Biot- CAC ACG CTT CCT CTC CAG AGT R: CAT TTC CGT GGC ACT AGG TG	108	S: GGACATCACCCTCACTTA[(1)]
GGH (C452T)	NG_028126.1	8p12.3	rs11545078	F: GAG CTT TCA CTG CTG ATT AGT GG S: Bio-TAC TTA CTA ATC CTG CCC AGC AA	142	S: ATTAGTGGAGAGTGCTTAT [(1)]
MTHFR (A1298C)	NG_013351.1	1p36.3	rs1801133	F: GGGAGGAGCTGACCAGTG R: Biot-CGCAGCAGCTCCTCCT	422	S: AGGAGCTGACCAGTGAA-3[(1)]
MTHFR (C677T)			rs1801131	F: AAG CAC TTG AAG GAG AAG GTG TC R: CCT CAA AGA AAA GCT GCG TGA	64	VIC-ATGAAATCGGCTCCC-MGB[(2)] FAM-ATGAAATCGACTCCCGC-MGB[(2)]
TSER (2R, 3R, 4R)	NG_028255.1.	18p11.32	Not applicable	F: CTG GCG CAC GCT CTC T R: FAM-TTG GAT CTG CCC CAG GT	315	Not applicable
TPMT (G460A and/or A719G)	NM_000367.2	6p22.3	rs1800460 rs1142345	F: GGAAGACATATGCTTGTGAGACA R: AAAAACATGTCAGTGTGATTTTATTTT	818	Not applicable
ITPA (C94A)	NC_018931.2	20p13	rs1127354	F: Biot- CTGAGTTGGTAAGCTTTAGGAGA R: CCAATATTAACTGAACCCTAGAGG	483	GCCACCAAAGTGCAT

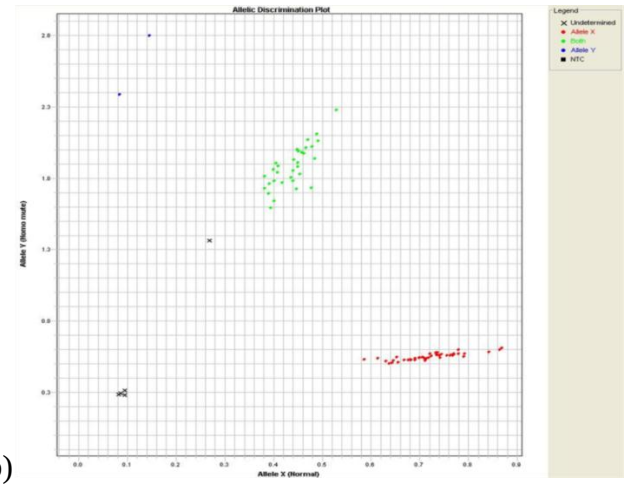
Biot: biotine ; Fam: mutated probe; MGB : Minor Groove Binding; NCBI: National Center for Biotechnology Information; VIC: wild type probe

Taqman real-time PCR assay using MGB probes

The MTHFR- C677T polymorphism was studied with two Taqman probes added together in one polymerase chain reaction (PCR) tube, each consisting of an oligonucleotide with a fluorescent reporter dye, a non-fluorescent quencher and an MGB group (Applied Biosystems, Foster City, CA, USA). VIC and FAM reporter dyes were used for the WT and mutated probes, respectively. A distinct emission wavelength maximum for each probe allowed the detection of allele-specific cleavage. PCR primers and MGB probes are detailed in Table 2. The reaction was carried out on the ABI PRISM 7900 machine with SDS 2.4.0 software (Applied Biosystems) in a final volume of 25 µl with 900 nM of forward and reverse primers, 200 nM of probes labeled with VIC and FAM, 12.5 µl of TaqMan® 2XUniversal Master Mix (Applied Biosystems), and 2.5 µl (~76 – 100 ng) of DNA sample.



(a) TaqMan technical



(b)

Figure 16: Taqman real-time PCR assay using MGB probes.

(a) TaqMan technical (source on medicine.tcd.ie/ Applied Biosystems)

(b) Result of MTHFR C677T polymorphism with real-time PCR

Polymerase chain reaction amplification

TYMS-TSER polymorphisms (TSER*2 and TSER*3) were analyzed by PCR using: buffer 1x, 1.5 mM $MgCl_2$, 2U Taq Gold polymerase, 200 µM dNTP, 10 pmol of each primer, 2.5 µL DMSO (Dimethylsulfoxyde) and H_2O to reach a final volume of 50 µL. The cycling conditions were 95°C for 5 min, followed by 35 cycles at 95°C for 40 s, 60°C for 40 s and 72°C for 1 min 20 s with a final extension step at 72°C for 7 min. The forward and reverse primers used for classic PCR of the TYMS-TSER gene are described in Table 2. DNA fragments for TSER*2 and *3 genotypes were run in a 2.5% agarose gel with ethidium bromide and visualized on a UV transilluminator. Sequencing analysis was performed in the assay validation phase as well as to confirm any unusual genotype (TSER*4). The DNA sequence of the purified product was determined on an automated ABI 3130 Genetic Analyzer using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

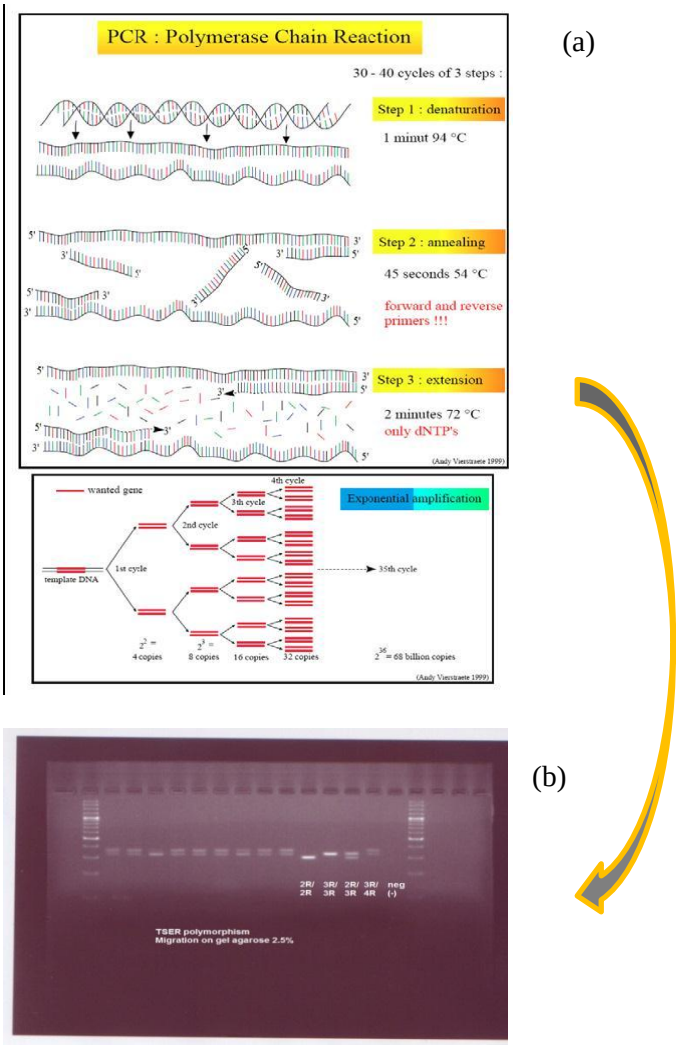
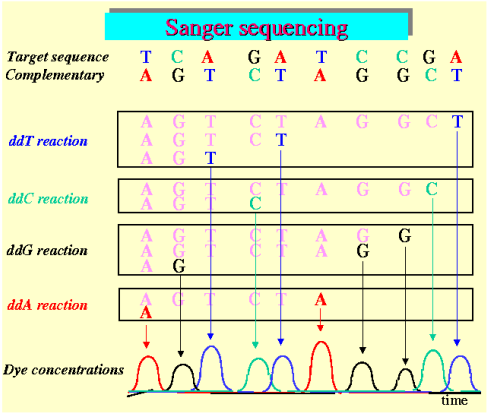


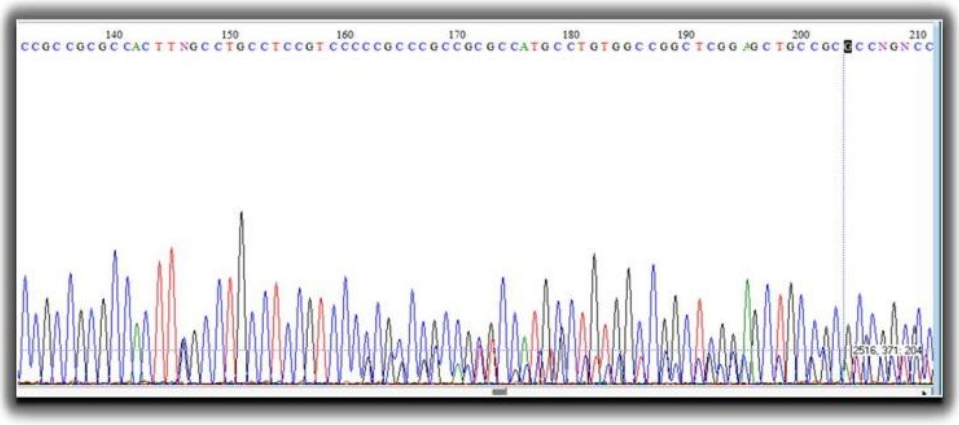
Figure 17: Polymerase chain reaction amplification. (a) PCR methods (b) Result of TSER polymorphism: migration on gel agarose 2.5%.

TPMT cDNA amplification

Total RNA was isolated from venous blood using Trizol reagent (Roche) and retro-transcribed by SuperscriptTM II RNase H-reverse transcriptase (Invitrogen) according to the manufacturers' instructions. TPMT complementary DNA (cDNA) was amplified by PCR using Ampli Taq DNA polymerase (Applied Biosystems) and a pair of primers that anneal to sequences within exon 4 and exon 10 of the published sequence of the TPMT cDNA (NCBI: accession number BC009596) (Table 2), as previously reported (Dewit, Moreels et al. 2011). cDNA sequencing was performed on both strands with the Big Dye® terminator cycle sequencing kit (Applied Biosystems), using an automated ABI3130 capillary sequencer. Sequences were compared with the wild-type sequence using the SeqScap version 2.0 software, which identifies variant and sequence matches from an allele library. In addition, sequences identify variant and sequence matches from an allele library.



(a)



(b)

Figure 18: TPMT cDNA amplification.

(a) Sequencing method (b) Result of sequencing

Statistical Analysis

Statistical analysis was performed using R 2.15.0 (http://www.r-project.org).

Within the Caucasian series, all patients were diagnosed for ALL after the beginning of the study in Belgium (2001). In contrast, some patients within the Vietnamese series were diagnosed for ALL before the beginning of the study in Vietnam (2009). Time between the diagnosis of ALL and inclusion into the series was introduced in the modelling as a left-truncated time in all time-to-event analyses in order to avoid overestimation of post-diagnostic survival probabilities and likely underestimation of hazard ratios in the Cox regression models.

Considering that Vietnamese families confronted with a relapsing child can rarely afford further drug therapy because of the financial burden of leukemia care, this study was not pursued after the first relapse. The RFS time was computed for each patient as the time from diagnosis of disease until first relapse or death.

Study I

The Caucasian and the Vietnamese clinical series were compared for clinical and biological variables using the chi-square test for discrete variables and the Student t test for continuous variables. RFS was estimated using the Kaplan-Meier product limit method. Curves were compared using the log-rank test. Multivariate Cox regression models were utilized for modeling the relationship between factors and RFS in the Vietnamese series and the white series and in the pooled series.

Regression models were adjusted for nationality, sex, age at diagnosis, WBC initial count, FAB classification, abnormal cytogenetics, immunophenotype, and corticosensitivity at day 8.

Study II

A multilocus genetic risk score (MGRS) was computed for each patient by summing the number of risk genotypes among the seven tested polymorphisms. Survival free of relapse curves were compared between low (≤ 3) and high (≥ 4) MGRS using the log-rank test. The effect of the MGRS was adjusted for clinical variables including race, age at diagnosis, WBC initial count, abnormal cytogenetics, corticosensitivity at day 8 (D8) using a multivariate Cox proportional hazards regression model.

All statistical analyses were adjusted for multiple testing using Bonferroni correction, with the exception of regression analyses as recommended.

4. Results and Discussion

STUDY 1: Comparison of Long-term Outcome between Caucasian and Vietnamese Children Treated for Acute Lymphoblastic Leukemia According to the FRALLE 2000 Protocol.

Aims of Study: To analyse therapeutic effects of protocol FRALLE 2000 on two different ethnic groups, we compared the clinical and biological parameters as well as the respective RFS of 2 series of Asian patients in Vietnam (n=141) and Caucasian patients in Belgium (n=94) treated according to the same protocol.

Study Introduction

The strong correlation of higher incidence of common ALL in early childhood with higher levels of socioeconomic development suggests that environmental factors linked to wealth contribute significantly to its etiology (Viana, Fernandes et al. 2001, Stiller 2004, Kroll, Stiller et al. 2012, Lightfoot, Johnston et al. 2012). The survival of children with ALL is lower in developing than in developed countries (Redaniel, Laudico et al. 2010). Indeed, the cure rate of childhood ALL is about 80% in resource-rich countries (Schrappe, Reiter et al. 2000, Pui and Evans 2006), whereas it remains as low as 40% to 50% in developing countries owing to the limited wealth resources and weak primary health care delivery system (Howard, Pedrosa et al. 2004, Gao, Lu et al. 2006).

Moreover, relapse related and treatment-related toxicity with fatal outcome also contribute to a decrease in the survival rate (Metzger, Howard et al. 2003, Mostert, Sitaresmi et al. 2006, Nguyen, Devidas et al. 2008). However, socioeconomic factors are not the only causal factors. A study from the US National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program from 1998 to 2008 showed that a poorer prognosis was observed in East Asians, specially Vietnamese and Filipinos, living in the United States, compared with non-Hispanic whites (Goggins and Lo 2012). This finding was interesting in that Asian ethnic groups do not seem to be socioeconomically disadvantaged (Goggins and Wong 2009, Goggins and Lo 2012).

In the current study, we compared the clinical and biological parameters as well as the respective RFS of 2 series of white and Vietnamese children with ALL treated according to the same FRALLE 2000 protocol. Considering that MTX is one of the most active agents for the treatment of ALL and is, as such, an essential component of most modern ALL frontline treatments worldwide, attention was also paid to the dose intensity of MTX and 6-MP and the

occurrence of adverse reactions. HD-MTX offers the advantage of targeting EM leukemia by producing cytotoxic concentrations in sanctuary sites where low-dose MTX does not readily distribute (eg, testes and CSF) (Barredo, Devidas et al. 2006, von Stackelberg, Hartmann et al. 2008).

Study Results and Discussion

The similarities and differences between two groups, Asian patients in Vietnam and Caucasian patients in Belgium, about the characteristics of patients and the application of protocol FRALLE 2000:

Clinical and biological data of both Caucasian and Vietnamese series were compared. There was no statistically significant difference in terms of sex, age at diagnosis, initial WBC count, CSF involvement at diagnosis, abnormal cytogenetic, and corticosensitivity at day 8. In contrast, Caucasian and Vietnamese clinical series differed significantly in terms of cytology (more L2-ALL in Vietnamese children (P<0.001)), flow cytometry (more T-ALL in Caucasian children (P=0.004)), a higher mean dose intensity of MTX and 6-MP, and more frequent grade 3 or 4 adverse reactions in Vietnamese children (P<0.001) (Table 3).

The distribution of the dose intensity of MTX and 6-MP, relative to the protocol recommendations, between both groups was significantly different (Figure 19) . The mean relative dose intensity was 0.88 in Belgium and was 0.94 in Vietnam. The number of patients who received a dose-intensity below 0.8 was much higher in Belgium. The difference is highly significant, also due to the high number of patients studied.

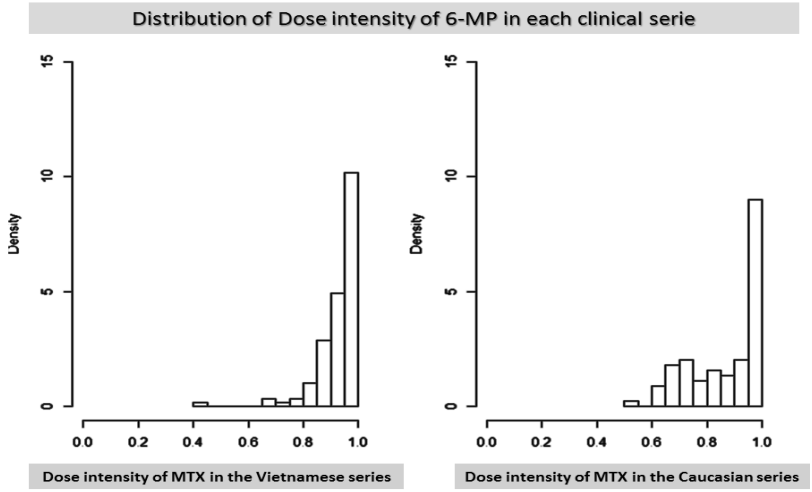


Figure 19: Dose intensity distribution of 6-MP (or MTX) between Vietnamese and Caucasian patients

The number of patients who received a dose-intensity below 0.8 was much higher in Belgium. The difference is highly significant, also due to the high number of patients studied.

Table 3: Clinical and Biological Characteristics of Patients.

Variables	Caucasian (n=94)	Vietnamese (n=141)	χ2 value	p value
Sex – no. (%)				
Boys	65 (69.1)	84 (59.6)		
Girls	29 (30.9)	57 (40.4)	1.835	0.176
Age at diagnosis – yr.				
Mean	6.67	5.57		0.09
Median	5.0	5.0		
Range	1-20	1-15		
≤ 10 years – no. (%)	72 (76.6)	113 (80.1)		
> 10 years – no. (%)	22 (23.4)	18 (12.8)		
Missing	0	10		
WBC initial count– no. (%)				
<50.000/mm³	73 (77.7)	103 (73.1)	<0.001	0.99
≥50.000/mm³	21 (22.3)	28 (19.9)		
Missing	0	10		
CSF involvement – no. (%)				
No	3 (3.2)	3 (2.1)		
Yes	91 (96.8)	138 (97.9)	0.257	0.613
FAB classification – no. (%)				
Type L1	51 (54.3)	18 (12.8)		
Type L2 and other type	43 (45.7)	113 (80.1)	**40.367**	**< 0.001**
Missing	0	10		
Abnormal cytogenetics (*) – no. (%)				
Good prognosis	88 (93.6)	127 (90.1)		
Poor prognosis	6 (6.4)	4 (2.8)	0.752	0.386
Missing	0	10		
Flow cytometry – no. (%)				
B precursor	75 (79.8)	122 (86.5)		
T precursor	19 (20.2)	8 (5.7)	**8.89**	**0.004**
Missing	0	11		
Corticosensitivity at D8 – no. (%)				
Sensitive	85 (90.4)	111 (78.7)		
Resistant	9 (9.6)	20 (14.2)	1.113	0.291
Missing	0	10		

Variables	Caucasian (n=94)	Vietnamese (n=141)	χ2 value	p value
Dose intensity of MTX or 6-MP (%)				
Min	52	43		
Max	100	100		
Mean	88	94	**9.529**	**<0.001**
Median	94	95		
ADR of HD-MTX (grade 3 or 4)–no. (%)				
No	88 (93.6)	89 (63.1)		
Yes	6 (6.4)	42 (29.8)	**19.99**	**< 0.001**
Missing	0	10		

(*) Abnormal cytogenetics: good prognostic factors include hyperdiploidy with more than 55 chromosomes, presence of t(1;19) or t(12;21), and normal karyotype. Poor prognostic factors include: hypodiploidy with fewer than 45 chromosomes, MLL-rearrangement and presence of t (4; 11).

Bold values emphasise significant results.

ADR indicates adverse drug reaction; CSF: cerebrospinal fluid; FAB: French-American-British classification; HD-MTX: high-dose methotrexate; MTX: methotrexate; 6-MP: 6-mercaptopurine.

Impact factors on Relapse Free Survival (RFS) of these two groups:

Relapse Free Survival was significantly higher in Caucasian:

RFS at 18 months and 5 years from diagnosis was 95.7% (95% confidence interval (CI), 91.7%-99.9%) and 83.8% (95% CI, 76.3%-92.0%) for Caucasian children, respectively, versus 70.4% (95% CI, 60.4%-82.0%) and 47.8% (95% CI, 35.6%-64.2%) for Vietnamese children, respectively (Fig. 21).

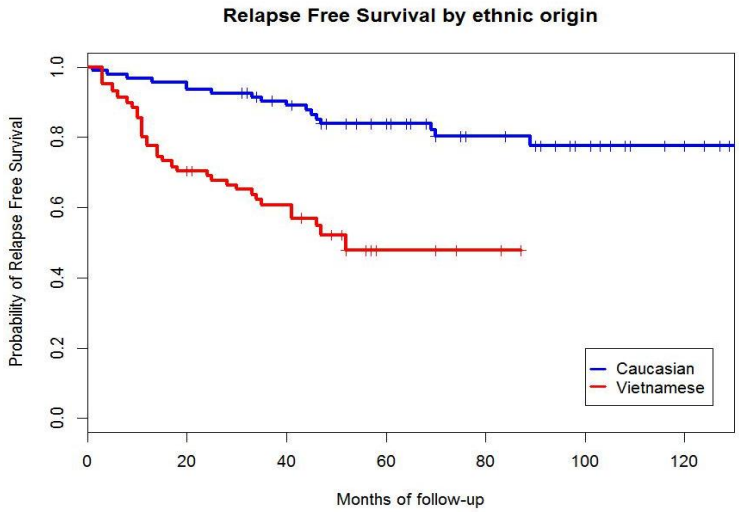


Figure 20: Relapse-free survival by ethnic origin.

Cox proportional hazards regression models were built for each clinical series (Caucasian, n=94; Vietnamese, n=141) and in the pooled series (n=235). Adjusted HRs is shown in Table 4.

Table 4: Clinical and Biological Characteristics of ALL Patients: Hazard Ratios from Multivariate Cox Regression Models for RFS.

	Caucasian Patients (n=94) Adjusted HR	Vietnamese Patients (n=141) Adjusted HR	Total Patients (n=235) Adjusted HR	***P***
Nationality				
Caucasian				
Vietnamese			**4.48 (2.16 - 9.3)**	**< 0.001**
Sex				
Boys	1.51 (0.49 - 4.66)	1.13 (0.55 - 2.33)	1.16 (0.64 - 2.09)	0.62
Girls				
Age at diagnosis (yrs)				
< 10 years				
≥ 10 years	1.19 (0.36 - 4.0)	1.33 (0.49 - 3.66)	1.22 (0.59 - 2.52)	0.59
WBC initial count				
<50.000/mm3				
≥50.000/mm3	2.48 (0.66 - 9.33)	1.45 (0.58 - 3.63)	1.69 (0.83 - 3.46)	0.15
FAB classification				
Type L1				
Type L2 and other type	1.02 (0.36 - 2.94)	0.64 (0.23 - 1.8)	0.82 (0.39 - 1.71)	0.59
Abnormal Cytogenetic				
Good prognosis				
Poor prognosis	2.07 (0.3 - 14.19)	3.69 (0.92 - 14.88)	**3.26 (1.14 - 9.31)**	**0.03**
Flow Cytometry				
B precursor				
T precursor	1.39 (0.35 - 5.58)	0.61 (0.1 - 3.71)	1.13 (0.46 - 2.79)	0.79
Corticosensitivity at D8				
Sensitive				
Resistant	1.88 (0.4 - 8.8)	1.52 (0.58 - 3.99)	1.54 (0.7 - 3.41)	0.28

Ethnic origin was found to be significant in Vietnamese children compared with Caucasian children (adjusted HR, 4.48; P<0.001). Vietnamese and Caucasian children with poor prognostic cytogenetic abnormalities had a higher risk of relapse with an HR of 3.69 and 2.07, respectively. An HR of pooled series was 3.26 (P=0.03). In Vietnam, BM and CSF relapses occurred very early, mostly within 18 months after diagnosis (Fig. 22). Compared with the Caucasian series, Vietnamese children had a higher risk of CSF relapse (HR=5.06; P=0.015) and BM relapse (HR=2.86; P=0.023). No significant difference was found between Caucasian and Vietnamese children in terms of EM relapse and combined BM and EM relapse.

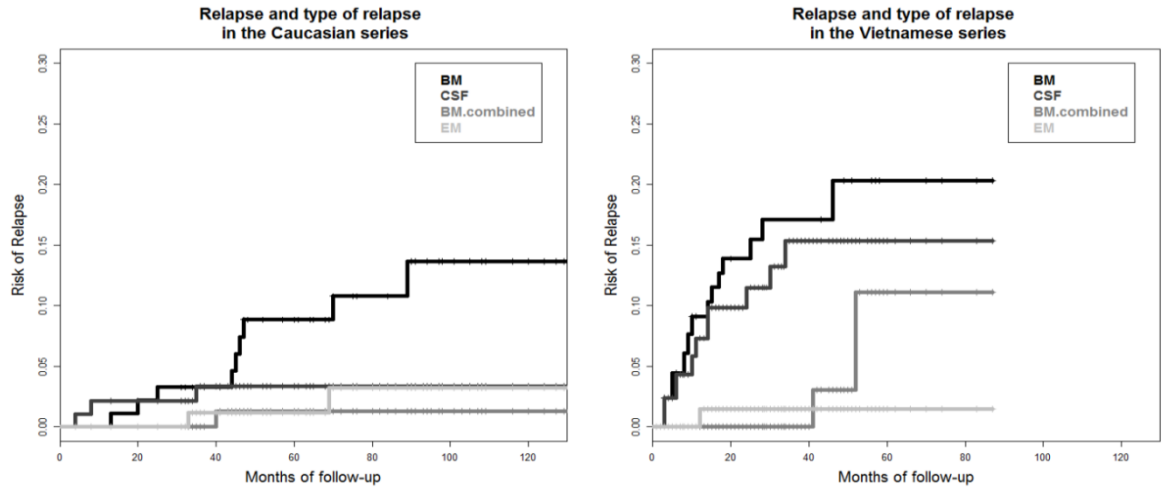


Figure 21: Type of Relapse in white and Vietnamese series.

BM: bone marrow; CSF: cerebrospinal fluid; EM: extramedullary.

In this comparative study of childhood ALL between Caucasian patients living in Belgium and Vietnamese patients living in Vietnam, the RFS was poorer in Vietnamese children (HR=4.48, P<0.001). Despite being treated with the same first-line protocol, Vietnamese children relapsed much earlier in BM and CSF than Caucasian children. The RFS at 18 months and at 5 years from diagnosis was significantly higher in Caucasian than in Vietnamese children.

Survival of children with ALL in developing countries is lower than in developed countries:

Survival of children with ALL in developing countries is reported to be lower than in developed countries. The most common causes of treatment failure reported are as follows: treatment discontinuation, lack of antibiotic prophylaxis, poor social status, and insufficient

high-quality health care system (Viana, Fernandes et al. 2001, Ribeiro and Pui 2005, Gao, Lu et al. 2006, Tang, Xu et al. 2008, Redaniel, Laudico et al. 2010, Wiemels 2012).

In Vietnam, a developing country, the costs for diagnostic and follow-up tests and for drugs in children under the age of 6 are covered at 100% by health insurance, whereas figures drop to 80% for children older than 6 years. Families hospitalizing a child have to pay for hospital beds (including for the person accompanying the patient) and for meals. Therefore, only patients with sufficient financial resources (ie, middle and upper class) were included and treated with FRALLE 2000 protocol. Patients who discontinued the treatment were excluded from the study. Supportive therapy was similar in both countries except for the additional use of acyclovir in Belgium. However, Vietnamese families confronted with a relapsing child could rarely afford further drug therapy because of the financial burden of leukemia care. That is the reason why we did not compare the outcome after the first relapse.

Available data were assessed to approach the reasons for such differences. Both series were comparable as far as age, sex, initial WBC count, and abnormal cytogenetic at diagnosis were concerned.

Impact factors on RFS in the application of protocol FRALLE 2000

Corticosensitivity at day 8 of treatment was similar in both series. L2-ALL was more frequent in Vietnamese children ($P<0.01$), whereas T-ALL was less frequent in the same series ($P<0.01$). T-ALL was present in 5.6% of the Vietnamese patients. This is similar to the 5.8% reported in the SEER study (Goggins and Lo 2012). The reason for such differences has not been determined but should not explain the worse prognosis observed in Vietnamese children. In contrast, T-ALL is known to have a worse prognosis. The SEER study reports a survival probability in the United States of 66% in East Asians with T-ALL as compared with 80% for B-ALL. As expected, poor prognostic cytogenetic aberrations had a negative impact on both groups. In that respect, RFS in Vietnamese children with poor cytogenetic abnormalities had an HR value nearly twice that found in Caucasian children (3.69 vs. 2.07, respectively). It implies a much more marked RFS difference in Vietnamese children with poor-risk and good-risk cytogenetic than observed in their Caucasian counterparts. Considering the worse RFS in Vietnamese children, the same observation applies with a strikingly higher difference between Vietnamese and Caucasian children with poor prognostic abnormalities compared with the difference observed in their respective counterparts with good prognostic abnormalities.

With regard to the first IT therapy, there was a substantial difference between both series. In the Belgium series, the first lumbar puncture was combined with the first IT injection. In the Vietnamese series, the first IT injection was delayed for a few days after diagnosis when the WBC count was high or when the lumbar puncture appeared to be traumatic. Both situations could have a negative impact on RFS as an intervening CSF contamination by leukemic cells may then not be ruled out (Rech, de Carvalho et al. 2005). Furthermore, CNS involvement at diagnosis may be underestimated as the first lumbar puncture is performed when the WBC count is already reduced by 50%.

Side effects of high dose Methotrexate and Mercaptopurine, two main drugs in protocole FRALLE 2000, between two groups:

A higher incidence of toxic events was observed in children aged 10 years and over at diagnosis, as well as in children being corticoresistant at day 8 (data not shown). In both situations, a dose-intensified treatment was administered inducing more toxicity. Even though HD-MTX administration was similar in both series, the incidence of HD-MTX-related grade 3 and 4 adverse events was significantly higher in Vietnamese children ($P<0.01$). One of the reasons might be the lack of pharmacokinetic monitoring of HD-MTX in Vietnamese children, which precludes any dosage adjustment, hence increasing the risk of MTX toxicity. An additional contribution of socioeconomic factors and/or variations in pharmacogenetic polymorphisms in Vietnamese patients cannot be ruled out, and this may also affect the metabolism of the drug and the outcome of Vietnamese children (Bhatia 2004, Cheok and Evans 2006, Kager and Evans 2006, Przekop, Tulgan et al. 2006, Goggins and Wong 2009, Goggins and Lo 2012).

Except for the first IT injection and the assessment of HD-MTX clearance, induction and consolidation phases were strictly comparable, whereas maintenance therapy differed in both series in terms of management of drug toxicity. In the latter case, Caucasian children received a reduced dose of 6-MP and MTX and discontinued the treatment after 18 or 24 months according to the protocol. In contrast, the Vietnamese children interrupted treatment in case of toxicity but resumed it later. Accordingly and compared with Caucasians, each Vietnamese patient received the same total dose of each drug but over a longer period of time, which ultimately resulted in a significantly higher dose intensity. In that respect, giving 6-MP every week has been reported to improve survival. The drug efficacy and survival benefit of 6-MP indeed appear associated with chronic systemic exposure to active metabolites of this agent

(Koren, Ferrazini et al. 1990, Relling, Hancock et al. 1999). It is therefore recommended to reduce the dose according to the WBC count rather than interrupting therapy, irrespective of the potential contribution of pharmacogenetics in the metabolism of 6-MP.

Study Conclusion

In this study, Vietnamese children with ALL have a worse prognosis than Caucasian children, despite receiving the same FRALLE 2000 protocol. It is of course somehow challenging to evaluate exactly the impact of socioeconomic factors and health care system differences in both series of patients. Nevertheless, only Vietnamese children from families with sufficient financial resources were included in this study. The treatment was administered in a single university hospital in Vietnam. Lessons to be learned from this study are as follows: firstly, the importance to initiate IT therapy as early as possible and to minimize the risk of traumatic lumbar puncture; secondly, to point out the need for a pharmacokinetic monitoring of HD-MTX with dosage adjustment to reduce the MTX-related toxicity; thirdly, to consider dose reduction during maintenance therapy rather than treatment interruption as is currently being done in Vietnam. There is no doubt whatsoever that increasing government spending on health, intensifying the medical training, and improving the disease management by educating the parents are concrete measures that are worth being taken into consideration in Vietnam.

Whether genetic differences in the metabolism and bioavailability of drugs used in the treatment of ALL (Cheok and Evans 2006, Kager and Evans 2006), especially MTX and 6-MP, might also affect the prognosis of ALL in Vietnamese children remains to be determined. This justifies further evaluation and comparison of both series of patients.

STUDY 2: Comparative pharmacogenetic analysis of risk polymorphisms in Caucasian and Vietnamese children with Acute Lymphoblastic Leukemia: prediction of therapeutic outcome?

Aims of Study: Aside from predisposing to ALL, polymorphisms could also be associated with poor outcome. Indeed, genetic variations involved in drug metabolism could, at least partially, be responsible for heterogeneous responses to standardized leukemia treatments, hence requiring more personalized therapy. The aims of this study were to (a) to determine the prevalence of seven common genetic polymorphisms including those that affect the folate and/or thiopurine metabolic pathways, i.e. CCND1 (CCND1-G870A), GGH-C452T, MTHFR-C677T and MTHFR-A1298C, TYMS-TSER, TPMT*3A and TPMT*3C and ITPA-C94A, in Caucasian (n = 94, age < 20) and Vietnamese (n = 141, age < 16 years) childhood ALL and (b) to assess the impact of a MGRS on RFS.

Study Introduction

Although current treatment protocols can cure approximately 80% of paediatric ALL patients, a number of children will relapse (Pui, Robison et al. 2008), with a relapse rate shown to vary within a group of patients with similar risk even though their leukemia blasts carry the same characteristics. Besides tumour and patient related factors, inter-individual variations in treatment responses and toxicity could also be attributable to genetic polymorphisms influencing drug disposition and treatment intensity.

While multiple drugs are used to treat children with ALL, mounting evidence indicates that gene polymorphisms may alter therapy responses, as they participate in folate and 6-MP/AZA metabolism, steroid response, drug transport and detoxification (Kager and Evans 2006). Polymorphisms in genes interacting with folate metabolism and MTX activity such as the MTHFR and the promoter enhancer region of TS (TYMS-TSER), or intervening in the 6-MP metabolic pathway such as TPMT and ITPA could explain at least part of the inconsistent clinical outcome in children with ALL receiving similar treatments (Aplenc, Thompson et al. 2005, Rocha, Cheng et al. 2005, Stanulla, Schaeffeler et al. 2005).

However, data regarding the Caucasian and Vietnamese allelic distribution of polymorphic variants involved in MTX metabolism in children with ALL are scarce. Polymorphisms

within MTHFR, TS-TSER, TPMP and ITPA, as well as CCND1, GGH genes were assessed in this study.

Study Results and Discussion

Distribution of genotypes of folate and thiopurine associated polymorphisms in Caucasian and Vietnamese children with ALL.

The distribution of each genotype studied in 94 Caucasian and 141 Vietnamese children with ALL is listed in Table 5.

Table 5: Genotype frequencies of folate and thiopurine pathways associated polymorphisms in Caucasian and Vietnamese children with ALL.

Polymorphic loci	Genotype	Protective vs Risk	Caucasian (N=94) n (%)	Vietnamese (N=141) n* (%)	Raw P value £	Adjusted P value ¥
CCND1 G870A					0.24	1.00
	G/G	P	28 (29.8)	22 (18.6)		
	G/A	P	45 (47.9)	60 (50.9)		
	A/A	R	21 (22.3)	36 (30.5)		
GGH C452T					0.13	0.97
	C/C	P	74 (78.7)	103 (87.3)		
	C/T	R	20 (21.3)	15 (12.7)		
MTHFR C677T					**0.001**	**0.008**
	C/C	P	42 (44.7)	80 (67.8)		
	C/T	R	43 (45.7)	36 (30.5)		
	T/T	R	9 (9.6)	2 (1.7)		
MTHFR A1298C					0.153	1.00
	A/A	P	54 (57.4)	55 (46.6)		
	A/C	R	31 (33)	54 (45.8)		
	C/C	R	9 (9.6)	9 (7.6)		
TS(TSER)					**< 0.001**	**<0.001**
	*2R/*2R	P	20 (21.3)	3 (2.5)		
	*2R/*3R	P	41 (43.6)	35 (29.7)		
	*3R/*3R	R	33 (35.1)	79 (67.0)		
	*3R/*4R	R	0	1 (0.8)		
TPMT(*3A and *3C)					0.31	1.00
	No mutation	P	90 (95.7)	116 (91.3)		
	Mutation	R	4 (4.3)	11 (8.7)		
	TPMT*3A		4 (4.3)	0		
	TPMT*3C		0	11(8.7)		
ITPA A94C					**0.003**	**0.02**
	A/A	P	1 (1.1)	4 (3.4)		
	A/C	P	13 (14.0)	36 (30.5)		
	C/C	R	79 (84.9)	78 (66.1)		

* Sum of n not equals to 141 because of missing data. £Chi-square test to compare reference with associated genotype between Caucasian and Vietnamese. ¥Bonferroni adjusted P values for multiple (n=7) testing.

The prevalence of *CCND1*-A870G, *GGH*-C452T, *MTHFR*A1298C and *TPMT* polymorphisms between Caucasian and Vietnamese series was not statistically different. Although *TMPT*3A* and *TMPT*3C* were detected in the Caucasian and Vietnamese series, respectively, their frequency was very low. Accordingly, they were pooled together within the TPMT group to allow the statistical analysis.

The prevalence of *MTHFR*-677TT genotype was significantly higher in the Caucasian (9.6%) than in the Vietnamese (1.7%) series ($P = 0.008$). Conversely, the prevalence of *TYMS-TSER*3R/3R* and *ITPA*-94AC/AA genotypes were significantly higher ($P < 0.001$ and $P = 0.02$, respectively) in Vietnamese (67.0% and 33.9%, respectively) than in Caucasian (35.1% and 15.1%, respectively) children with ALL.

This study showed differences between Caucasian and Vietnamese children with ALL in the frequency distribution of MTHFR-C677T, TYMS-TSER and ITPA-C94A polymorphisms. Indeed, Vietnamese children had a much higher proportion of TYMS-TSER*3R/3R and of ITPA- 94AC/AA genotypes, whereas the prevalence of the MTHFR-677TT genotype was very low. No significant difference was found between both series in the frequency distribution of CCND1-G870A, GGH-C452T, MTHFR-A1298C and TPMT polymorphisms. However TPMT*3A (G460A and A719G) was only found in Caucasian children while TPMT*3C (A719G) was only found in Vietnamese children. TPMT*3B (G460A) was found neither in Caucasian nor in Vietnamese children.

Vietnamese children had a two-fold higher prevalence of TSER*3R/3R than Caucasian children. These results are in line with data from Dutch (de Jonge, Hooijberg et al. 2005) and Indonesian children with and without leukemia (Giovannetti, Ugrasena et al. 2008). It is of note that a very rare TSER*3R/4R genotype was identified in a single case from the Vietnamese clinical series (one out of 141 children) and was associated with a poor short term outcome after chemotherapy. The prevalence of MTHFR-677TT genotype in Caucasian and Vietnamese ALL children was 9.6% and 1.7%, respectively. While not found in Caucasian children, TPMT*3C is known to be more prevalent among Vietnamese (~10%) and Thai (11%) than among other Asian patients (i.e. Chinese, Japanese, Indian and Malaysian) where prevalence ranges between 0.8% and 3% (Sasaki, Goto et al. 2006, Zhou 2006). While GGH-452TT genotype was not found in this study, this may well be biased by the moderate sample size. It is indeed a rare variant being only infrequently reported in a Japanese population (one out of 269) (Hayashi, Fujimaki et al. 2007), in Chinese leukemia children (one in 203) (Chen, Wen et al. 2012), but not found in Mexican paediatric patients with ALL (n = 70) (Organista-

Nava, Gómez-Gómez et al. 2010). The prevalence of ITPA-94AC/AA genotypes in Caucasian (15.1%) and Vietnamese (33.9%) children with ALL was in line with previous studies (Marsh, Ameyaw et al. 2000, Stocco, Cheok et al. 2009, Farfan, Salas et al. 2014).

Correlations between 7 polymorphisms and RFS.

The univariate analyses show that the individual impact of each variant was not statistically significant probably due to a limited association with RFS and a small sample size. All P values were adjusted for multiple testing with Bonferroni correction because of the number of hypothesis tests (n = 21) which were simultaneously conducted. The Bonferroni adjusted P value obtained with the TPMT*3A genotype in the Caucasian series was not statistically significant and should therefore be confirmed in larger clinical series.

Whereas the association between TPMT polymorphisms and 6-MP cytotoxicity is widely accepted (Lennard, Lilleyman et al. 1990, Krynetski, Tai et al. 1996, Zhou 2006), reports on a potential association between TPMT mutations and RFS are extremely scarce. In a hospital-based cohort study of 172 childhood ALL patients (Lilleyman and Lennard 1994), a 6-TGN concentration lower than the median concentration value (i.e. 284 pmol per 8 × 108 erythrocytes) was associated with a 63% RFS at 5 years whereas RFS was 84% at 5 years in patients with the median concentration value (RFS difference 21%; 95% CI = 3, 39%; P = 0.0018). As the 6-MP metabolic pathway is influenced both by TPMT and ITPA genotypes, the effect of ITPA genotyping on RFS was also assessed in this study.

Regarding ITPA variants, it is worth emphasizing that evidence on their impact in terms of toxicity and survival are scarce and often controversial (Adam de Beaumais, Fakhoury et al. 2011, Kim, Kang et al. 2012), which explains why the impact of ITPA on RFS remains so far unknown. ITPA- 94CA/AA genotypes appeared both in the Caucasian and Vietnamese series as a protective genotype in this study, whereas the wild-type ITPA-94CC genotype was associated with a significantly poorer survival. ITPA-94CC was therefore considered as a risk factor in univariate analysis of RFS, as well as in the computation of the MGRS. While the pharmacologic explanation of the effect of ITPA-C94A on RFS is so far unclear, it could be related to the unfavourable effect on the pool of 6-TGN. 6-TGN is indeed incorporated in nucleic acids or with phosphate in 6-TITP, a process that determines the clinical efficacy but can be reversed by ITPA (Adam de Beaumais, Fakhoury et al. 2011).

Regarding the impact of the folate pathway, many but not all studies have reported the association of genetic variations in the selected genes with the effect of MTX and relapsed

risk. In a cohort study (Krajinovic, Lemieux-Blanchard et al. 2004) and case-control study (Aplenc, Thompson et al. 2005) of 201 and 137 paediatric patients with ALL, respectively, MTHFR-C677T and MTHFR-A1298C variants were indeed associated with an increased risk of relapse.

In a prospective (Rocha, Cheng et al. 2005) and case-control (Haferlach 2003) study of 247 and 80 childhood ALL patients, respectively, homozygous TSER*3R/3R variants showed a greater risk of haematological relapse related to drug resistance. However, the assessment of 20 polymorphisms located in 11 genes (i.e. GSTM1, GSTT1, GSTP1, NQO1, MTHFR, MTHFD1, SLC19A1, ABCB1, TSER, CRC5 and IL15) in a cohort study of 463 paediatric ALL patients failed to demonstrate any association between MTHFR and TSER variants with event-free survival (EFS). Conversely, ABCB1 3435TT and CCR5 246GA were found to predict lower EFS probability than their 3435CC (HR = 2.496; P = 0.012) and 246GG (HR = 1.82; P = 0.033) genotype counterparts (Lu, Kham et al. 2011). In a Cox regression model of EFS adjusted for clinical variables such as ethnicity, gender, age, lineage, initial WBC and cytogenetic subtypes, a significant difference (P = 0.0015) was found only with cytogenetic subtypes. This is in agreement with our own findings, i.e., a strong association between abnormal cytogenetic (associated with a poor prognosis group), Vietnamese ethnicity and the risk of relapse (Hoang, Ambroise et al. 2014).

Multilocus Genetic Risk Score improve the predictive accuracy of the clinical model.

The MGRS was computed for each patient as the number of risk genotypes. In our study, MGRS was based on seven genotypes at multiple single nucleotide polymorphisms. It was used to identify the association between genetics and risk of relapse in each patient. For example: If MGRS was scored at 3 (or 4), it means that the patient had 3 (or 4) risk of relapse polymorphisms (Table 6). Because of the limited number of patients, the original MGRS ranging between 0 and 6 were dichotomized in two categories (≤3 and ≥4). Compared with children with a low MGRS (≤3), a high MGRS (≥4) significantly increased the risk of relapse (univariate HR = 2.06; 95% CI = 1.01, 4.21; log rank *P* value = 0.042) (Figure 23).

Table 6 : Example of MGRS in patients (R = risk, P = protective)

Gene	CCND1 G870A	GGH C452T	MTHFR A1298C	MTHFR C677T	TS	TPMT (*3A et*3C)	ITPA A94C	MGRS
Patient 1	R	R	R	P	P	P	P	**3**
Patient 2	R	R	R	R	P	P	P	**4**

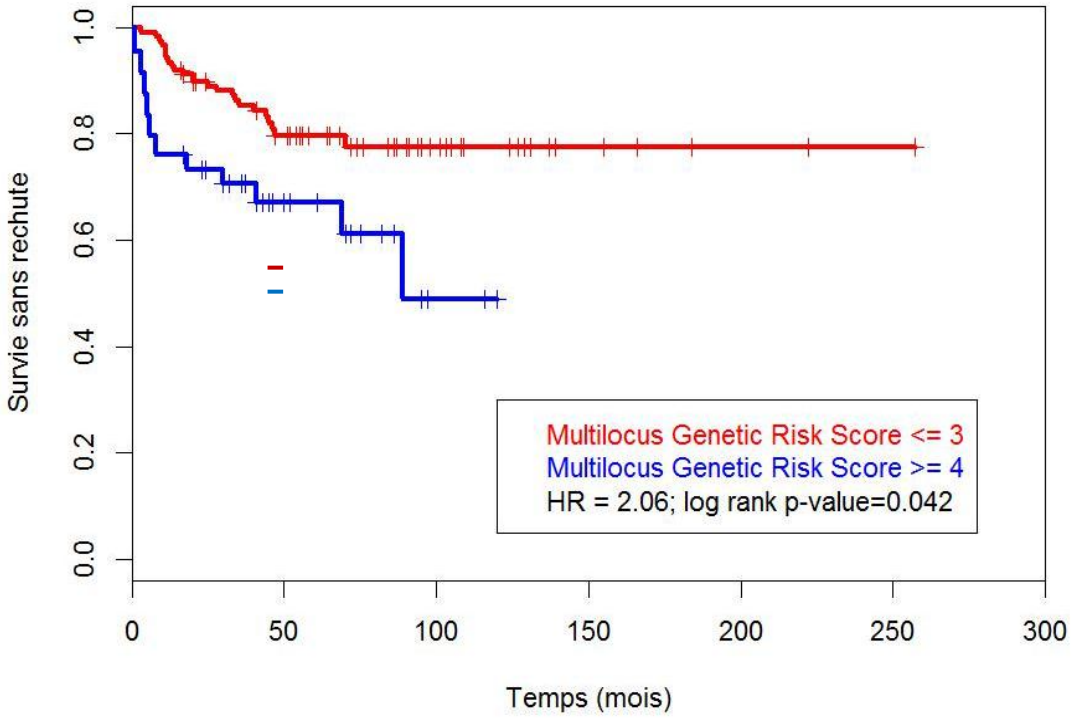


Figure 22: Relapse free survival according to multilocus genetic risk score.

Compared with those with a low MGRS (≤ 3), ALL children with a high MGRS (≥ 4) presented a significantly increased risk of relapse (HR=2.06; 95%CI = 1.01 - 4.21; log- rank P value = 0.042)

The adjusted HR of MGRS between children with a low (≤3) and high (≥4) MGRS computed from the multivariate model integrating race/ethnicity and four clinical variables was equivalent (HR = 2.09, 95% CI = 1.06, 4.13, *P* value = 0.03) to the univariate HR (Table 7). Adding MGRS to the model only slightly impacted on the highly significant HR of the single race/ethnicity variable. Compared with Caucasian children, the risk of relapse in Vietnamese

children was increased by 4.39 (95% CI = 2.28, 8.46, P value <0.001) times, irrespective of MGRS.

Table 7: Multivariate Cox proportional-hazard regression model in the pooled series with and without multilocus generic risk score.

	Cox PH model without MGRS		Cox PH model with MGRS	
	Hazard ratios (95%CI)	p value	Hazard ratios (95%CI)	p value
Race				
Belgian	1		1	
Vietnamese	**4.19 (2.20 – 7.97)**	**<0.001**	**4.39 (2.28 – 8.46)**	**<0.001**
Age at diagnosis				
<10 years	1		1	
≥ 10 years	1.26 (0.60 – 2.65)	0.54	1.37 (0.64 – 2.92)	0.42
Initial WBC count				
< 50.000/mm³	1		1	
≥ 50.000/mm³	1.82 (0.91 – 3.64)	0.09	1.57 (0.77 – 3.17)	0.21
Abnormal cytogenetics				
Good prognosis	1		1	
Poor prognosis	**3.66 (1.31 – 10.24)**	**0.01**	**3.51 (1.23 – 9.99)**	**0.02**
Corticosensitive at day 8				
Sensitive	1		1	
Resistant	1.41 (0.66 – 3.00)	0.36	1.38 (0.64 – 2.98)	0.42
MGRS				
≤ 3			1	
≥ 4			**2.09 (1.06 – 4.13)**	**0.03**

Predictive accuracy of the model was higher with than without MGRS on the whole range of tested time points. The predictive accuracy of both Cox regression models slightly decreased with longer time points (Figure 24).

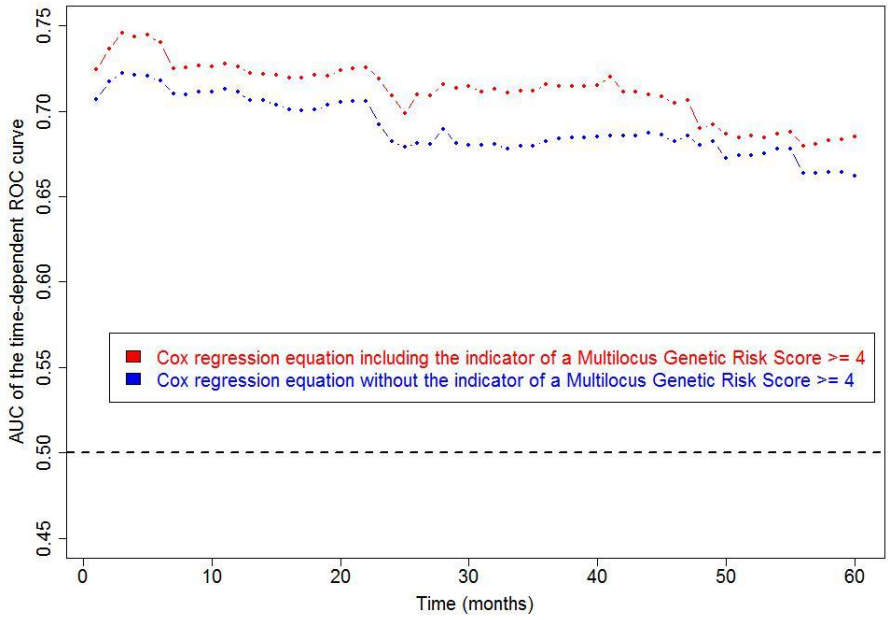


Figure 23: Predictive accuracy of Cox regression models. AUC of the time-dependent ROC curve obtained on the cross-validated predictions of the model with (red) and without (blue) MGRS.

In the current study, the univariate analysis of RFS showed a significant impact of MGRS and multivariate analysis indicated being Vietnamese, having a high MGRS and having abnormal cytogenetic significantly increased the risk of relapse. MGRS improved the predictive accuracy of the clinical model integrating ethnicity and four relevant clinical variables. The MGRS benefit was particularly marked during the first 4 years but tended to decrease with longer time points. However, there were a restricted number of polymorphisms and patients assessed in both clinical case series. Furthermore, the association between selected polymorphisms and adverse drug reaction to MTX and 6-MP was not assessable.

What special results do we get out from this study?

*TSER*3R/4R*

It is of note that a very rare TSER*3R/4R genotype was identified in a single case from the Vietnamese clinical series (one out of 141 children) and was associated with a poor short term outcome after chemotherapy.

ITPA 94AA

Vietnamese children had a higher prevalence of ITPA 94CA and AA than Caucasian children. ITPA 94AA genotype in our series was only present in 4/118 Vietnamese children and in 1/93 Caucasian child. It is of note that it is a rare genotype in the literature. The ITPA

94AA genotype was reported in the literature in 2/100 children in Korea (Kim, Kang et al. 2012), 0 /103 in Chile (Farfan, Salas et al. 2014) and 0/200 in Japan (Kudo, Saito et al. 2008).

Study Conclusion

Although patients in both series were treated according to the same protocol, there were major differences in the therapeutic management (Hoang, Ambroise et al. 2014). Limitations concerned the day of first IT therapy and the way MTX toxicity was managed (resulting in a higher dose intensity in Vietnamese children), which could explain in part the difference of RFS between the series. However, it is of note that significant differences in the prevalence of the MTHFR, TYMS-TSER and ITPA polymorphism were also detected, suggesting that MGRS may improve the predictive accuracy of the clinical model. To strengthen the current observation, the impact of pharmacogenetic determinants affecting the folate and/or thiopurine pathway genes on RFS in different ethnic groups deserves further study in a larger and more homogeneous clinical series of patients.

Additional SNPs, such as those previously reported (i.e. ATP-binding cassette subfamily B member 1 transporter (ABCB1), chemokine receptor 5 (CCR5)) (Lu, Kham et al. 2011), could also be integrated as potential predictive factors when further assessing and evaluating the strength of association between well determined pharmacogenotypes and outcome of paediatric ALL.

5. Conclusion and Perspective

Conclusions and Perspectives:

There is very little research and articles in the literature who study childhood ALL in Vietnam. Our prospective studies compared Vietnamese (n=141) and Caucasian (n=94) children with ALL. Based on our findings, the following conclusions could be made:

- RFS was significantly worse in Vietnamese patients with HR= 4.48, $p<0.01$, despite receiving the same FRALLE 2000 protocol as Caucasian patients.
- Vietnamese patients had a higher risk of CSF and BM relapse, which occurred earlier than in Caucasian patients.
- The prevalence of MTHFR 677TT, TSER*3R/3R and ITPA 94AA/AC genotypes differed significantly between Vietnamese and Caucasian clinical series.
- Our pooled data suggest evidences for a role of polymorphisms in the MTX and thiopurine pathway and risk of relapse.

Lessons from this study are as follows:

Firstly, the importance to initiate IT therapy as early as possible and to minimize the risk of traumatic lumbar puncture.

Secondly, the need of performing a pharmacokinetic monitoring for HD-MTX, with dosage adjustment in order to reduce the MTX-related toxicity.

Thirdly, to consider dose reduction during maintenance therapy rather than treatment interruption as is currently being done in Vietnam.

In order to improve the results and to reduce the high rate of early relapses, we need (1) to train our pediatricians to do the first IT injection as soon as possible, with a minimal risk of traumatic lumbar puncture, (2) to monitor the pharmacokinetic of HD-MTX in order to adjust the dose and to administer the leucovorin at the right dose, at the right time. The choice of MTX and leucovorin doses may be regarded as an intricate balance between effect and counter-effect and (3) to consider a dose reduction during maintenance therapy rather than treatment interruption. Indeed, the drug efficacy and survival benefit of 6-MP appear to be associated with chronic systemic exposure to active metabolites of this agent. Even if the relative dose intensity is higher in Vietnam due to prolonged administration, the efficacy is probably reduced by the regular interruption of the maintenance therapy

Furthermore, significant differences in the prevalence of the MTHFR, TSER and ITPA polymorphism were also detected in our study, suggesting that MGRS may improve the

predictive accuracy of the clinical model. The higher MGRS have contributed to relapse of childhood ALL in this cohort, but we also have to acknowledge that our statistical power was limited by the relatively small number of patients included in this study.

To strengthen these current observations, further study deserves confirmation on a larger cohort of patients. Additional SNPs, such as those previously reported (i.e., ABCB1, CCR5, RFC1), could usefully be integrated as potential predictive factors when further assessing and evaluating strength of association between pharmacogenotypes and outcome of pediatric ALL. A resulting drug dose adjustment, if feasible, will be beneficial to the patients. A regular review of the literature on the subject will be of upmost importance to keep up to date in this very quickly moving field.

In conclusion, it will probably be necessary to make choices among all these suggestions. Improving every day's clinical practice is essential. Well-trained pediatric hematologists/oncologists play an important role in applying the treatment protocol.

To join the leaders in the field of ALL, rethinking together the protocol, is also a way to better understand all the requirements. This will help to apply them all with more accuracy. The inclusion of patients in a protocol rather than just following it, is also very important in order to follow accurately the guidelines.

Furthermore, the local specialists are responsible to identify correctable defects in the health care system and to implement cost-effective changes. Support from a private foundation could help to improve the health care system, to support patients and families who are in need, and to raise money to be able to implement new techniques like genotyping and to do further research in this very interesting field.

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APPENDIX

STUDY I (published)

ORIGINAL ARTICLE

Comparison of Long-term Outcome Between White and Vietnamese Children Treated for Acute Lymphoblastic Leukemia According to the FRALLE 2000 Protocol

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Aim of this Study: To compare the relapse-free survival (RFS) in Vietnamese (n = 141) and white (n = 94) children living in Vietnam and Belgium, respectively, and treated in their own country for acute lymphoblastic leukemia according to the same FRALLE 2000 protocol.

Results: RFS was significantly worse in Vietnamese children (hazards ratio = 4.48; 95% confidence interval [CI], 2.16-9.3; $P < 0.01$). The 5-year RFS was 83.8% (95% CI, 76.3%-92.0%) and 47.8% (95% CI, 35.6%-64.2%) for white and Vietnamese children, respectively. In the latter group, relapses occurred in bone marrow and cerebrospinal fluid at a much earlier stage. The outcome was compared at first relapse only because of different treatments afterward, according to the country. Both series were similar for sex, age at diagnosis, initial white blood cell count, cytogenetic abnormalities, and corticosensitivity at day 8. Higher frequency of L2-acute lymphoblastic leukemia ($P < 0.001$) but lower frequency of T-acute lymphoblastic leukemia ($P = 0.004$) were observed in Vietnamese children.

Conclusions: Several factors may contribute to the poor RFS in Vietnamese children, which include the time interval before the first intrathecal therapy and differences in the management of drug-related toxicity. However, additional contribution of socioeconomic factors and/or variations in pharmacogenetic polymorphisms in Vietnamese patients cannot currently be ruled out.

Key Words: leukemia, children, racial disparity

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Acute lymphoblastic leukemia (ALL) is the most common cancer diagnosed in children, representing over 25% of the childhood cancer cases.[1] Its annual incidence rate is approximately 9 to 10 cases per 100,000 population in children, with a peak incidence between 2 to 5 years of age.[2] The strong correlation of higher incidence of common ALL in early childhood with higher levels of socioeconomic development suggests that environmental factors linked to wealth contribute significantly to its etiology.[3–6] The survival of children with ALL is lower in developing than in developed countries.[7] Indeed, the cure rate of childhood ALL is about 80% in resource-rich countries,[8,9] whereas it remains as low as 40% to 50% in developing countries owing to the limited wealth resources and weak primary health care delivery system.[10,11] In that respect, it is worth noting that >60% of the children worldwide have little or no access to effective cancer therapy[12]; among those who have access to therapies in developing countries, the most common cause of treatment failure is treatment discontinuation, accounting for at least 30% to 40% of the patients, even in dedicated treatment centers.[13,14] Moreover, relapse-related and treatment-related toxicity with fatal outcome also contribute to a decrease in the survival rate.[13–15]

However, socioeconomic factors are not the only causal factors. A study from the US National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program from 1998 to 2008 showed that a poorer prognosis was observed in East Asians, specially Vietnamese and Filipinos, living in the United States, compared with non-Hispanic whites.[16] This finding was interesting in that Asian ethnic groups do not seem to be socioeconomically disadvantaged in the United States relative to non-Hispanic whites, as shown by the US Census data.[17] Although a strong limitation of the SEER study consisted of unavailable prognostic factors such as white blood cell (WBC) initial count and chromosomal abnormalities,[17] several studies have recently confirmed the impact of racial and ethnic differences in the survival of children with ALL, without clear explanation for such observation.[7,18,19]

In the current study, we compared the clinical and biological parameters as well as the respective RFS of 2 series of white and Vietnamese children with ALL treated according to the same FRALLE 2000 protocol. Considering that methotrexate (MTX) is one of the most active agents for the treatment of ALL and is, as such, an essential component of most modern ALL frontline treatments worldwide, attention was also paid to the dose intensity of MTX and 6-mercaptopurine (6-MP) and the occurrence of adverse reactions. High-dose MTX (HD-MTX) offers the

advantage of targeting extramedullary leukemia by producing cytotoxic concentrations in sanctuary sites where low-dose MTX does not readily distribute (eg, testes and cerebrospinal fluid [CSF]).[20,21]

PATIENTS AND METHODS

White and Vietnamese Series of Patients

The white series included 98 patients diagnosed under the age of 20 years between 2000 and 2011 and who were followed up at the Cliniques universitaires Saint-Luc, Brussels, Belgium. The Vietnamese series included 166 patients diagnosed under the age of 16 years between 2005 and 2011 at the Blood Transfusion and Hematology Hospital, University of Medicine Pham Ngoc Thach (UPNT) at Ho Chi Minh City. Before 2005, Vietnamese patients were treated according to previous protocols (FRALLE 93 and 97). Consequently, they were not included in this study. Only patients able to afford the burden of chemotherapy, supportive therapy, and hospitalization were eligible to enter this study.

Four and 25 patients were excluded from the white and Vietnamese series, respectively, either because of a very young age (children under the age of 1 y) or the presence of the Philadelphia chromosome. In this case, the treatment protocol indeed differed between the Belgium and Vietnam cohorts. Furthermore, the treatment protocol also differed for relapsing patients. Accordingly, the current study compared the RFS between both series of ALL children.

ALL Features in Both Series

The diagnosis of ALL was based on bone marrow (BM) and blood morphology according to the French-American-British (FAB) classification, immunology, cytogenetics, and molecular biology. Karyotype was performed in 50% of the Vietnamese patients and 100% of the white patients. With regard to the cytogenetic studies, abnormalities were categorized as good and poor prognostic factors. Good prognostic factors include hyperdiploidy with >55 chromosomes, presence of t (12; 21), t (1; 19), and normal karyotype. Poor prognostic factors include hypodiploidy with <45 chromosomes, MLL-rearrangement, or t (4;11) (Table 1).

FRALLE Protocol and Treatment Follow-up

A summary of the FRALLE 2000 protocol is shown in Figure 1 and detailed in Annexes 1 (Supplemental Digital Content 1, http://links.lww.com/JPHO/A64) and 2 (Supplemental Digital Content 2, http://links.lww.com/JPHO/A65). Patients were assigned to subgroups A1/A2/A3, B1/B2, or T1/T2 according to the results of flow cytometry, myelogram of day 21, and/or minimal residual disease at day 35.

To assess the treatment response, BM examination and minimal residual disease evaluation were carried out at day 35 of the induction phase in both the groups. CSF was studied at each intrathecal (IT) injection. After completion of therapy, BM and CSF were examined on a regular basis during 3 years. At the end of this time interval, children were considered to be cured. In contrast, treatment failure was classified as isolated central nervous system (CNS) relapse, isolated BM relapse, combined BM and extramedullary (EM) relapse, and isolated EM relapse.

Drug-related Toxicities

Mucositis, neutropenia, diarrhea, skin rash, liver failure, central neurotoxicity, and leukoencephalopathy were recorded. Only grade 3 or 4 were considered.

Differences in the Application of the FRALLE 2000 Protocol Between Belgium and Vietnam Cohorts

Only children with the possibility to comply with therapy were considered. A group of Vietnamese children unable to afford the costs of full therapy was not included in this study. A bias concerning children coming from families with better socioeconomic factors was thus introduced.

The first IT therapy was delayed in Vietnamese children when the WBC count was $\geq 100.000/mm^3$. The IT therapy was performed when the WBC count was reduced by 50%. Although this occurred only in a minority of children (14/141), CNS involvement at diagnosis might be underestimated. Another reason of delaying the first IT injection was a traumatic lumbar puncture and the uneasiness of administering an IT injection in such condition. In patients from Belgium, the first IT injection was always administered with the first lumbar puncture, within the first 24 hours.

During induction and consolidation therapies, treatments were applied accurately in both countries. However, in Vietnamese children, it is not possible to measure the MTX level in the blood and to follow the clearance of the medication. Twelve doses of lederfoline ($12\,mg/m^2$/injection every 6 h) were given systematically and 2 or 3 doses were added in case of toxicity. There was no reduction in MTX dose.

During maintenance therapy, when toxicity occurred in Vietnamese children, the treatment was stopped and resumed afterward. Each patient received ultimately 100% of the total dose but during a longer time period. The duration of therapy was prolonged by 2 to 35 weeks (median, 6 wk; mean, 8 wk). In patients from Belgium cohort, the dose was reduced to two third and rarely stopped. The duration of the maintenance therapy was 18 to 24 months according to the protocol. Therefore, the dose intensity was computed for each patient from both series as the ratio between the total dose received and the duration of treatment.

The supportive care consisted of cotrimoxazole in both Vietnam and Belgium cohorts with the addition of acyclovir in the Belgium cohort.

Statistical Analysis

The white and the Vietnamese clinical series were compared for clinical and biological variables using the χ^2 test for discrete variables and the Student t test for continuous variables.

RFS was estimated using the Kaplan-Meier product limit method. Curves were compared using the log-rank test. Multivariate Cox regression models were utilized for modeling the relationship between factors and RFS in the Vietnamese series and the white series and in the pooled series. Regression models were adjusted for nationality, sex, age at diagnosis, WBC initial count, FAB classification, abnormal cytogenetics, immunophenotype, and corticosensitivity at day 8.

The RFS time was computed for each patient as the time from diagnosis of disease until first relapse or death. Within the white series, all patients were diagnosed after the

TABLE 1. Clinical and Biological Characteristics of Patients

Variables	White (n = 94)	Vietnamese (n = 141)	χ^2 value	P
Sex (n [%])				
Boys	65 (69.1)	84 (59.6)	1.835	0.176
Girls	29 (30.9)	57 (40.4)		
Age at diagnosis (y)				
Mean	6.67	5.57		0.09
Median	5.0	5.0		
Range	1-20	1-15		
≤ 10 y (n [%])	72 (76.6)	113 (80.1)		
> 10 y (n [%])	22 (23.4)	18 (12.8)		
Missing	0	10		
White blood cell (WBC) initial count (n [%]) (/mm^3)				
< 50,000	73 (77.7)	103 (73.1)	< 0.001	0.99
≥ 50,000	21 (22.3)	28 (19.9)		
Missing	0	10		
CSF involvement (n [%])				
No	3 (3.2)	3 (2.1)	0.257	0.613
Yes	91 (96.8)	138 (97.9)		
FAB classification (n [%])				
Type L1	51 (54.3)	18 (12.8)	**40.367**	**< 0.001**
Type L2 and other type	43 (45.7)	113 (80.1)		
Missing	0	10		
Abnormal cytogenetics (n [%])*				
Good prognosis	88 (93.6)	127 (90.1)	0.752	0.386
Poor prognosis	6 (6.4)	4 (2.8)		
Missing	0	10		
Flow cytometry (n [%])				
B precursor	75 (79.8)	122 (86.5)	**8.89**	**0.004**
T precursor	19 (20.2)	8 (5.7)		
Missing	0	11		
Corticosensitivity at D8 (n [%])				
Sensitive	85 (90.4)	111 (78.7)	1.113	0.291
Resistant	9 (9.6)	20 (14.2)		
Missing	0	10		
Dose intensity of MTX or 6-MP (%)				
Min	52	43		**< 0.001**
Max	100	100		
Mean	88	94		
Median	94	95		
ADR of HD-MTX (grade 3 or 4) (n [%])				
No	88 (93.6)	89 (63.1)	**19.99**	**< 0.001**
Yes	6 (6.4)	42 (29.8)		
Missing	0	10		

*Abnormal cytogenetics: good prognostic factors include hyperdiploidy with >55 chromosomes, presence of t (1;19) or t (12;21), and normal karyotype. Poor prognostic factors include: hypodiploidy with fewer than 45 chromosomes, MLL-rearrangement, and presence of t (4; 11).

Bold values emphasise significant results.

ADR indicates adverse drug reaction; CSF, cerebrospinal fluid; FAB, French-American-British classification; HD-MTX, high-dose methotrexate; MTX, methotrexate; 6-MP, 6-mercaptopurine.

beginning of the study in Belgium (2001). In contrast, some patients within the Vietnamese series were diagnosed before the beginning of the study in Vietnam (2009). Time between the diagnosis of ALL and inclusion into the series was introduced in the modeling as a left-truncated time in all time-to-event analyses in order to avoid overestimation of postdiagnostic survival probabilities and likely underestimation of hazard ratios (HRs) in the Cox regression models.[22]

Statistical analysis was performed using the "Epi"[23] and the "Survival"[24] package of the R 2.15.0 project.

RESULTS

Clinical and Biological Data

Clinical and biological data of both white and Vietnamese series were compared. There was no statistically significant difference in terms of sex, age at diagnosis, initial WBC count, CSF involvement at diagnosis, abnormal cytogenetics, and corticosensitivity at day 8.

In contrast, white and Vietnamese clinical series differed significantly in terms of cytology (more L2-ALL in Vietnamese children [$P < 0.001$]), flow cytometry (more T-ALL in white children [$P = 0.004$]), a higher mean dose intensity of MTX and 6-MP, and more frequent grade 3 or 4 adverse reactions in Vietnamese children ($P < 0.001$) (Table 1).

RFS Analysis

RFS at 18 months and 5 years from diagnosis was 95.7% (95% confidence interval [CI], 91.7%-99.9%) and 83.8% (95% CI, 76.3%-92.0%) for white children, respectively, versus 70.4% (95% CI, 60.4%-82.0%) and 47.8% (95% CI, 35.6%-64.2%) for Vietnamese children, respectively (Fig. 2).

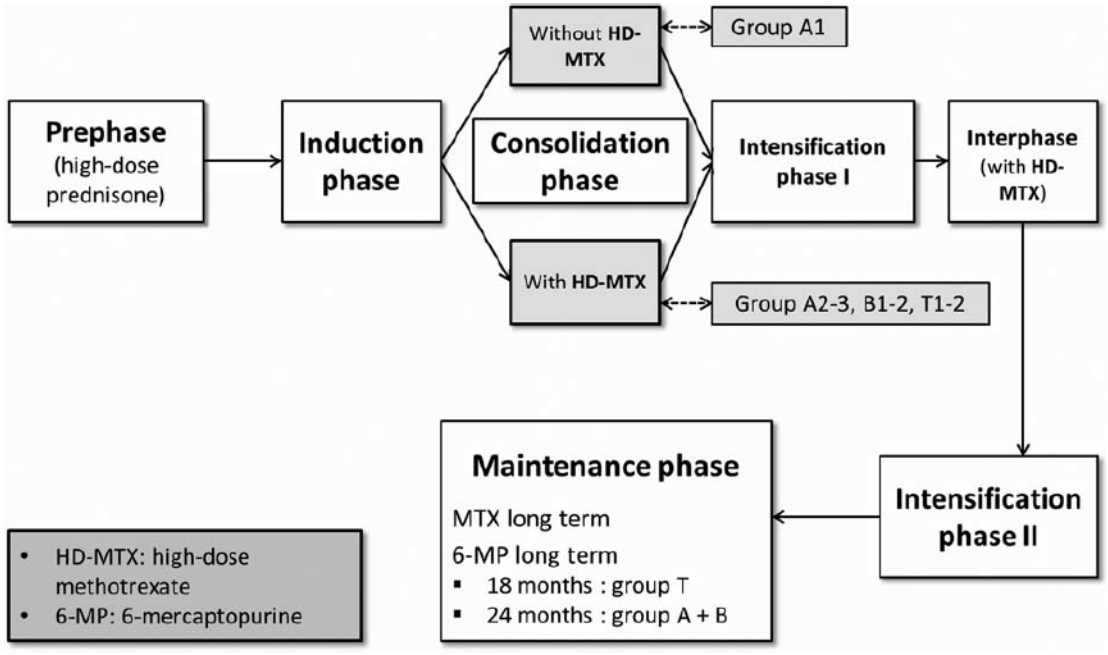

FIGURE 1. Diagram of FRALLE 2000 protocol. HD-MTX indicates high-dose methotrexate; MTX, methotrexate; 6-MP, 6-mercaptopurine.

Cox proportional hazards regression models were built for each clinical series (whites, n = 94; Vietnamese, n = 141) and in the pooled series (n = 235). Adjusted HRs are shown in Table 2. Ethnic origin was found to be significant in Vietnamese children compared with white children (adjusted HR, 4.48; $P < 0.001$). Vietnamese and white children with poor prognostic cytogenetic abnormalities had a higher risk of relapse with an HR of 3.69 and 2.07, respectively. An HR of pooled series was 3.26 ($P = 0.03$).

In Vietnam, BM and CSF relapses occurred very early, mostly within 18 months after diagnosis (Fig. 3). Compared with the white series, Vietnamese children had a higher risk of CSF relapse (HR = 5.06; $P = 0.015$) and BM relapse (HR = 2.86; $P = 0.023$). No significant difference was found between white and Vietnamese children in terms of EM relapse and combined BM and EM relapse.

DISCUSSION

In this comparative study of childhood ALL between white patients living in Belgium and Vietnamese patients living in Vietnam, the RFS was poorer in Vietnamese children (HR = 4.48, $P < 0.001$). Despite being treated with the same first-line protocol, Vietnamese children relapsed much earlier in BM and CSF than white children. The RFS at 18 months and at 5 years from diagnosis was significantly higher in white than in Vietnamese children.

Survival of children with ALL in developing countries is reported to be lower than in developed countries. The most common causes of treatment failure reported are as follows: treatment discontinuation, lack of antibiotic prophylaxis, poor social status, and insufficient high-quality health care system.[4,7,11–12,25,26]

In Vietnam, the costs for diagnostic and follow-up tests and for drugs in children under the age of 6 are covered at 100% by health insurance, whereas figures drop to 80% for children older than 6 years. Families hospitalizing a child have to pay for hospital beds (including for the person accompanying the patient) and for meals. Therefore, only patients with sufficient financial resources (ie, middle and upper class) were included and treated with FRALLE 2000 protocol. Patients who discontinued the treatment were excluded from the study. Supportive therapy was similar in both countries except for the additional use of acyclovir in Belgium. However, Vietnamese families confronted with a relapsing child could rarely afford further

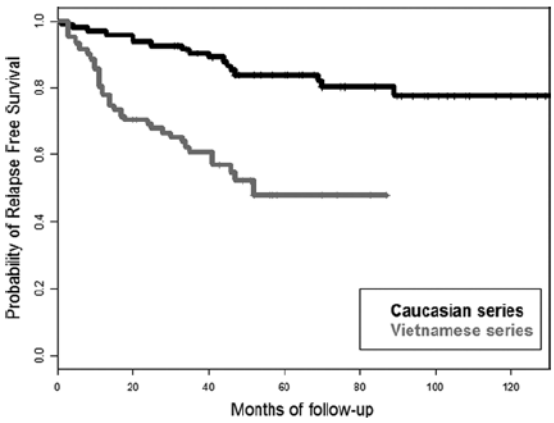

FIGURE 2. Relapse-free survival by ethnic origin.

TABLE 2. Clinical and Biological Characteristics of ALL Patients: Hazard Ratios (HR) From Multivariate Cox Regression Models for RFS

	White Patients (n = 94)	Vietnamese Patients (n = 141)	Total Patients (n = 235)	
	Adjusted HR	Adjusted HR	Adjusted HR	*P*
Nationality				
White				
Vietnamese			**4.48 (2.16-9.3)**	**< 0.001**
Sex				
Boys				
Girls	1.51 (0.49-4.66)	1.13 (0.55-2.33)	1.16 (0.64-2.09)	0.62
Age at diagnosis (y)				
< 10 y				
≥ 10 y	1.19 (0.36-4.0)	1.33 (0.49-3.66)	1.22 (0.59-2.52)	0.59
White blood cell (WBC) initial count (/mm^3)				
< 50,000				
≥ 50,000	2.48 (0.66-9.33)	1.45 (0.58-3.63)	1.69 (0.83-3.46)	0.15
FAB classification				
Type L1				
Type L2 and other type	1.02 (0.36-2.94)	0.64 (0.23-1.8)	0.82 (0.39-1.71)	0.59
Abnormal cytogenetics				
Good prognosis				
Poor prognosis	2.07 (0.3-14.19)	3.69 (0.92-14.88)	**3.26 (1.14-9.31)**	**0.03**
Flow cytometry				
B precursor				
T precursor	1.39 (0.35-5.58)	0.61 (0.1-3.71)	1.13 (0.46-2.79)	0.79
Corticosensitivity at D8				
Sensitive				
Resistant	1.88 (0.4-8.8)	1.52 (0.58-3.99)	1.54 (0.7-3.41)	0.28

ALL indicates acute lymphoblastic leukemia; FAB, French-American-British classification; RFS, relapse-free survival.

drug therapy because of the financial burden of leukemia care. That is the reason why we did not compare the outcome after the first relapse.

Available data were assessed to approach the reasons for such differences. Both series were comparable as far as age, sex, initial WBC count, and abnormal cytogenetics at diagnosis were concerned.

Corticosensitivity at day 8 of treatment was similar in both series. L2-ALL was more frequent in Vietnamese children ($P < 0.01$), whereas T-ALL was less frequent in the same series ($P < 0.01$). T-ALL was present in 5.6% of the Vietnamese patients. This is similar to the 5.8% reported in the SEER study.[16] The reason for such differences has not been determined but should not explain the worse prognosis observed in Vietnamese children. In contrast, T-ALL is known to have a worse prognosis. The SEER study reports a survival probability in the United States of 66% in East Asians with T-ALL as compared with 80% for B-ALL. As expected, poor prognostic cytogenetic aberrations had a negative impact on both groups. In that

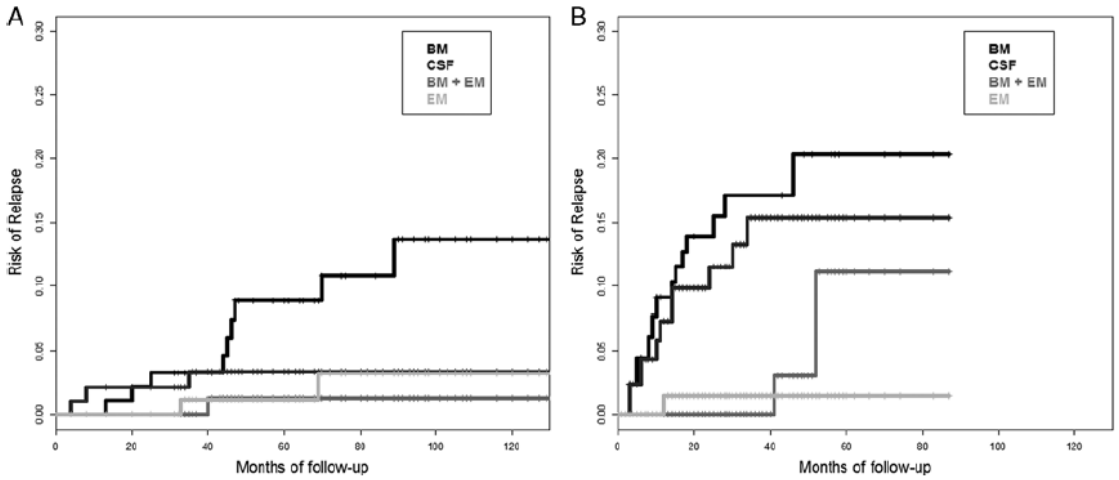

FIGURE 3. Type of relapse in white series (A) and in Vietnamese series (B). BM indicates bone marrow; CSF, cerebrospinal fluid; EM, extramedullary.

respect, RFS in Vietnamese children with poor cytogenetic abnormalities had an HR value nearly twice that found in white children (3.69 vs. 2.07, respectively). It implies a much more marked RFS difference in Vietnamese children with poor-risk and good-risk cytogenetics than observed in their white counterparts. Considering the worse RFS in Vietnamese children, the same observation applies with a strikingly higher difference between Vietnamese and white children with poor prognostic abnormalities compared with the difference observed in their respective counterparts with good prognostic abnormalities.

With regard to the first IT therapy, there was a substantial difference between both the series. In the Belgium series, the first lumbar puncture was combined with the first IT injection. In the Vietnam series, the first IT injection was delayed for a few days after diagnosis when the WBC count was high or when the lumbar puncture appeared to be traumatic. Both situations could have a negative impact on RFS as an intervening CSF contamination by leukemic cells may then not be ruled out.[27] Furthermore, CNS involvement at diagnosis may be underestimated as the first lumbar puncture is performed when the WBC count is already reduced by 50%.

A higher incidence of toxic events was observed in diagnosed children aged 10 years and over, as well as in children being corticoresistant at day 8 (data not shown). In both situations, a dose-intensified treatment was administered inducing more toxicity. Even though HD-MTX administration was similar in both series, the incidence of HD-MTX-related grade 3 and 4 adverse events was significantly higher in Vietnamese children ($P < 0.01$). One of the reasons might be the lack of pharmacokinetic monitoring of HD-MTX in Vietnamese children, which precludes any dosage adjustment, hence increasing the risk of MTX toxicity. An additional contribution of socioeconomic factors and/or variations in pharmacogenetic polymorphisms in Vietnamese patients cannot be ruled out, and this may also affect the metabolism of the drug and the outcome of Vietnamese children.[16–18,28–30]

Except for the first IT injection and the assessment of HD-MTX clearance, induction and consolidation phases were strictly comparable, whereas maintenance therapy differed in both series in terms of management of drug toxicity. In the latter case, white children received a reduced dose of 6-MP and MTX and discontinued the treatment after 18 or 24 months according to the protocol. In contrast, the Vietnamese children interrupted treatment in case of toxicity but resumed it later. Accordingly and compared with Caucasians, each Vietnamese patient received the same total dose of each drug but over a longer period of time, which ultimately resulted in a significantly higher dose intensity. In that respect, giving 6-MP every week has been reported to improve survival. The drug efficacy and survival benefit of 6-MP indeed appear associated with chronic systemic exposure to active metabolites of this agent.[31,32] It is therefore recommended to reduce the dose according to the WBC count rather than interrupting therapy, irrespective of the potential contribution of pharmacogenetics in the metabolism of 6-MP.

In this study, Vietnamese children with ALL have a worse prognosis than white children, despite receiving the same FRALLE 2000 protocol. It is of course somehow challenging to evaluate exactly the impact of socioeconomic factors and health care system differences in both series of patients. Nevertheless, only Vietnamese children from families with sufficient financial resources were included in this study. The treatment was administered in a single university hospital in Vietnam. Lessons to be learned from this study are as follows: firstly, the importance to initiate IT therapy as early as possible and to minimize the risk of traumatic lumbar puncture; secondly, to point out the need for a pharmacokinetic monitoring of HD-MTX with dosage adjustment to reduce the MTX-related toxicity; thirdly, to consider dose reduction during maintenance therapy rather than treatment interruption as is currently being done in Vietnam. There is no doubt whatsoever that increasing government spending on health, intensifying the medical training, and improving the disease management by educating the parents are concrete measures that are worth being taken into consideration in Vietnam.

Whether genetic differences in the metabolism and bioavailability of drugs used in the treatment of ALL,[28,29] especially MTX and 6-MP, might also affect the prognosis of ALL in Vietnamese children remains to be determined. This justifies further evaluation and comparison of both series of patients.

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STUDY II (published)

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Comparative pharmacogenetic analysis of risk polymorphisms in Caucasian and Vietnamese children with acute lymphoblastic leukemia: prediction of therapeutic outcome?

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Current treatment protocols can cure approximately 80% of paediatric acute lymphoblastic leukemia (ALL) patients.
- Several studies have shown the impact of racial and ethnic differences in the survival of children with ALL.
- Some polymorphisms in genes involved in drug metabolism are associated with the clinical response in ALL children.

WHAT THIS STUDY ADDS

- Large differences were found between Caucasian and Vietnamese ALL children in the frequency distribution of *MTHFR*-C677T, *TYMS-TSER* and *ITPA*-C94A polymorphisms.
- A multilocus genetic risk score (MGRS) computed from seven polymorphisms (*CCND1*-G870A, *GGH*-C452T, *MTHFR*-C677T, *MTHFR*-A1298C, *TYMS-TSER*, *TPMT*, *ITPA*-C94A) identifies children with poor prognosis, irrespective of the racial/ethnic group.
- Including MGRS into the clinical model improves the predictive accuracy of short and medium term prognosis.

AIMS

Acute lymphoblastic leukemia (ALL) is the most common of all paediatric cancers. Aside from predisposing to ALL, polymorphisms could also be associated with poor outcome. Indeed, genetic variations involved in drug metabolism could, at least partially, be responsible for heterogeneous responses to standardized leukemia treatments, hence requiring more personalized therapy. The aims of this study were to (a) to determine the prevalence of seven common genetic polymorphisms including those that affect the folate and/or thiopurine metabolic pathways, i.e. cyclin D1 (*CCND1*-G870A), γ-glutamyl hydrolase (*GGH*-C452T), methylenetetrahydrofolate reductase (*MTHFR*-C677T and *MTHFR*-A1298C), thymidylate synthase promoter (*TYMS-TSER*), thiopurine methyltransferase (*TPMT**3A and *TPMT**3C) and inosine triphosphate pyrophosphatase (*ITPA*-C94A), in Caucasian ($n = 94$, age < 20) and Vietnamese ($n = 141$, age < 16 years) childhood ALL and (b) to assess the impact of a multilocus genetic risk score (MGRS) on relapse-free survival (RFS) using a Cox proportional-hazards regression model.

RESULTS

The prevalence of *MTHFR*-677TT genotype was significantly higher in Caucasians ($P = 0.008$), in contrast to the prevalence of *TYMS-TSER**3R/3R and *ITPA*-94AA/AC genotypes which were significantly higher in Vietnamese ($P < 0.001$ and $P = 0.02$, respectively). Compared with children with a low MGRS (≤3), those with a high MGRS (≥4) were 2.06 (95% CI = 1.01, 4.22; $P = 0.04$) times more likely to relapse. Adding MGRS into a multivariate Cox regression model with race/ethnicity and four clinical variables improved the predictive accuracy of the model (AUC from 0.682 to 0.709 at 24 months).

CONCLUSION

Including MGRS into a clinical model improved the predictive accuracy of short and medium term prognosis, hence confirming the association between well determined pharmacogenotypes and outcome of paediatric ALL. Whether variants on other genes associated with folate metabolism can substantially improve the predictive value of current MGRS is not known but deserves further evaluation.

Introduction

Nearly one-third of all paediatric cancers are acute lymphoblastic leukemia (ALL), making it the most common malignancy affecting children [1]. Pharmacogenetics, which has become a major field of research in recent years [2–4], investigates genetic variations that affect pharmacokinetics and pharmacodynamics of drugs by changing the structure of proteins involved in drug absorption, distribution, metabolism, excretion and cellular transport, and their influence on drug response phenotypes [5]. Although current treatment protocols can cure approximately 80% of paediatric ALL patients, a number of children will relapse [6], with a relapse rate shown to vary within a group of patients with similar risk even though their leukemia blasts carry the same characteristics. Children with ALL are most often homogeneously treated within collaborative studies according to the risk groups and the body surface area. Besides tumour and patient related factors, inter-individual variations in treatment responses and toxicity could also be attributable to genetic polymorphisms influencing drug disposition and treatment intensity.

While multiple drugs are used to treat children with ALL, mounting evidence indicates that gene polymorphisms may alter therapy responses, as they participate in folate and 6-mercaptopurine/azathioprine (6-MP/AZA) metabolism, steroid response, drug transport and detoxification [3]. Polymorphisms in genes interacting with folate metabolism and methotrexate (MTX) activity such as the methylenetetrahydrofolate reductase (*MTHFR*) and the promoter enhancer region of *TYMS* (*TYMS-TSER*), or intervening in the 6-mercaptopurine (6-MP) metabolic pathway such as thiopurine methyltransferase (*TPMT*) and inosine triphosphate pyrophosphatase (*ITPA*) could explain at least part of the inconsistent clinical outcome in children with ALL receiving similar treatments [7–9]. However, data regarding the Caucasian and Vietnamese allelic distribution of polymorphic variants involved in MTX metabolism in children with ALL are scarce. Polymorphisms within *MTHFR*, *TYMS-TSER*, *TPMP* and *ITPA*, as well as cyclin D1 (*CCND1*), γ-glutamyl hydrolase (*GGH*) genes were assessed in this study.

The *CCND1* gene codes for cyclin D1, which is a key protein for the cellular division, as it regulates the transition from the G1 to the S phase of the cell cycle. The *CCND1* gene harbours a G > A polymorphism (G870A) in exon 4. The *CCND1*-870AA genotype has been reported to increase the risk for childhood ALL in Chinese and Egyptian populations [10, 11]. It has been associated with a lower probability of event free survival [12] but also with a reduced MTX toxicity [13]. A lower MTX efficacy in patients with *CCND1*-870AA genotype could explain these effects. The reported prevalence of 870AA homozygotes in Caucasian and in Asian children with ALL ranges from 22.3 to 25.7% and 32.4 to 38%, respectively [14–17].

GGH is a lysosomal peptidase that catalyzes the removal of γ-linked polyglutamates of folates and antifolates such as MTX, thus allowing folates to be exported from the cells or converting long chain MTX polyglutamates (MTXPG) into short chain MTXPGs and ultimately to MTX [18, 19]. It has been previously shown that the T variant allele of *GGH* may result in decreased GGH activity, hence increasing intracellular MTXPGs. These observations suggest that *GGH*-C452T polymorphism monitoring could be used to predict side effects and resistance to MTX [20]. Little is known however about MTX resistance in relapsed childhood ALL. Cells from relapsed precursor-B ALL have been shown to be threefold more resistant to MTX than those from newly diagnosed ALL [21]. It can therefore be hypothesized that the *GGH*-C452T polymorphism could play a role in ALL relapse through its interaction with MTX metabolism. While the reported frequency of the *GGH*-452CT genotype in Caucasian and in Asian ALL patients varies from 6 to 15.5% and 10 to 19%, respectively, it ranges between 0 and 4% or 0.4 and 1.5% for the *GGH*-452TT homozygote variant in both ALL populations, respectively [16, 20, 22, 23].

MTHFR catalyzes the reduction of 5,10-methylene-THF to 5,10-methyl-THF, being therefore a major determinant of the amount of folate available in the cell for DNA synthesis and methylation. The accumulation of 5,10-methyl-THF resulting from the *MTHFR* genetic polymorphism may play a role in MTX response [24]. Specific *MTHFR* genotypes have allegedly been associated with MTX toxicity in leukemia patients [25, 26] and with an increased risk of relapse in paediatric ALL patients [27]. Recent meta-analyses and literature reviews were recently carried out regarding the relationship between *MTHFR*-C677T and -A1298C polymorphisms, and ALL patients outcome including MTX toxicity [28], relapse [29] and overall survival [30]. Both polymorphisms were reported to be unsatisfactory predictors of MTX-related toxicity in paediatric ALL [28] whereas *MTHFR*-677CT/TT genotypes were possibly associated with higher relapse [29] and mortality rates [30]. The prevalence of *MTHFR*-C677T and the A1298C variant alleles varies with ethnicity. The prevalence of 677TT genotype ranges between 9 to 11% and 2.5 to 8.5% in Caucasian and Asian ALL children, respectively [24, 31, 32], *vs*. 9% to 16.5% and 0% to 9%, respectively, for the prevalence of the 1298CC genotype [27, 31, 33].

Thymidylate synthase (TYMS) catalyzes the conversion of deoxyuridylate monophosphate (dUMP) to deoxythymidylate monophosphate (dTMP) which is required for DNA synthesis and repair [34]. A 28-bp polymorphic tandem repeat sequence in the 5′ untranslated enhancer region of the human *TSER* gene has been shown to influence *TYMS* expression. Whereas homozygous *TSER*3R* correlates with increased *TYMS* expression in childhood ALL [35], this variant may also be associated with MTX resistance and with a higher risk of relapse [24, 35].

Accordingly, determining the *TYMS-TSER* genotype may help predict the patient response to cytotoxic treatment [36]. The prevalence of homozygous *TYMS-TSER*3R* lies between 28 to 30% and 50 to 77% in Caucasian and Asian ALL children, respectively [24, 27, 37].

TPMT catalyzes the S-methylation of thiopurine drugs which are used within the routine treatment for patients with malignant (ALL) and inflammatory diseases (rheumatoid arthritis) [38–40]. TPMT enzyme activity in human tissues is influenced by genetic polymorphisms [38–40]. Variations in TPMT activity impact on treatment toxicity and efficacy. Patients with low or undetectable levels of TPMT activity have the highest risk of developing severe myelosuppression when treated with standard doses of thiopurines whereas patients with very high TPMT activity are more likely to have a reduced clinical response to these cytotoxic agents [41–44]. Four common alleles have been identified in the *TPMT* gene (i.e. *TPMT*2*, *TPMT*3A*, *TPMT*3B* and *TPMT*3C*) which account for approximately 80% of Caucasians with low or intermediate TPMT activity. The *TPMT*3A* allele is mutated at both nucleotides G460A and A719G whereas *TPMT*3B* and *TPMT*3C* only have the G460A or A719G mutation, respectively [45–47]. The prevalence of the *TPMT*3A* mutation ranges from 0.9 to 5.7% in Caucasians but is usually not found in Asians [48–50] whereas the prevalence of the *TPMT*3C* mutation varies between 0.6 and 3% in Asians *vs*. 0.3 and 1.6% in Caucasians [48, 50–52].

Like TPMT, ITPA is an enzyme affecting 6-MP metabolism. ITPA catalyzes the hydrolysis of inosine triphosphate (ITP) to inosine monophosphate (IMP), protecting cells from the accumulation of harmful nucleotides such as ITP and deoxyinosine triphosphate [4, 53]. ITPA shows genetically determined polymorphic activity. The *ITPA*-C94A transversion reduces ITPA enzymatic activity to 25% and ~0% in heterozygote and homozygote carriers, respectively [54]. For patients without dose reduction of 6-MP according to *TPMT* genotype, those with *ITPA*-94AC/AA genotype were reported to have a higher probability of severe febrile neutropenia [54]. Conversely, *TPMT* has been shown to have a higher impact than *ITPA* on 6-MP toxicity when the dose of 6-MP was not adjusted for *TPMT* genotype [55]. In line with these data, it has been suggested that *ITPA* variants may have a stronger impact in Asians than in Caucasians as the prevalence of the *TPMT*3A* variant in Asians is lower than in Caucasians whereas the reverse is observed for the prevalence of *ITPA* variants [56, 57]. In contrast, Kim *et al*. showed that toxicity of 6-MP was related neither to *TMPT* nor to *ITPA* polymorphisms, but reported a lower event-free survival (EFS) rate in patients carrying the *ITPA*-94AC/AA genotypes [57]. The effect of *ITPA* on relapse free survival (RFS) remains so far unclear. The prevalence of the *ITPA*-C94A transversion varies with ethnicity, ranging from 3.3% in Africans to 8.3% in Caucasians, 11–19% in Asians [54, 56] and 3% in Chileans [58].

Accordingly, the first aim of this study was to compare the allele frequency distribution of the seven aforementioned loci in Caucasian and Vietnamese children with ALL. All these polymorphisms were common variants associated with drug toxicity and/or response to therapy. Except for *CCND1*, *GGH* and *ITPA*, they are all routinely assessed with validated methods in clinical practice. The second aim was to identify their effects on the clinical outcome of these children in both series, as measured by RFS.

Methods

Ethics statement

The comparative study of the long term outcome between Caucasian and Vietnamese children treated for acute ALL according to the FRALLE 2000 Protocol was approved by Professor J.M. Maloteaux, Chairman of the Faculty of Medicine and Health Sciences Research Ethics Committee of the Université catholique de Louvain (Belgium) and by Phan Nguyen Thanh Van, M.D., Ph.D., Chairman of the Health Sciences Research Ethics Committee of the Blood Transfusion and Hematology Hospital, Ho Chi Minh city, Vietnam. Following approval by both Ethics Committees, an informed consent was provided by each patient and samples were anonymized prior to analysis, as requested.

Caucasian and Vietnamese series of patients

The Caucasian series included 94 ALL patients diagnosed under the age of 21 (mean age 6.66 ± 4.57 years with a median of 5.0 years, range 1–20 years) between 2000 and 2011, and followed at the Cliniques Universitaires Saint-Luc (UCL), Brussels, Belgium. The Vietnamese series included 141 ALL patients diagnosed under the age of 16 (mean age 5.57 ± 3.52 years with a median of 5.0 years, range 1–15 years) between 2005 and 2011, and followed at the Blood Transfusion and Hematology Hospital, University of Medicine Pham Ngoc Thach (UPNT) at Ho Chi Minh city, Vietnam.

Treatment protocol

In both series, the patients were treated according to the FRALLE 2000 protocol (Figure 1, Supplementary files 1 and 2). The description of both clinical series and their respective clinical outcomes assessment has been thoroughly described previously [59]. Comparison of groups originating in countries with differences in socio-economic and health care systems is highly challenging. Accordingly, efforts were made to limit this potential bias. Only Vietnamese children from families with sufficient financial resources were included in this study and the same treatments were applied accurately in both countries during induction and consolidation therapies. Patients who discontinued the treatment were excluded from the study.

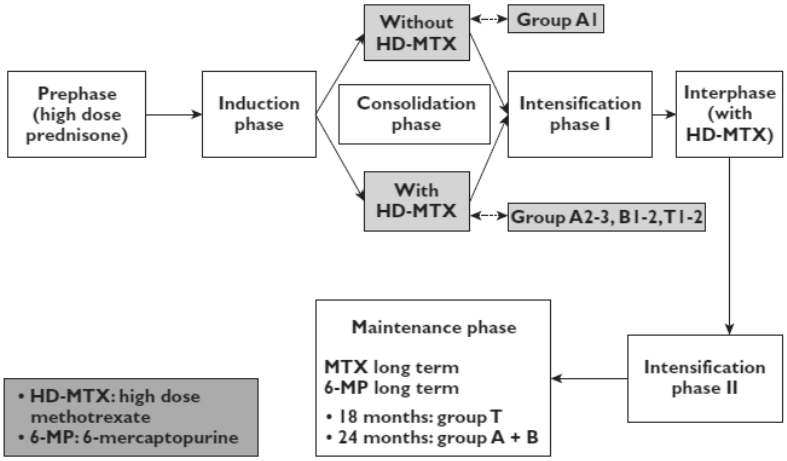

Figure 1

Diagram of FRALLE 2000 protocol. HD-MTX indicates high dose methotrexate; MTX, methotrexate; 6-MP, 6-mercaptopurine

Comparing respective therapeutic management between both series highlighted, however, some major differences as previously detailed [59]. Firstly, both series differed in terms of timing of the first intrathecal (IT) therapy. In Belgium, the first lumbar puncture was combined with the first IT injection. In Vietnam, the first IT injection was delayed for a few days after diagnosis when the WBC count was high or the lumbar puncture traumatic. Secondly, the lack of pharmacokinetic monitoring of HD-MTX in Vietnam precluded any dosage adjustment during consolidation therapy. There was therefore no reduction in MTX dose but lederfoline was systematically administered to Vietnamese patients. During maintenance therapy, the Vietnamese children interrupted the treatment in case of toxicity but resumed it later and for a longer period whereas Caucasian children received a lower dose of 6-MP and MTX and discontinued the treatment according to the protocol. Irrespective of these socio-economic factors, health care system and therapeutic management differences, each Vietnamese patient received, however, the same FRALLE 2000 protocol and the total dose of each drug, but over a longer period of time which ultimately resulted in a significantly higher dose intensity ($P < 0.001$) [59].

DNA and RNA isolation

For each subject, two venous blood samples (7.5 ml) were collected in EDTA tubes. The extraction of genomic DNA was performed from peripheral blood lymphocytes using the EZ1 DNA Blood kit and BioRobot EZ1 (Qiagen, Leusden, The Netherlands), according to the manufacturer's protocol.

Total RNA was isolated from whole blood over Ficoll Hypaque and extracted with TRIzol® reagent (Invitrogen Life Sciences, Merelbeke, Belgium) according to the manufacturer's protocol.

Concentrations of extracted DNA and RNA were measured both using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and were stored at −20°C and −80°C until use, respectively.

Pyrosequencing

Identification of selected polymorphisms in *CCNDA*-G870A, *GGH*-C452T, *MTHFR*-A1298C and *ITPA*-C94A was performed by polymerase chain reaction (PCR) followed by pyrosequencing (PyroMark Q96 ID Sequencer from Qiagen). Forward and reverse primers for each of the four SNPs, as well as primers for pyrosequencing were designed using the Pyrosequencing™ Assay Design or the Bionumerics software and synthesized by Eurogentec (Liège, Belgium). Each pyro-sequencing run included a negative control. The cumulated genotype call rate was 100%. The primers used in PCR and pyrosequencing (QIAGENE GmbH, Hilden, Germany), and size of corresponding amplicons are given in Table 1. The PCR reaction mixture was as follows: 5 µl buffer (10 mM Tris hydrochloride, and 50 mM potassium hydrochloride, pH 8.3), 3 mM $MgCl_2$, 1U Taq Gold polymerase, 200 µM of each deoxynucleotide triphosphate (dNTP), 10 pmol of each primer and 5 µl (~150–200 ng) of genomic DNA in a final volume of 50 µl. The cycling conditions were 95°C for 5 min, followed by 40 cycles at 95°C for 40 s, 60°C for 40 s, and 72°C for 80 s, with a final extension step at 72°C for 7 min.

Table 1

Target genes and related single nucleotide polymorphisms: primers and probes used in the various pharmacogenetic assays

Target gene and related polymorphism	Genbank (NCBI) accession	Location	RS number	Forward and reverse primers (5′ – 3′) used in polymerase chain reaction	Target size (bp)	Pyrosequencing primer, Taqman probes (5′–3′)
***CCND1* (*G870A*)**	NG_007375.1	11q13	rs603965	F: Biot- CAC ACG CTT CCT CTC CAG AGT R: CAT TTC CGT GGC ACT AGG TG	108	S: GGACATCACCCTCACTTA
***GGH* (*C452T*)**	NG_028126.1	8p12.3	rs11545078	F: GAG CTT TCA CTG CTG ATT AGT GG S: Bio-TAC TTA CTA ATC CTG CCC AGC AA	142	S: ATTAGTGGAGAGTGCTTAT
***MTHFR* (*A1298C*)**	NG_013351.1	1p36.3	rs1801133	F: GGGAGGAGCTGACCAGTG R: Biot-CGCAGCAGCTCCTCCT	422	S: AGGAGCTGACCAGTGAA-3
***MTHFR* (*C677T*)**			rs1801131	F: AAG CAC TTG AAG GAG AAG GTG TC R: CCT CAA AGA AAA GCT GCG TGA	64	VIC-ATGAAATCGGCTCCC-MGB FAM-ATGAAATCGACTCCCGC-MGB
***TSER* (*2R, 3R, 4R*)**	NG_028255.1.	18p11.32	Not applicable	F: CTG GCG CAC GCT CTC T R: FAM-TTG GAT CTG CCC CAG GT	315	Not applicable
***TPMT* (*G460A* and/ or *A719G*)**	NM_000367.2	6p22.3	rs1800460 rs1142345	F: GGAAGACATATGCTTGTGAGACA R: AAAAACATGTCAGTGTGATTTTATTTT	818	Not applicable
***ITPA* (C94A)**	NC_018931.2	20p13	rs1127354	F: Biot- CTGAGTTGGTAAGCTTTAGGAGA R: CCAATATTAACTGAACCCTAGAGG	483	GCCACCAAAGTGCAT

Biot, biotine; Fam, mutated probe; MGB, minor groove binding; NCBI, National Center for Biotechnology Information; VIC, wild type probe.

Taqman real-time PCR assay using Minor Groove Binding (MGB) probes

The *MTHFR*-C677T polymorphism was studied with two Taqman probes added together in one PCR tube, each consisting of an oligonucleotide with a fluorescent reporter dye, a non-fluorescent quencher and an MGB group (Applied Biosystems, Foster City, CA, USA). VIC and FAM reporter dyes were used for the WT and mutated probes, respectively. A distinct emission wavelength maximum for each probe allowed the detection of allele-specific cleavage. PCR primers and MGB probes are detailed in Table 1. The reaction was carried out on the ABI PRISM 7900 machine with SDS 2.4.0 software (Applied Biosystems) in a final volume of 25 µl with 900 nM of forward and reverse primers, 200 nM of probes labelled with VIC and FAM, 12.5 µl of TaqMan® 2XUniversal Master Mix (Applied Biosystems) and 2.5 µl (~76–100 ng) of DNA sample as detailed previously [60].

Polymerase chain reaction amplification

TYMS-TSER polymorphisms were analyzed by PCR using: buffer 1x, 1.5 mM $MgCl_2$, 2U Taq Gold polymerase, 200 µM dNTP, 10 pmol of each primer, 2.5 µl DMSO (dimethylsulfoxide) and H_2O to reach a final volume of 50 µl. The cycling conditions were 95°C for 5 min, followed by 35 cycles at 95°C for 40 s, 60°C for 40 s and 72°C for 1 min 20 s with a final extension step at 72°C for 7 min. The forward and reverse primers used for classic PCR of the *TYMS-TSER* gene are described in Table 2. DNA fragments for *TSER*2R* and **3R* genotypes were run in a 2.5% agarose gel with ethidium bromide and visualized on a UV transilluminator. Sequencing analysis was performed in the assay validation phase as well as to confirm any unusual genotype (*TSER*4R*). The DNA sequence of the purified product was determined on an automated ABI 3130 Genetic Analyzer using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

TPMT *cDNA amplification*

Total RNA was isolated from venous blood using Trizol reagent (Roche) and retro-transcribed by Superscript™ II RNase H-reverse transcriptase (Invitrogen) according to the manufacturers' instructions. TPMT cDNA was amplified by PCR using Ampli Taq DNA polymerase (Applied Biosystems) and a pair of primers that anneal to sequences within exon 4 and exon 10 of the published sequence of the *TPMT* cDNA (NCBI: accession number BC009596) (Table 2), as previously reported [61]. cDNA sequencing was performed on both strands with the Big Dye® terminator cycle sequencing kit (Applied Biosystems), using an automated ABI3130 capillary sequencer. Sequences were compared with the wild-type sequence using the SeqScap version 2.0 software, which identifies variant and sequence matches from an allele library. In addition, sequences identify variant and sequence matches from an allele library.

Statistical analysis

The Caucasian and Vietnamese series were compared for seven polymorphisms using the chi-squared test. RFS was computed for each patient as the time from diagnosis of disease until first relapse or death. Considering that Vietnamese families confronted with a relapsing child can rarely afford further drug therapy because of the financial burden of leukemia care, this study was not pursued after the first relapse. Within the Caucasian series, all patients were diagnosed for ALL after the beginning of the study in Belgium (2001). In contrast, some patients within the Vietnamese series were diagnosed for ALL before the

Table 2

Genotype frequencies of folate and thiopurine pathways associated polymorphisms in Caucasian and Vietnamese children with ALL

Polymorphic loci	Genotype	Protective *vs.* risk	Caucasian (*n* = 94) *n* (%)	Vietnamese (*n* = 141) *n** (%)	Raw *P* value £	Adjusted *P* value ¥	Reported distribution
***CCND1* G870A**					0.24	1.00	[14–17]
	G/G	P	28 (29.8)	22 (18.6)			
	G/A	P	45 (47.9)	60 (50.9)			
	A/A	R	21 (22.3)	36 (30.5)			
***GGH* C452T**					0.14	0.97	[16, 20, 22, 23]
	C/C	P	74 (78.7)	103 (87.3)			
	C/T	R	20 (21.3)	15 (12.7)			
***MTHFR* C677T**					**0.001**	**0.008**	[24, 31, 32]
	C/C	P	42 (44.7)	80 (67.8)			
	C/T	R	43 (45.7)	36 (30.5)			
	T/T	R	9 (9.6)	2 (1.7)			
***MTHFR* A1298C**					0.153	1.00	[27, 31, 33]
	A/A	P	54 (57.4)	55 (46.6)			
	A/C	R	31 (33)	54 (45.8)			
	C/C	R	9 (9.6)	9 (7.6)			
TYMS (TSER)					**<0.001**	**<0.001**	[24, 31, 37]
	*2R/*2R	P	20 (21.3)	3 (2.5)			
	*2R/*3R	P	41 (43.6)	35 (29.7)			
	*3R/*3R	R	33 (35.1)	79 (67.0)			
	*3R/*4R	R	0	1 (0.8)			
TPMT(*3A and *3C)					0.31	1.00	[49, 51]
	No mutation	P	90 (95.7)	116 (91.3)			
	Mutation	R	4 (4.3)	11 (8.7)			
	TPMT*3A		4 (4.3)	0			
	TPMT*3C		0	11(8.7)			
***ITPA* A94C**					**0.003**	**0.02**	[54, 56, 59]
	A/A	P	1 (1.1)	4 (3.4)			
	A/C	P	13 (14.0)	36 (30.5)			
	C/C	R	79 (84.9)	78 (66.1)			

*Sum of *n* not equals to 141 because of missing data. £Chi-squared test to compare reference with associated genotype between Caucasian and Vietnamese. ¥Bonferroni adjusted *P* values for multiple (*n* = 7) testing.

beginning of the study in Vietnam (2009). Time between the diagnosis of ALL and inclusion into the series was introduced in the modelling as a left-truncated time in all time-to-event analyses in order to avoid overestimation of post-diagnostic survival probabilities and likely underestimation of hazard ratios in the Cox regression models [62]. A multilocus genetic risk score (MGRS) was computed for each patient by summing the number of risk genotypes among the seven tested polymorphisms. Survival free of relapse curves were compared between low (≤3) and high (≥4) MGRS using the log-rank test. The effect of the MGRS was adjusted for clinical variables including race, age at diagnosis, white blood cell (WBC) initial count, abnormal cytogenetics, corticosensitivity at day 8 (D8) using a multivariate Cox proportional hazards regression model. The predictive accuracy of the models was compared with and without the MGRS for time points ranging from 1 to 60 months. Accuracy was assessed by computing area under the time-dependent ROC curves after leave-one-out cross-validation [63]. All statistical analyses were adjusted for multiple testing using Bonferroni correction, with the exception of regression analyses as recommended [62]. Statistical analysis was performed using R 2.15.0 (http://www.r-project.org) [64].

Results

The distribution of each genotype studied in 94 Caucasian and 141 Vietnamese children with ALL is listed in Table 2.

The prevalence of *CCND1*-A870G, *GGH*-C452T, *MTHFR*-A1298C and *TPMT* polymorphisms between Caucasian and Vietnamese series was not statistically different. Although *TMPT*3A* and *TMPT*3C* were detected in the Caucasian and Vietnamese series, respectively, their frequency was very low. Accordingly, they were pooled together within the TPMT group to allow the statistical analysis.

The prevalence of *MTHFR*-677TT genotype was significantly higher in the Caucasian (9.6%) than in the Vietnamese (1.7%) series ($P = 0.008$). Conversely, the prevalence of *TYMS-TSER*3R/3R* and *ITPA*-94AC/AA genotypes were

Table 3

Univariate analysis of relapse free survival (RFS) according to pharmacogenotypes

Polymorphisms	Caucasian series (n = 17/94)* Hazard ratios (95% CI)	Raw P value	Adjusted P value¥	Vietnamese series (n = 33/141)* Hazard ratios (95% CI)	Raw P value	Adjusted P-value¥	Pooled series (n = 50/235)* Hazard ratios (95% CI)	Raw P value	Adjusted P value¥
***CCND1* G870A**									
G/G – G/A	1			1			1		
A/A	0.70 (0.20, 2.42)	0.57	1.00	2.05 (0.77, 5.45)	0.15	1.00	1.20 (0.57, 2.53)	0.63	1.00
***GGH* C452T**									
C/C	1			1			1		
C/T	1.79 (0.63, 5.09)	0.28	1.00	0.73 (0.17, 3.19)	0.67	1.00	1.32 (0.57, 3.06)	0.51	1.00
***MTHFR* C677T**									
C/C	1			1			1		
C/T – T/T	1.09 (0.41, 2.84)	0.87	1.00	1.05 (0.39, 2.8)	0.93	1.00	1.08 (0.54, 2.15)	0.83	1.00
***MTHFR* A1298C**									
A/A	1			1			1		
A/C – C/C	1.18 (0.45, 3.06)	0.73	1.00	1.14 (0.45–2.91)	0.78	1.00	1.14 (0.59, 2.23)	0.69	1.00
TSER									
2R/2R – 2R/3R	1			1			1		
3R/3R – 3R/4R	1.08 (0.40, 2.91)	0.89	1.00	2.37 (0.68–8.27)	0.18	1.00	1.48 (0.72, 3.05)	0.28	1.00
***TPMT* (*3A and *3C)**									
No mutation	1			1			1		
Mutation	6.07 (1.36, 27.0)	0.02	0.37	0.66 (0.09–5.04)	0.69	1.00	1.51 (0.45, 5.05)	0.50	1.00
ITPA									
A/A A/C	1			1			1		
CC	2.91 (0.40, 22.0)	0.30	1.00	1.39 (0.5–3.91)	0.53	1.00	1.69 (0.69, 4.14)	0.25	1.00

*Number of relapse/number of children. ¥Bonferroni adjusted P values for multiple (n = 21) testing.

significantly higher ($P < 0.001$ and $P = 0.02$, respectively) in Vietnamese (67.0% and 33.9%, respectively) than in Caucasian (35.1% and 15.1%, respectively) children with ALL. The hazard ratio of each genetic variant was computed within each clinical series and for the pooled series by using Cox proportional hazards (Cox PH) models (Table 3). For the pooled series, the hazard ratio of each genetic variant was adjusted for race/ethnicity. As shown in Table 3, the individual impact of each variant was not statistically significant, except for TPMT mutation in the Caucasian series (HR = 6.07; 95% CI = 1.36, 27.0; raw P value = 0.02) before Bonferroni correction for multiple testing.

The MGRS was computed for each patient as the number of risk genotypes. Because of the limited number of patients, the original MGRS ranging between 0 and 6 was dichotomized in two categories (≤3 and ≥4). Compared with children with a low MGRS (≤3), a high MGRS (≥4) significantly increased the risk of relapse (univariate HR = 2.06; 95% CI = 1.01, 4.21; log rank P value = 0.042) (Figure 2). The adjusted hazard ratio of MGRS between children with a low (≤3) and high (≥4) MGRS computed from the multivariate model integrating race/ethnicity and four clinical variables was equivalent (HR = 2.09, 95% CI = 1.06, 4.13, P value = 0.03) to the univariate hazard ratio (Table 4). Adding MGRS to the model only slightly impacted on the highly significant hazard ratio of the single race/ethnicity variable. Compared with Caucasian children, the risk of relapse in Vietnamese children was increased by 4.39 (95% CI = 2.28, 8.46, P value <0.001) times, irrespective of MGRS.

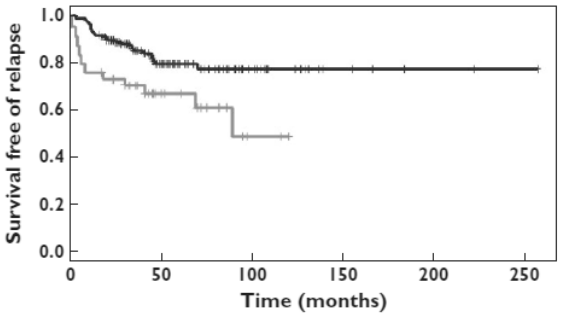

Figure 2

Relapse free survival according to multilocus genetic risk score (MGRS). Compared with those with a low MGRS (≤3), ALL children with a high MGRS (≥4) presented a significantly increased risk of relapse (HR = 2.06; 95% CI = 1.01, 4.21; log rank P value = 0.042). ■, multilocus genetic risk score <=3; ■, multilocus genetic risk score >=4

Predictive accuracy of the model was higher with than without MGRS on the whole range of tested time points. The predictive accuracy of both Cox regression models slightly decreased with longer time points (Figure 3).

Table 4

Multivariate Cox proportional-hazards regression model in the pooled series with and without multilocus genetic risk score (MGRS)

	Cox PH model without MGRS		Cox PH model with MGRS	
	Hazard ratios (95% CI)	*P* value	Hazard ratios (95% CI)	*P* value
Race				
Belgian	1		1	
Vietnamese	**4.19 (2.20, 7.97)**	**<0.001**	**4.39 (2.28, 8.46)**	**<0.001**
Age at diagnosis				
< 10 years	1		1	
≥ 10 years	1.26 (0.60, 2.65)	0.54	1.37 (0.64, 2.92)	0.42
Initial WBC count				
< 50.000/mm³	1		1	
≥ 50.000/mm³	1.82 (0.91, 3.64)	0.09	1.57 (0.77, 3.17)	0.21
Abnormal cytogenetics				
Good prognosis	1		1	
Poor prognosis	**3.66 (1.31, 10.24)**	**0.01**	**3.51 (1.23, 9.99)**	**0.02**
Corticoresistance on D8				
Sensitive	1		1	
Resistant	1.41 (0.66, 3.00)	0.36	1.38 (0.64, 2.98)	0.42
Multilocus genetic risk score				
≤ 3			1	
≥ 4			**2.09 (1.06, 4.13)**	**0.03**

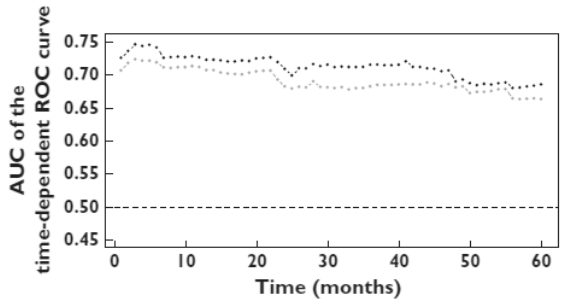

Figure 3

AUC of the time-dependent ROC curves obtained on the cross-validated predictions of the model with (black) and without (grey) multilocus genetic risk score. ■, Cox reression equation including the indicator of a multilocus genetic risk score >=4; ■, Cox reression equation without the indicator of a multilocus genetic risk score >=4

Discussion

This study showed differences between Caucasian and Vietnamese children with ALL in the frequency distribution of *MTHFR*-C677T, *TYMS-TSER* and *ITPA*-C94A polymorphisms. Indeed, Vietnamese children had a much higher proportion of *TYMS-TSER*3R/3R* and of *ITPA*-94AC/AA genotypes, whereas the prevalence of the *MTHFR*-677TT genotype was very low. No significant difference was found between both series in the frequency distribution of *CCND1*-G870A, *GGH*-C452T, *MTHFR*-A1298C and *TPMT* polymorphisms. However *TPMT*3A* (G460A and A719G) was only found in Caucasian children while *TPMT*3C* (A719G) was only found in Vietnamese children. *TPMT*3B* (G460A) was found neither in Caucasian nor in Vietnamese children.

Vietnamese children had a two-fold higher prevalence of *TSER*3R/3R* than Caucasian children. These results are in line with data from Dutch [31] and Indonesian children with and without leukemia [24]. It is of note that a very rare *TSER*3R/4R* genotype was identified in a single case from the Vietnamese clinical series (one out of 141 children) and was associated with a poor short term outcome after chemotherapy. The prevalence of *MTHFR*-677TT genotype in Caucasian and Vietnamese ALL children was 9.6% and 1.7%, respectively. While not found in Caucasian children, *TPMT*3C* is known to be more prevalent among Vietnamese (~10%) and Thai (11%) than among other Asian patients (i.e. Chinese, Japanese, Indian and Malaysian) where prevalence ranges between 0.8% and 3% [50, 51]. While *GGH*-452TT genotype was not found in this study, this may well be biased by the moderate sample size. It is indeed a rare variant being only infrequently reported in a Japanese population (one out of 269) [20], in Chinese leukemia children (one in 203) [65], but not found in Mexican paediatric patients with ALL (n = 70) [23]. The prevalence of *ITPA*-94AC/AA genotypes in Caucasian (15.1%) and Vietnamese (33.9%) children with ALL was in line with previous studies [37, 54, 58].

The univariate analyses show that the individual impact of each variant was not statistically significant probably due to a limited association with RFS and a small sample size. This univariate analysis model showed, however, the association between heterozygous *TPMT*3A* and a six-fold increase in RFS of Caucasian paediatric ALL.

Nevertheless, it should be pointed out that this finding was only based on four cases heterozygous for *TPMT*3A* and 17 ALL relapses, all from the Caucasian series. All *P* values were adjusted for multiple testing with Bonferroni correction because of the number of hypothesis tests ($n = 21$) which were simultaneously conducted. The Bonferroni-adjusted *P* value obtained with the *TPMT*3A* genotype in the Caucasian series was not statistically significant and should therefore be confirmed in larger clinical series. Whereas the association between *TPMT* polymorphisms and 6-MP cytotoxicity is widely accepted [40, 41, 50], reports on a potential association between *TPMT* mutations and RFS are extremely scarce. In a hospital-based cohort study of 172 childhood ALL patients [44], a 6-thioguanine nucleotide (6-TGN) concentration lower than the median concentration value (i.e. 284 pmol per 8×10^8 erythrocytes) was associated with a 63% RFS at 5 years whereas RFS was 84% at 5 years in patients with the median concentration value (RFS difference 21%; 95% CI = 3, 39%; $P = 0.0018$). As the 6-MP metabolic pathway is influenced both by *TPMT* and *ITPA* genotypes, the effect of *ITPA* genotyping on RFS was also assessed in this study. Regarding *ITPA* variants, it is worth emphasizing that evidence on their impact in terms of toxicity and survival are scarce and often controversial [53, 57], which explains why the impact of *ITPA* on RFS remains so far unknown. *ITPA*-94CA/AA genotypes appeared both in the Caucasian and Vietnamese series as a protective genotype in this study, whereas the wild-type *ITPA*-94CC genotype was associated with a significantly poorer survival. *ITPA*-94CC was therefore considered as a risk factor in univariate analysis of RFS, as well as in the computation of the MGRS (Tables 2 and 3). While the pharmacologic explanation of the effect of *ITPA*-C94A on RFS is so far unclear, it could be related to the unfavourable effect on the pool of 6-TGN. 6-TGN is indeed incorporated in nucleic acids or with phosphate in 6-thioinosine triphosphate, a process that determines the clinical efficacy but can be reversed by ITPA [53].

Regarding the impact of the folate pathway, many but not all studies have reported the association of genetic variations in the selected genes with the effect of MTX and relapse risk. In a cohort study [27] and case-control study [8] of 201 and 137 paediatric patients with ALL, respectively, *MTHFR*-C677T and *MTHFR*-A1298C variants were indeed associated with an increased risk of relapse. In a prospective [9] and case-control [66] study of 247 and 80 childhood ALL patients, respectively, homozygous *TSER*3R/3R* variants showed a greater risk of haematological relapse related to drug resistance. However, the assessment of 20 polymorphisms located in 11 genes (i.e. *GSTM1*, *GSTT1*, *GSTP1*, *NQO1*, *MTHFR*, *MTHFD1*, *SLC19A1*, *ABCB1*, *TSER*, *CRC5* and *IL15*) in a cohort study of 463 paediatric ALL patients failed to demonstrate any association between *MTHFR* and *TSER* variants with event-free survival (EFS). Conversely, *ABCB1* 3435TT and *CCR5* 246GA were found to predict lower EFS probability than their 3435CC (HR = 2.496; $P = 0.012$) and 246GG (HR = 1.82; $P = 0.033$) genotype counterparts [67]. In a Cox regression model of EFS adjusted for clinical variables such as ethnicity, gender, age, lineage, initial WBC and cytogenetic subtypes, a significant difference ($P = 0.0015$) was found only with cytogenetic subtypes. This is in agreement with our own findings, i.e., a strong association between abnormal cytogenetics (associated with a poor prognosis group), Vietnamese ethnicity and the risk of relapse [59].

In the current study, the univariate analysis of RFS showed a significant impact of MGRS and multivariate analysis indicated being Vietnamese, having a high MGRS and having abnormal cytogenetics significantly increased the risk of relapse. MGRS improved the predictive accuracy of the clinical model integrating ethnicity and four relevant clinical variables. The MGRS benefit was particularly marked during the first 4 years but tended to decrease with longer time points. However, there was a restricted number of polymorphisms and patients assessed in both clinical case series. Furthermore, the association between selected polymorphisms and adverse drug reaction to MTX and 6-MP was not assessable.

Although patients in both series were treated according to the same protocol, there were major differences in the therapeutic management [59]. Limitations concerned the day of first intrathecal therapy and the way MTX toxicity was managed (resulting in a higher dose intensity in Vietnamese children), which could explain in part the difference of RFS between the series. However, it is of note that significant differences in the prevalence of the *MTHFR*, *TYMS-TSER* and *ITPA* polymorphism were also detected, suggesting that MGRS may improve the predictive accuracy of the clinical model. To strengthen the current observation, the impact of pharmacogenetic determinants affecting the folate and/or thiopurine pathway genes on RFS in different ethnic groups deserves further study in a larger and more homogeneous clinical series of patients. Additional SNPs, such as those previously reported (i.e. *ABCB1*, *CCR5*) [67], could also be integrated as potential predictive factors when further assessing and evaluating the strength of association between well determined pharmacogenotypes and outcome of paediatric ALL.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare no support from any organization for the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years and no other relationships or activities that could appear to have influenced the submitted work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Annex 1
Protocol FRALLE 2000: details of successive treatments (dose and schedule)
Annex 2
FRALLE 2000 protocol: definition of groups and subgroups