Universidad del País Vasco
Euskal Herriko Unibertsitatea

Faculty of Medicine & Odontology

Department of Genetics, Physical Anthropology and Animal Physiology.

Doctoral Thesis

New genetic markers for treatment personalization in pediatric Acute Lymphoblastic Leukemia

Elixabet López López

Leioa, 2012

Este trabajo ha sido posible gracias a la concesión de una beca predoctoral para Personal Investigador en Formación otorgada por el Departamento de educación, universidades e investigación del Gobierno Vasco.

Lo que sabemos es una gota de agua, lo que ignoramos es el océano

Isaac Newton

A mis padres y a mi hermana

A Héctor

AGRADECIMIENTOS

Quisiera aprovechar estas líneas para agradecer el apoyo de todos los que han estado a mi lado y han hecho posible que esta tesis sea una realidad.

En primer lugar, a África, por haberme ofrecido la oportunidad de realizar esta tesis, por haber movido los hilos y haber tirado del carro para que todo saliera adelante.

A Aurora, por haber tenido la puerta de tu despacho siempre abierta y porque, a pesar de que el trabajo se te amontonase, siempre has conseguido hacernos un hueco a mí y a este proyecto.

A los doctores y doctoras Mª Ángeles Piñán, Purificación García-Miguel, Nagore García de Andoin, Jose Javier Uriz, José Sánchez de Toledo, Ana Isabel Carboné, por vuestra ayuda en la recogida de muestras y datos. Sin ellos, está claro que no podría haber hecho este trabajo.

A Javier Ballesteros y a Borja por vuestra ayuda y explicaciones de los análisis estadísticos.

A Anna Puiggros, el Dr. Francesc Solé y al resto de miembros del grupo de investigación translacional en neoplasias hematológicas del IMIM, por haberme enseñado a manejarme con el ChAS y porque con vosotros viví mi primer lipdub.
A Beatriz Ramírez y la Dra. Lourdes Ortiz del CIMA, por haberme abierto la puerta del laboratorio y haberme enseñado el trabajo que hacéis con los arrays.

Thanks to Dr. Michel Zwaan, for opening for me the door to Rotterdam and to all the people in the AML group for turning my 3 months there in an unforgettable experience: Dr. Marry van den Heuvel-Eibrink, Andrica, Trudy, Eva, Anna, Jenny, Malou, Susan, Lonneke, Nicola, Daria and especially Jasmijn, for being my keeper. Also thanks to Marissa for her enthusiasm and to all the people in the Pediatric Oncology Department.

A Idoia, porque no sólo has sido mi compañera en el laboratorio, has sido mi "gurú" en la investigación desde el principio. A todos los demás compañeros del laboratorio. Ángela, Nerea Bilbao, Icíar, María Pombar, María Constantin, Nerea Paz, Elena, Maialen, Espe, Maite, Nora, Aitor, Silvia, Eddy, Cristina, Maitane, Mikel, Ale, Lorea. Espero no olvidarme a nadie (y si lo he hecho, lo siento mucho, ha sido por mala cabeza). Gracias a vosotros el trabajo ha sido mucho más llevadero.

A todos los miembros del Departamento de Genética, Antropología Física y Fisiología Animal, del laboratorio de Bioquímica de la Facultad de Medicina, así como a los Servicios Generales de Investigación de la universidad, en especial a la Unidad de Genómica, por vuestra ayuda en el día a día.

A mi familia, porque está claro que sin vosotros no habría podido llegar tan lejos. Gracias por todos los esfuerzos que habéis hecho a lo largo de estos años y por todo el apoyo y comprensión que me dais.

A Héctor, porque llegaste a mi vida cuando yo ya estaba metida en este proyecto pero sin ti esta tesis no habría sido lo mismo.

Y a todos los que me han ayudado de alguna manera pero que, por culpa de mi mala memoria, no están aquí mencionados.

MUCHAS GRACIAS A TODOS

PUBLICATIONS

The work of this thesis is reflected in the following publications:

- Lopez-Lopez E, Martin-Guerrero I, Ballesteros J, Piñan MA, Garcia-Miguel P, Navajas A, Garcia-Orad A. Polymorphisms of the SLCO1B1 gene predict methotrexate-related toxicity in childhood acute lymphoblastic leukemia. Pediatr Blood Cancer 2011; 57: 612-619.

- Lopez-Lopez E, Ballesteros J, Garcia-Orad A. MTHFR 677TT genotype and toxicity of methotrexate: controversial results. Cancer Chemother Pharmacol 2011; 68: 1369-1370.

- Lopez-Lopez E, Martin-Guerrero I, Ballesteros J, Garcia-Orad A. A systematic review and meta-analysis of MTHFR polymorphisms in methotrexate toxicity prediction in pediatric acute lymphoblastic leukemia. (Submitted to The Pharmacogenomics Journal).

- Lopez-Lopez E, Ballesteros J, Piñan MA, Sanchez de Toledo J, Garcia de Andoin N, Garcia-Miguel P, Navajas A, Garcia-Orad A. Polymorphisms in the methotrexate transport pathway: a new tool for toxicity prevention in pediatric acute lymphoblastic leukemia. (Submitted to Haematologica).

- López-López e, Puiggros A, Piñan MA, Navajas A, Solé F, García-Orad A. Copy number alterations as risk stratification and prognosis markers in pediatric acute lymphoblastic leukemia. (In preparation).

- Lopez-Lopez E, Bilbao N, Garcia-Orad A. Pharmacogenetics update on MTHFR and methotrexate toxicity. Methotrexate: Pharmacology, Clinical Uses and Adverse Effects. Authors/Editors: Valentina S. Castillo and Laura A. Moyano. 2012

ABBREVIATIONS

3UTR: 3'UTR regulation
5UTR: 5'UTR regulation
6-MP: 6-mercaptopurine
ABC: ATP-binding cassette
ABL1: c-abl oncogene 1
ADD3: adducin 3 (gamma)
AF4: AF4/FMR2 family, member 1
AF9: myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3
ALB: albumin
ALL: Acute lymphoblastic leukemia
AML1/RUNX1: runt-related transcription factor 1
ANKS1B: ankyrin repeat and sterile alpha motif domain containing 1B
APC: anaphase-promoting complex
AraC: Cytarabine
ARID2: AT rich interactive domain 2
ASO: allele specific oligos
ASP: L-asparaginase
AST/ALT: Aspartate amino transferase / Alanine amino transferase
ATP: Adenosine-5'-triphosphate
AUC: Area under the curve
B- ALL: B-cell lineage acute lymphoblastic leukemia
BCR: breakpoint cluster region
BIB: Bibliographic
BTLA: B and T lymphocyte associated protein
BUBR1: budding uninhibited by benzimidazoles 1 homolog beta (yeast)
C/T: cytosine/thymine
CCR5: chemokine (C-C motif) receptor 5
CDC27: cell division cycle 27 homolog

CDKN2A/B: cyclin-dependent kinase inhibitor 2A/B

CeGen: Spanish National Genotyping Center

CFM: cyclophosphamide

CG: CpG site

CHAS: Chromosome Analysis Suite

Chrom: Chromosome

CIMA: Centre for Applied Medical Research

CN State: Copy Number state

CNOT: CCR4-NOT transcription complex

CNS: central nervous system

CNVs: copy number variations

cSNPs: coding SNPs

CYP: cytochrome P450

del : deletion

DEXA: dexamethasone

DGCR8: DiGeorge syndrome critical region gene 8

DHF: dihydrofolate

DHFR: dihydrofolate reductase

DICER: ribonuclease type III

DNA: Deoxyribonucleic acid

DNR: daunorubicine

dNTPs: Deoxynucleotide Triphosphates

DO: Design and optimization in our laboratory

DPF3: D4, zinc and double PHD fingers, family 3

DR: Downstream regulation

DROSHA: double-stranded RNA-specific endoribonuclease

DTT: Ditiotreitol

E2A: transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)

EBF1: early B-cell factor 1

EDTA: Ethylenediamine-tetraacetic acid

EFS: event free survival

EIF2C2: eukaryotic translation initiation factor 2C

ENL: myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 1

epiADR: epiadriamycin

ERG: v-ets erythroblastosis virus E26 oncogene homolog

ESE: Exonic splicing enhancer

ESS: Exonic splicing silencer

ETV6: ets variant 6

FAM71C: family with sequence similarity 71, member C

FDA: Food and Drug Administration

FDR: false discovery rate

FHIT: fragile histidine triad

GEMIN3: DEAD (Asp-Glu-Ala-Asp) box polypeptide 20

GEMIN4: gem (nuclear organelle) associated protein 4

GEMIN5: gem (nuclear organelle) associated protein 5

GSTs: glutathione-S-transferases

HDMTX: high-dose methotrexate

HIWI: piwi-like 1

HPRT: hypoxanthine phosphoribosyl-transferase

HR: High risk

HWE: Hardy-Weinberg equilibrium

IGL@: immunoglobulin lambda locus

IKZF1: IKAROS gene

IMIM: Institut de Recerca Hospital del Mar

IRF2BP2: interferon regulatory factor 2 binding protein 2

ISE: Intronic splicing enhancer

ISS: Intronic splicing silencer

K2HPO4: Potassium Hydrogen Phosphate

KCl: Potassium chloride

KHCO3: Potassium Hydrogen Carbonate

LBL: lymphoblastic lymphoma

LD: Linkage disequilibrium

LDL: Low density lipoprotein receptor

LEF1: lymphoid enhancer factor 1

LOD: logarithm of the odds of linkage

LSO: locus specific oligo

Mad2: mitotic arrest deficient-like 1

MAF: Minor Allele Frequency

Max: maximum

MeMP: methyl-mercaptopurine

Met: Methyonine

MgCl2: magnesium chloride

Min: minimum

miRNA: MicroRNA

MIRTS: miRNA target site

ML: malignant lymphoma.

MLL: myeloid/lymphoid or mixed-lineage leukemia

MRD: minimal residual disease

mRNA: Messenger RNA

MRP2: multidrug resistance protein 2

MRP4: multidrug resistance protein 4

MTHFR: methylene-tetrahydrofolate reductase

MTX: methotrexate

MTXPGs: polyglutamated forms of methotrexate

N.E.: Not Estimable

N.S.: non-significant

NA: not available

Na2HPO4: Disodium hydrogen phosphate

NaAc: sodium acetate

NaCl: Sodium Chloride

NFQ: non-fluorescent quencher.

NH4Cl: ammonium chloride

NQO1: NAD (P) H: quinone oxidoreductase

NR3C1: nuclear receptor subfamily 3, group C, member 1

NS: Non-synonimous

On: Over night

OR: Odds ratio

OS: overall survival

p55CDC: cell division cycle 20 homolog

PAX5: paired box 5 gene

PBS: Phosphate Buffered Saline

PBX1: pre-B-cell leukemia homeobox 1

PCR: polymerase chain reaction

PCR-RFLP: Polymerase Chain Reaction - Restriction Fragment Length Polymorphism

PDN: prednisone.

PIP4K2A: phosphatidylinositol-5-phosphate 4-kinase, type II, alpha

PTDR: Post-traductional regulation

PTEN: tumor suppressor and cell cycle regulatory genes

Pter: p terminus

Qter: q terminus

RAN : member RAS oncogene family

RB1: retinoblastoma 1

RFC1: reduced folate carrier

RNA: RiboNucleic Acid

S100A11: S100 calcium binding protein A11

SD: Standard Deviation.

SDS: sodium dodecyl sulfate

SHMT1: serine hydromethyl transferase

SLC: solute carrier family

SND1: staphylococcal nuclease and tudor domain containing 1

SNP: single nucleotide polymorphisms

SR: Splicing regulation

SR: Standard risk

TAG: tagSNP

TBL1XR1: transducin (beta)-like 1 X-linked receptor 1

TBXAS1: thromboxane A synthase 1

TCR: Transcriptional regulation

TEL: ets variant 6

TEMED: Tetramethyl-ethylene-diamine

TGNs: thioguanine nucleotides

THF: tetrahydrofolate.

TIT: intrathecal treatment

TNR: Tenascin-R

TNRC6A/B: trinucleotide repeat containing 6A/B

TOX: toxicity

TPMT: thiopurine methyltransferase

TR: transcriptional regulation

TRBP: TAR (HIV-1) RNA binding protein 2

TRB: T-cell receptor beta

TRG@: T cell receptor gamma locus

TS: thymidylate synthase

Tyr: Tyrosine

UR: Upstream regulation

UTR: untranslated region

VCR: vincristine

VHR: Very high risk

WGA: whole genome amplification

WHO: World Health Organization

XPO5: exportin 5

RESUMEN

INTRODUCCIÓN

La leucemia linfoblástica aguda (LLA) es el cáncer pediátrico más común y la principal causa de muerte por enfermedad en niños. Es un desorden de las células linfoblásticas, que son las precursoras de los linfocitos, y se caracteriza por la acumulación en médula ósea y sangre de pequeñas células blásticas con poco citoplasma y cromatina dispersa. El 80-85% de todas las LLAs son de linaje de células B (B-ALL).

Una de las principales características de esta enfermedad es su gran heterogeneidad, con marcadas diferencias entre los individuos en el momento del diagnóstico, el comportamiento clínico y la respuesta al tratamiento. Ciertas características presentes al diagnóstico como la edad, el recuento de glóbulos blancos o la presencia de enfermedad extramedular se asocian con un pronóstico adverso. Las alteraciones citogenéticas se encuentran entre los marcadores de mayor valor para la predicción del pronóstico.

En las últimas décadas, la supervivencia ha mejorado mucho, en parte debido a la implantación de terapias combinadas y la adecuación de la terapia a grupos de riesgo. Los pacientes se separan en tres grupos de riesgo, Riesgo Estándar, Alto Riesgo y Muy Alto Riesgo, en base a marcadores pronósticos y se intensifica el tratamiento en los grupos en los que, a priori, se espera una peor respuesta. De este modo, se aumenta la probabilidad de supervivencia en los grupos de mal pronóstico, mientras que se reduce la toxicidad en los pacientes con mejor pronóstico.

Sin embargo, uno de los problemas que se presentan durante el tratamiento es que algunos pacientes en los grupos de riesgo estándar y alto riesgo no responden bien al tratamiento y se convierten en alto riesgo y muy alto riesgo, respectivamente. Esto significa que la clasificación de los grupos de riesgo no es totalmente exacta y es susceptible de mejora. El riesgo inherente a una clasificación errónea es que algunos pacientes podrían recibir un tratamiento menos intensivo del que necesitan. En este contexto, gracias a los avances en tecnología citogenética, hoy en día se pueden identificar nuevas alteraciones crípticas, tales como deleciones y duplicaciones, que no se podían detectar hasta ahora. Algunas de estas alteraciones podrían ser útiles para mejorar la clasificación en grupos de riesgo.

Una vez que se establecen los grupos de riesgo, se ajusta el tratamiento. Hoy en día, se aplican protocolos de tratamiento complejos y bien establecidos. En concreto, uno de los protocolos utilizados para el tratamiento de la LLA es el aprobado por la Sociedad Española de Hematología y Oncología Pediátrica, LAL/SHOP. En estos protocolos, el metotrexato (MTX) y la 6-mercaptopurina (6MP) son muy importantes.

Uno de los problemas más importantes asociados con estos protocolos de tratamiento es que, a pesar del éxito clínico, algunos pacientes experimentan toxicidad grave, que puede requerir reducción de dosis o suspensión del tratamiento. Por lo tanto, sería de gran interés reconocer de antemano qué pacientes van a sufrir de estos efectos secundarios, con el fin de ajustar el tratamiento desde el principio. En este sentido, los estudios farmacogenéticos

están proporcionando una base importante para mejorar la eficacia del tratamiento y reducir las complicaciones.

La farmacogenética es el estudio de la base genómica de las diferencias interindividuales en la absorción, distribución, metabolismo y excreción de los fármacos (farmacocinética) y su relación con los efectos farmacológicos terapéuticos o adversos (farmacodinámica). Los estudios farmacogenéticos tratan de desarrollar modelos para predecir con exactitud la respuesta a los fármacos y la toxicidad en cada paciente y utilizar esta información para personalizar de forma prospectiva el tratamiento con el fin de mejorar su eficacia y seguridad.

Los estudios farmacogenéticos pueden ser muy útiles en el contexto de la LLA infantil por varias razones:

- Los protocolos de tratamiento están estandarizados y bien establecidos.
- Los fármacos utilizados en el tratamiento tienen un rango terapéutico muy estrecho. Es decir, hay poca diferencia entre la dosis efectiva y la dosis que produce toxicidad. En consecuencia, pequeños cambios en la función de los genes implicados en sus vías pueden tener un gran impacto en la respuesta al tratamiento.
- Los genes que influyen en la respuesta a estos medicamentos son muy variables.

Como el metotrexato y 6-mercaptopurina son la columna vertebral de la terapia, ha habido un gran interés en el análisis de polimorfismos en los genes implicados en su metabolismo y vías de transporte. Además, como los pacientes

de LLA infantil se tratan con regímenes complejos de múltiples fármacos, también se han estudiado polimorfismos en genes que codifican enzimas que afectan a la detoxificación de varios fármacos.

Sin embargo, las asociaciones encontradas entre polimorfismos y toxicidad no se suelen confirmar. Esta falta de replicación podría ser debida a diferencias entre protocolos de tratamiento o al uso de poblaciones pequeñas o no homogéneas o criterios de toxicidad no homogéneos o poco objetivos.

Por otro lado, la mayoría de los estudios farmacogenéticos realizados hasta el momento se centran en regiones codificantes. No obstante, estas regiones corresponden sólo a un 1,5% de la totalidad del genoma. En consecuencia, un importante hito en los estudios recientes es el análisis de las regiones que no codifican proteínas pero pueden tener una función reguladora, como los microRNAs (miRNAs), pequeños RNAs endógenos que participan en la regulación de la expresión génica a nivel post-transcripcional. Los SNPs relacionados con los miRNAs (SNPs en los genes de miRNA, en sitios de unión y en la vía de biogénesis de miRNAs) pueden tener una importante función reguladora y podrían jugar un papel importante en la respuesta al tratamiento.

HIPÓTESIS

Un gran reto en el tratamiento del cáncer es que la combinación de variaciones genómicas adquiridas (somáticas) y heredadas (línea germinal) van a influir en la eficacia y la toxicidad de la terapia.

Si tenemos en cuenta que el tratamiento de la leucemia linfoblástica aguda infantil tiene un rango terapéutico estrecho y que la administración de la terapia

más intensiva que se pueda tolerar aumenta la supervivencia, debemos tener en cuenta que:

Por un lado, algunos pacientes no responden bien al tratamiento y deben ser cambiados a grupos de mayor riesgo. Esto puede querer decir que los grupos de riesgo no están completamente bien definidos. Por lo tanto, sería de interés caracterizar a los pacientes que desde el principio deberían haber sido considerados como de mayor riesgo y tratarlos con una terapia más intensiva.

Por otro lado, un alto porcentaje de pacientes experimentan toxicidad, que puede llegar a ser muy grave en algunos casos, siendo necesario interrumpir el tratamiento. En consecuencia, sería altamente beneficioso reconocer a los pacientes que van a ser más sensibles al tratamiento, con el fin de ajustar las dosis.

Por estas razones, proponemos que se podría aumentar la supervivencia y reducir la toxicidad con un tratamiento más individualizado. Nos planteamos la hipótesis de que la identificación de nuevos marcadores genéticos, utilizando nuevas estrategias y tecnologías, permitirá la caracterización de los tumores y de los individuos, lo que facilitará la personalización y ajuste del tratamiento en la LLA infantil.

OBJETIVOS

El objetivo principal de este trabajo fue mejorar el ajuste y la personalización del tratamiento de los niños con leucemia linfoblástica aguda mediante la identificación de nuevos marcadores genéticos que permitan hacerlo más seguro y eficaz.

Para ello, establecimos los siguientes objetivos específicos:

1) Mejorar la caracterización de los grupos de riesgo y ajuste del tratamiento mediante nuevos marcadores genéticos presentes en el tumor.

- Detectar nuevas regiones de deleción y amplificación mediante oligo-arrays.
- Definir su utilidad como marcadores para la caracterización de grupos de riesgo.

2) Predecir la toxicidad debida al tratamiento mediante polimorfismos en genes clave.

- Determinar si los polimorfismos en los genes más representativos de las vías metabólicas de los fármacos utilizados en el protocolo LAL/SHOP podrían ser utilizados como marcadores de toxicidad en LLA infantil.
- Determinar la implicación de los polimorfismos en miRNAs que regulan los genes de las rutas metabólicas de los fármacos en la respuesta al tratamiento de la LLA infantil.
- Determinar si los polimorfismos en los genes de procesamiento de miRNAs tienen un papel en la toxicidad del tratamiento de la LLA infantil.

RESULTADOS

En primer lugar, en este estudio hemos querido identificar nuevas deleciones y duplicaciones, crípticas para las técnicas tradicionales de citogenética, presentes en las células tumorales que podrían permitir una mejor clasificación de grupos

de riesgo. Con este objetivo, analizamos muestras de ADN de 23 pacientes con diagnóstico de B-ALL de los diferentes grupos de riesgo con Affymetrix Cytogenetics Whole-Genome 2.7M Array. Detectamos un alto número de anomalías genómicas por caso, incluyendo aberraciones recurrentes que podrían contribuir a implementar la diferenciación entre los grupos de riesgo estándar y alto riesgo (deleción en 7p14.1 y 12q23.1) o sustituir a marcadores tradicionales como el cariotipado cuando estos fallen. También detectamos alteraciones (ganancia en 1q21.3 y 1q25.1, ganancia o pérdida en 5q33.3 y pérdida en 10q25.1-q25.2 y 12q12) que podrían permitir una mejor caracterización de los grupos de riesgo, ya que podrían distinguir entre pacientes de riesgo estándar que permanecen en este grupo y aquellos que cambian a alto riesgo y, en consecuencia, deberían haber sido tratados como de alto riesgo desde el principio.

Por otro lado, con el fin de seleccionar marcadores para predecir el efecto tóxico del tratamiento con el protocolo LAL/SHOP, evaluamos la influencia de polimorfismos en genes clave en la toxicidad en un grupo de 115 niños con diagnóstico de B-ALL y tratados de acuerdo con el protocolo estándar LAL/SHOP.

En el grupo de las enzimas de detoxificación de fármacos, no encontramos ninguna asociación estadísticamente significativa entre los 5 polimorfismos analizados en GSTM1, GSTT1, GSTP1, CYP1A1 y NQO1 y toxicidad en inducción o consolidación.

En la ruta de la 6-mercaptopurina, no encontramos ningún paciente con el genotipo homocigoto deficiente para TPMT y no encontramos ninguna

asociación significativa entre el genotipo heterocigoto TPMT y cualquiera de los parámetros de toxicidad estudiados en la fase de consolidación.

En nuestro estudio, hemos encontrado una asociación significativa entre el genotipo SLCO1B1 rs11045879 CC y el aumento de los niveles plasmáticos de MTX. No se encontró ninguna asociación entre los polimorfismos en MTHFR, SHMT1, TS, ABCB1, ABCG2 y RFC1 y la toxicidad del MTX.

Los alelos MTHFR 677T y A1298C codifican proteínas con menor actividad enzimática. Estos polimorfismos se han propuesto como posibles marcadores de aumento de la toxicidad para la individualización de la dosis de MTX. En nuestro estudio, no encontramos ninguna asociación significativa entre estos polimorfismos y los niveles plasmáticos de MTX. De hecho, observamos una tendencia a la reducción de los niveles plasmáticos de MTX en el grupo de pacientes con el genotipo 1298CC. Los resultados publicados por otros autores son contradictorios. En este contexto, decidimos realizar una revisión y meta-análisis para evaluar su papel en la toxicidad del MTX en LLA infantil. Según los estudios publicados y el meta-análisis que hemos realizado, los alelos 677T y 1298C no parecen ser buenos marcadores de toxicidad MTX en los pacientes de LLA infantil. En todo caso, el alelo 1298C parece ser más probablemente un factor de protección que de riesgo.

El otro resultado interesante de este estudio fue que todos los pacientes con el genotipo SLCO1B1 rs11045879 CC tenían altas concentraciones plasmáticas de MTX a las 72 h después de la infusión con MTX. Por otra parte, el genotipo rs4149081 AA siempre estaba asociado con altas concentraciones plasmáticas de MTX, aunque esta asociación no alcanzó significación estadística (p=0,057).

Además, los 3 individuos con el genotipo rs11045879 CC y los 2 pacientes con el genotipo rs4149081 AA desarrollaron toxicidad durante el tratamiento de consolidación. Ambos SNPs, rs4149081 y rs1104579, están en desequilibrio de ligamiento. En consecuencia, el hecho de que ambos SNPs estén asociados con la toxicidad sugiere la implicación de estos SNPs o de otros SNPs en el bloque de ligamiento en el aclaramiento del MTX, así como un papel importante de SLCO1B1 en la toxicidad del MTX.

En este contexto, pensamos que sería de gran interés estudiar la implicación de otros polimorfismos en SLCO1B1 y otros genes relacionados en la toxicidad del MTX. Sin embargo, no existen estudios que analicen en profundidad los polimorfismos de los genes implicados en el transporte y la toxicidad de MTX. Esa es la razón por la cual decidimos evaluar la correlación de 384 polimorfismos en 12 genes clave implicados en la ruta de transporte con la toxicidad del metotrexato en un grupo mayor de 151 niños con diagnóstico de B-ALL y tratados de acuerdo con el protocolo estándar LAL/SHOP.

En este estudio, identificamos principalmente dos polimorfismos y un haplotipo significativos en dos genes transportadores de MTX, y ABCC4 ABCC2, asociados con el aclaramiento de metotrexato en pacientes de LLA infantil. La identificación de estos polimorfismos en niños con LLA podría ser una herramienta útil para el seguimiento de pacientes con riesgo de bajo aclaramiento de MTX para evitar la toxicidad relacionada con este fármaco.

También se ha propuesto que SNPs relacionados con los miRNAs pueden interferir con la función de los miRNAs y podrían conducir a la sensibilidad al tratamiento. De hecho, en nuestro estudio en la ruta del transporte de MTX

observamos que el polimorfismo con la asociación más fuerte con toxicidad del MTX en ABCC4 creaba un nuevo sitio de unión de miRNAs. Sin embargo, existen muy pocos estudios que analicen el papel de los polimorfismos en miRNAs y genes de procesamiento de miRNAs y, hasta ahora, ninguno de ellos ha sido llevado a cabo en la LLA infantil. Esa es la razón por la cual decidimos ampliar nuestro estudio y analizar polimorfismos en pre-miRNAs y en la vía de la biogénesis de miRNAs.

Hemos encontrado 30 asociaciones estadísticamente significativas con la toxicidad durante la fase de inducción del tratamiento (16 se encuentran en los genes de procesamiento y 14 en pre-miRNAs). También se encontraron 31 asociaciones estadísticamente significativas con toxicidad durante la fase de consolidación (23 en los genes de procesamiento y 8 en el pre-miRNAs). De éstos, la asociación entre rs639174 en DROSHA y vómitos se mantuvo estadísticamente significativa después de la corrección FDR.

CONCLUSIONES

En LLA infantil:

1. La deleción en 14q24.2 podría ser un nuevo marcador de riesgo estándar y la deleción en 12q23.1 de alto riesgo.

2. Un total de 5 nuevos marcadores (las ganancias en 1q21.3 y 1q25.1, las pérdidas en 10q25.1-q25.2 y 12q12 y la ganancia o pérdida en 5q33.3) podrían mejorar la caracterización del grupo de riesgo estándar.

3. No hay evidencia para apoyar el uso de de los SNPs MTHFR C677T o A1298C como marcadores de toxicidad del MTX.

4. SNPs en transportadores de MTX como rs11045879 en SLCO1B1, rs9516519 en ABCC4 y rs3740065 en ABCC2 podrían ser herramientas útiles para evitar la toxicidad relacionada con MTX.

5. El SNP rs56103835 en mir-453, que podría regular los genes ABCC1, ABCC2 y ABCC4, podría ser también un marcador de la toxicidad del MTX. rs639174 en DROSHA, un gen de procesamiento de miRNAs, está fuertemente asociado con vómitos. Estos dos resultados sugieren que los SNPs relacionados con miRNAs, podrían ser útiles en los estudios de toxicidad.

Conclusiones finales:

El tratamiento de la LLA-B infantil se puede mejorar utilizando nuevos marcadores genéticos para predecir la eficacia sobre el tumor y el riesgo de toxicidad en el individuo.

Abrimos un nuevo campo de investigación, que implica el estudio de polimorfismos relacionados con los miRNAs en el tratamiento de la LLA infantil.

INDEX

INTRODUCTION

ACUTE LYMPHOBLASTIC LEUKEMIA

DEFINITION

Lymphoid neoplasms constitute an heterogeneous group of neoplasms of the lymphoid system at various stages of differentiation, defined by distinct cells of origin, pathologies, risk factor profiles and prognoses [1]. These neoplasms together comprise the sixth most common group of malignancies worldwide [2].

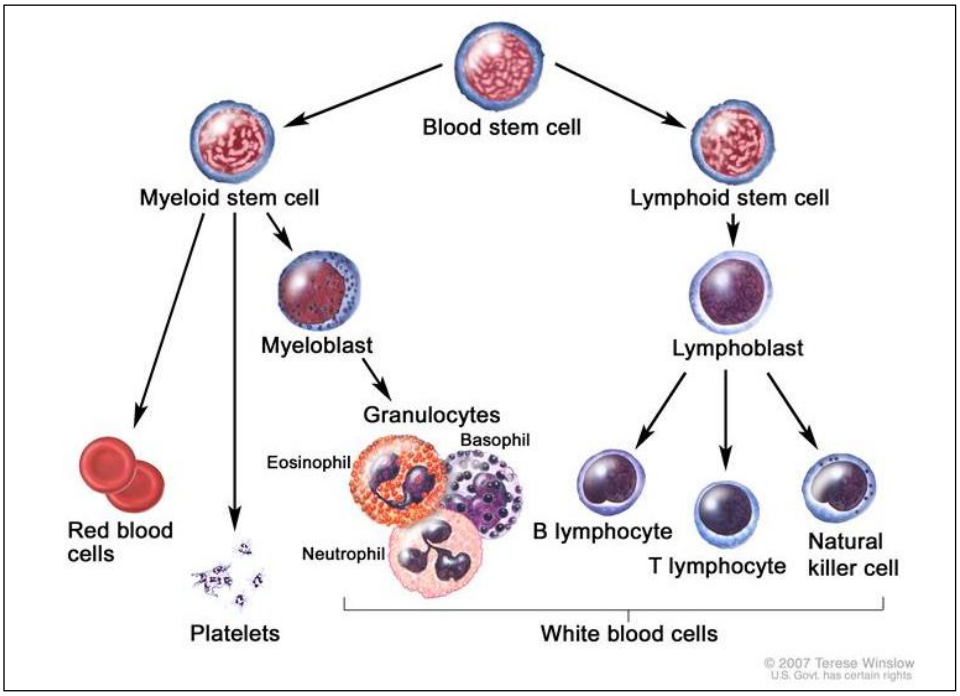


Figure 1. Normal blood cell development (http://www.cancer.gov/cancertopics/pdq/treatment/childALL/Patient/page1 NCI).

Acute lymphoblastic leukemia (ALL) is a neoplasm of precursor cells (lymphoblasts), committed to the B- or T-cell lineage. Acquisition by the precursor of a series of genetic abnormalities disturbs its normal maturation process (Figure 1), leading to differentiation arrest and proliferation of the transformed cell. As a consequence there is accumulation of an immature B- or T-cell clone, typically composed of small to medium-sized blast cells with scant cytoplasm, moderately condensed to dispersed chromatin and inconspicuous nucleoli. By definition, bone marrow is involved in all cases and peripheral blood is usually affected. Extramedullary involvement is frequent, with particular predilection for the central nervous system, lymph nodes, spleen, liver and testis in males [1].

EPIDEMIOLOGY

Acute lymphoblastic leukemia (ALL) is primarily a disease of children; 75% of cases occur in children under six years of age and there is a frequency peak between 2 and 5 years. In fact, ALL is the major pediatric cancer in developed countries, accounting for 30% of all malignancies in children [1,3-5]. The worldwide incidence is estimated at 1-4.75/100,000 per year [1,3]. Despite cure rates now exceeding 80% [6], ALL remains the leading cause of non-traumatic death in children and young adults [7,8].

The 80–85% of all the ALL are of B-cell lineage (B- ALL) [9]. From now on, we are going to center in this majority subtype.

ETIOLOGY

The precise pathogenetic events leading to development of acute lymphoblastic leukemia are unknown. Only a few cases (<5%) are associated with inherited, predisposing genetic syndromes, such as Down's syndrome, Bloom's syndrome, ataxia-telangiectasia, and Nijmegen breakage syndrome, or with ionizing radiation or exposure to specific chemotherapeutic drugs [6].

Some translocations associated with B-ALL have been detected in neonatal specimens long before the onset of leukemia, and monozygotic twins with concordant leukemia frequently share genetic abnormalities, suggesting a genetic component to at least some cases. In fact, many of these translocations appear to be primary initiating events [1,10,11].

There is very strong evidence that for the majority of childhood acute leukemia the first genetic event occurs prenatally in the fetus. Only 1% of preleukemia clones convert to overt leukemia and the most plausible explanation currently available is of an abnormal deregulated response to infection as proposed and investigated by Greaves [12]. Whether any of the other putative leukemogenic factors play any part in these secondary events is yet unclear.

Genetically determined responses certainly do provide a degree of individual susceptibility, e.g. to produce an abnormal immune response which may drive the secondary events to convert pre-leukemic clones into overt ALL [13].

CLINICAL FEATURES

Most patients with B-ALL present with evidence and consequences of bone marrow failure: thrombocytopenia, anemia and/or neutropenia. The leukocyte count may be decreased, normal or markedly elevated. Lymphadenopathy, hepatomegaly and splenomegaly are frequent. Bone pain and arthralgias may be prominent [1].

The lymphoblast in B-ALL in smeared imprint preparations vary from small blasts with scant cytoplasm, condensed nuclear chromatin and indistinct nucleoli to larger cells with moderate amounts of light blue to blue-grey cytoplasm occasionally vacuolated, dispersed nuclear chromatin and multiple variably prominent nucleoli. In most cases the morphology of the lymphoblast differs from that of normal B-cell precursors (hematogones) with which they may be confused [1].

In bone marrow biopsies, the lymphoblasts in B-ALL are relatively uniform in appearance with round to oval, indented or convoluted nuclei. Nucleoli range from inconspicuous to prominent. The chromatin is finely dispersed [1].

The lymphoblasts in B-ALL are almost always positive for the B-cell markers CD19, cytoplasmic CD79a and cytoplasmic CD22; while none of these by itself is specific, positivity in combination or at high intensity strongly supports the B lineage.

The degree of differentiation of B-lineage lymphoblasts has clinical and genetic correlates. In the earliest stage, so called early precursor B-ALL or proB-ALL, the

blasts express CD19, cytoplasmic CD79a, cytoplasmic CD22 and nuclear TdT. In the intermediate stage, so called common ALL, the blasts express CD10. In the most mature precursor B differentiation stage, so called pre-B-ALL, the blasts express cytoplasmic μ chains. The immunophenotype of precursor B-ALL differs in almost all cases from that seen in normal B-cell precursors. These differences can be very useful in evaluation of follow up bone marrow specimens for minimal residual disease [1,14].

CLASSIC PROGNOSTIC AND PREDICTIVE FACTORS

One of the main characteristics of this disease is its great heterogeneity, with marked differences between individuals at diagnosis, clinical behavior and response to chemotherapeutic agents.

Infancy, increasing age (>10 years) and higher white blood cell count are all associated with adverse prognosis. The presence of CNS disease at diagnosis is associated with adverse outcome, and requires specific therapy.

In this context, it is very remarkable that cytogenetic abnormalities are among the markers with the highest value for prognosis prediction. Cytogenetic alterations are seen in the majority of cases of B-ALL and, in many cases, they define specific leukemia subtypes with unique phenotypic and prognostic features (Table 1).

Table 1. Most common cytogenetic abnormalities in ALL and their prognostic value.

Prognosis	Cytogenetic abnormalities
Favorable or no unfavorable	Hyperdiploidy 51-81 chromosomes
	t(12;21) TEL-AML1+
	Normal Karyotype
Unfavorable	Hyperdiploidy 47-50 chromosomes
	Hypodiploidy 30-45 chromosomes
	Almost tetraploidy 82-94 chromosomes
	Other structural changes not included in the other groups
Very unfavorable	Almost haploidy 24-29 chromosomes
	t(9;22) BCR/ABL+
	t(4;11) MLL+

t(9;22)(q34;q11.2); BCR-ABL1:

BCR-ABL1 associated ALL accounts for only 2-4% of childhood ALL. The t(9;22) results from fusion of *BCR* at 22q11.2 and the cytoplasmic tyrosine kinase gene *ABL1* at 9q34, with production of a BCR-ABL1 fusion protein. There is some suggestion that the cell of origin of t(9;22) ALL is more immature than that of other B-ALL cases [15]. The presence of this translocation has the worst prognosis among patients with ALL [1].

t(v;11q23); MLL rearranged:

ALL with MLL rearrangements is the most common leukemia in infants <1 year of age. It is less common in older children and increases with age into adulthood. The *MLL* gene on chromosome 11q23, that encodes a histone methyl -transferase involved in epigenetic regulation, has many fusion partners. The most common partner genes are *AF4* transcription factor on chromosome 4q21, *ENL* on chromosome 19p13 and *AF9* on chromosome 9p22. Leukemias with the MLL-AF4 translocation have a poor prognosis [1].

t(12;21)(p13;q22); TEL-AML1 (ETV6-RUNX1):

TEL-AML1 leukemia is common in children accounting for about 25% of cases of B-ALL. It is not seen in infants and decreases in frequency in older children. The t(12;21)(p13;q22); ETV6-RUNX1 translocation results in the production of a fusion protein that interferes with normal function of the transcription factor RUNX1, involved in normal hematopoiesis. This leukemia appears to derive from a B-cell progenitor rather than from a hematopoietic stem cell. B-ALL with the TEL-AML1 translocation has a very favorable prognosis, especially if they have other favorable risk factors. Relapses often occur much later than those of other types of ALL. Children with this leukemia who also harbor adverse prognostic factors, such as age over 10 years or high white count do not have as good a prognosis, but may still fare better than other patients with these same adverse factors [1].

Hyperdiploidy:

This leukemia is common in children, accounting for about 25% of cases of B-ALL. It is not seen in infants, and decreases in frequency in older children. Hyperdiploid B-ALL contains a numerical increase in chromosomes, usually without structural abnormalities. Extra copies of chromosomes are non random, with chromosomes 21, X, 14 and 4 being the most common and chromosomes 1, 2 and 3 being the least often seen [16]. Specific chromosomes that appear as trisomies may be more important to prognosis than the actual number of chromosomes, with simultaneous trisomies of 4, 10 and 17 carrying the best prognosis [17]. Hyperdiploid B-ALL has a very favorable prognosis. Presence of adverse factors, such as advanced age or high white count may adversely affect the prognosis, but patients may not fare as badly as others without this favorable abnormality [1].

Hypodiploidy:

Hypodiploid ALL accounts for about 5% of ALL overall. All patients by definition show loss of one or more chromosomes, having from 45 chromosomes to near haploid (23-29 chromosomes). Structural abnormalities may be seen in the remaining chromosomes though there are no specific abnormalities that are characteristically associated. The diagnosis of near haploid or low hypodiploid B-ALL may be missed by standard karyotyping because the hypodiploid clone may undergo endoreduplication doubling the number of chromosomes, resulting in a near diploid or hyperdiploid karyotype. Hypodiploid B-ALL has a poor prognosis. The prognosis depends on the number of chromosomes: those with 44-45 chromosomes have the best prognosis and those with near haploid B-ALL fare worst in some but not in all studies [18,19]. There is some evidence that, in contrast to other types of B-ALL, patients may fare poorly even if they do not have minimal residual disease following therapy [1].

t(1;19)(q23;p13.3); E2A-PBX (TCF3-PBX1):

E2A-PBX ALL is relatively common in children, accounting for about 6% of cases of B-ALL. The E2A-PBX translocation results in the production of a fusion protein that has an oncogenic role as a transcriptional activator and also likely interferes with the normal function of the transcription factors coded by *E2A*, involved in lymphocyte development and *PBX1*. The functional fusion gene resides on chromosome 19, and there may be loss of the derivative chromosome 1 in some but not all cases, resulting in an unbalanced translocation. In early studies, E2A-PBX was associated with poor prognosis, but this is now readily being overcome with modern intensive therapy [1].

The treatment outcome for children with acute lymphoblastic leukemia (ALL) has improved substantially with the use of risk-directed treatment and improved supportive care. The 5-year event-free survival rates for ALL now range between 76% and 86% in children receiving protocol-based therapy in the developed countries [20-34].

Risk-directed treatment is based on the stratification of patients based on analysis of known prognostic markers, and the intensification of treatment in groups in which, a priori, a poor response is expected (Table 2). Thus, the probability of survival is increased in the groups of poor prognosis while the toxicity is reduced in those with better prognosis [35].

However, one of the most important problems during treatment is that some patients in the standard risk and high risk group do not respond well to treatment and become high risk and very high risk respectively, due to slow response to initial therapy as assessed by morphologic examination of bone marrow and the presence of minimal residual disease after therapy.

This means that risk group classification is not completely accurate and could be improved. The risk attached to a misclassification is that some patients may be undertreated. In this context, thanks to advances in cytogenetic technology, new cryptic alterations can be identified, such as deletions and duplications, which have not been detected until now. Some of these alterations could be useful for the implementation of risk group classification.

Table 2. Inclusion criteria in risk groups for ALL treatment.

STANDARD RISK (SR)
A patient must meet all the following criteria to be included in this group:
Age 1-9 years
Common ALL immunophenotype (CD19+, CD10+, cytoplasmic μ chains -)
White blood cell count at diagnosis <20x10^9/l
No extramedullary involvement (CNS, testis)
Absence of unfavorable cytogenetics
<5% blasts in bone marrow at day +14
<0.1% MRD at the end of the induction phase of treatment
HIGH RISK (HR)
The existence of at least one of these criteria determines the inclusion of the patient at high risk:
Age ≥ 10 years
Any immunophenotype except for the one indicated in SR
White blood cell count between 20 and 200x10^9/l
Extramedullary involvement (CNS, testis)
Unfavorable cytogenetics
≥ 5% blasts in bone marrow at day +14
≥ 0.1% MRD at the end of induction
VERY HIGH RISK (VHR)
The existence of at least one of these criteria determines the inclusion of the patient at high risk:
White blood cell count >200x10^9/l
Very unfavorable cytogenetics
HR with ≥ 5% blasts in bone marrow at day +14/+21
HR with ≥ 0.1% MRD at the end of consolidation

NEW CYTOGENETIC PROGNOSTIC FACTORS

As we have previously described, childhood ALL is divided in multiple subtypes defined by recurring cytogenetic alterations [36]. These alterations are widely used in the stratification of patients into risk groups in order to define the therapy [7]. Several observations indicate that these alterations are insufficient to explain the response to therapy. This suggests that the detection of additional genetic alterations is required in order to improve risk group classification and treatment adjustment [7,36].

Identification of these additional genetic alterations has been limited by conventional cytogenetic approaches, which typically can only detect gross rearrangements or structural alterations more than several megabases in size [37,38]. The completion of the human genome project and the development of microarray technologies to profile structural genetic alterations at high resolution have changed our ability to identify genetic alterations in cancer genomes [7].

Submicroscopic genetic alterations can define novel subgroups of acute lymphoblastic leukemia, cooperating with known cytogenetic alterations [7]. Over 50 regions of recurring genetic alteration have been detected in ALL [39]. Many of these are not evident on conventional cytogenetic analysis and commonly involved only a single or few genes that can be involved in leukemogenesis.

Many of the targets of alteration were logical candidates in leukemogenesis, including tumor suppressor and cell cycle regulatory genes (*CDKN2A/B*, *PTEN*,

RB1), transcription factors and transcriptional coactivators (*ETV6*, *ERG*, *TBL1XR1*), involved in lymphoid maturation and signaling that have not previously been studied (e.g., *BTLA/CD200*, *TOX*), and genes involved in drug responsiveness (e.g., the glucocorticoid receptor *NR3C1*). In addition, over two-thirds of B-progenitor ALL cases have genetic alterations that disrupt the normal process of lymphoid maturation [39,40]. Common targets of alteration are *PAX5* (paired box 5), *IKZF1* (IKAROS), *EBF1* (early B-cell factor 1) and *LEF1* (lymphoid enhancer factor 1). Alterations of *PAX5* are common in ALL but do not influence treatment outcome, whereas alterations of *IKZF1* are less common but strongly associated with poor outcome in multiple distinct subtypes of high-risk ALL [41].

Another important observation was that the nature and frequency of individual lesions varied significantly between B-ALL subtypes. Notably, MLL-rearranged leukemias harbor few additional genetic alterations, consistent with the notion that MLL-rearrangement may be sufficient to induce leukemia [42,43]. In contrast, ETV6-RUNX1 (TEL-AML1) and BCR-ABL1 rearranged leukemias harbor [36,44] additional copy number alterations per case.

These studies have provided clear evidence of the power of genome-wide profiling approaches to identify genes and pathways of central importance in establishment of the leukemic clone, and also in responsiveness to therapy. However, from the clinical perspective, despite remarkable progress in cataloging the molecular lesions, our understanding of how to integrate these findings into treatment adjustment and patient care is still rudimentary [20]. We think that the detection of new alterations, cryptic for the traditional technologies, could be of great help for risk group optimization. This

optimization would improve treatment, increasing the benefits of therapy while reducing secondary effects.

CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA TREATMENT: LAL/SHOP

Once risk groups are established according to classic prognostic factors, the therapy is adjusted. Nowadays, well established and complex treatment protocols are applied [6]. Specifically, one of the protocols used in Spain for treatment of ALL is the one approved by the Society of Pediatric Hematology and Oncology, LAL/SHOP. It has several versions (94, 99, 2005), with slight differences among them. LAL/SHOP 2005 is described below (Figure 2).

First, an induction phase is applied during about 5 weeks. In this phase, the number of leukemic cells in the bone marrow is to be reduced to 5% in order to restore normal hematopoiesis. For this, they use drugs such as prednisone (glucocorticoid), vincristine (interferes with microtubules of the mitotic spindle), L-asparaginase (inhibits activation of asparagine and interferes with the synthesis of proteins), daunorubicine (blocks topoisomerases) and cyclophosphamide (alkylating agent). Furthermore, intrathecal therapy (methotrexate, cytarabine and hydrocortisone) is begun to avoid the involvement of the central nervous system (CNS).

In the next phase of consolidation, which is maintained for around 8 weeks, the treatment is enhanced to prevent the onset of therapy-resistant clones. All the risk groups receive the same consolidation therapy. At this stage, methotrexate (MTX) and 6-mercaptopurine (6-MP), that are the backbone of therapy, are

used. Methotrexate is a folate analogue that exerts its antitumor effect by inhibiting the synthesis of purines, pyrimidines and proteins. MTX is given in 24 h infusion with folinic acid rescue. The 6-mercaptopurine, meanwhile, is analogous to purines and incorporated into DNA [45]. Furthermore, the treatment is completed with cytarabine (cytosine analogue inhibits DNA polymerase) and intrathecal therapy.

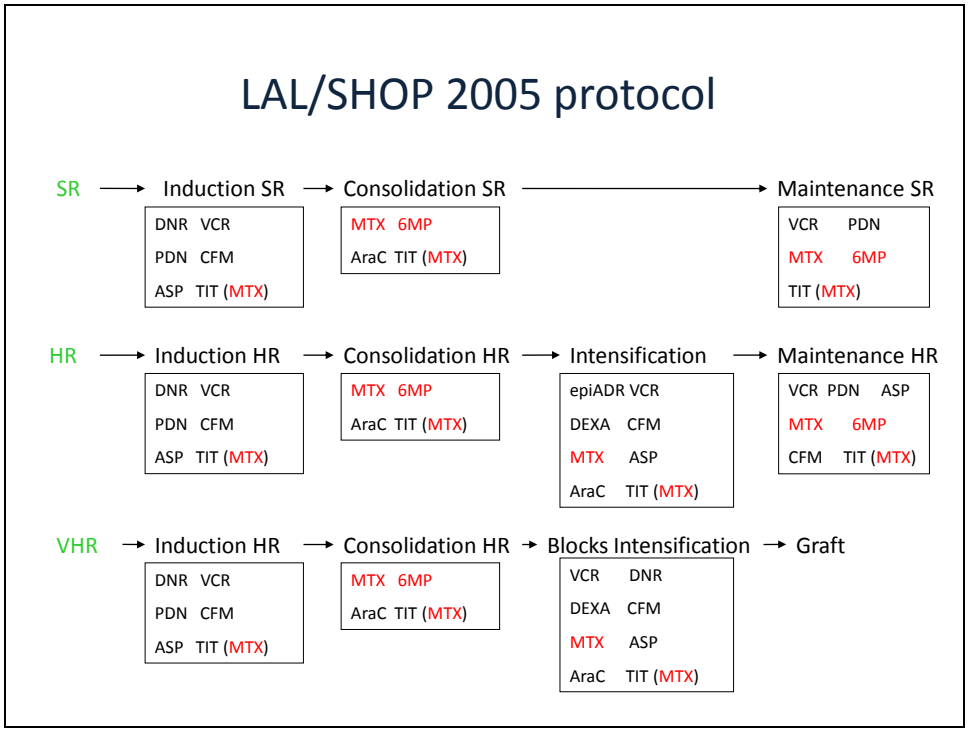


Figure 2. Diagram of LAL/SHOP-2005 treatment protocol

Finally, it conducts a maintenance phase, which can be extended for about two years, which is intended to maintain remission with reinductions. It still uses methotrexate and mercaptopurine in combination with other drugs [46].

In high risk (HR) and very high risk (VHR) groups, in addition, there is an

intensification phase before maintenance phase. In this phase, a variety of drugs are used including vincristine, dexamethasone, methotrexate, cytarabine or L-asparaginase. Patients who respond worse to treatment are undergoing hematopoietic cell transplantation.

Improvements in treatment protocols have diminished or eliminated the impact of many conventional prognostic factors in ALL, such as male sex and black race [27,31]. Thus current ALL trials have focused on improving not only the outcome of a few subtypes that remain refractory to treatment (e.g., infant ALL with *MLL* rearrangement, hypodiploid ALL, and poor early responders), but also the quality of life of the patient [20].

One of the most important problems associated with these treatment protocols is that, in spite of clinical success, some patients experience severe toxicity, which can necessitate dose reduction or treatment cessation. Therefore, it would be of great interest to recognize in advance which patients are going to suffer from these side effects in order to adjust the treatment. In this regard, pharmacogenetic studies are providing an important basis for enhancing the effectiveness of treatment and reduce complications [47].

PHARMACOGENETICS

Pharmacogenetics is the study of the genomic basis for interindividual differences in the absorption, distribution, metabolism, excretion of drugs (pharmacokinetics) and the relationship to pharmacologic effects, either therapeutic or adverse (pharmacodynamics) [48].

Pharmacogenetic studies try to develop models that accurately predict drug response and toxicity for individual patients, and to use this information to prospectively personalize treatment regimens with the goal of enhancing efficacy and safety [49].

The inherited interindividual differences in pharmacokinetics and pharmacodynamics of drugs can be due to genetic polymorphisms affecting the gene function or expression [48,50]. Common genetic variations include single-nucleotide polymorphisms (SNPs), genomic insertions and deletions, and genetic copy number variations (CNVs) [49].

The polymorphisms most often used in the pharmacogenetic studies are the SNPs. SNPs are single base substitutions of one nucleotide with another (Figure 3), observed in the general population at a frequency greater than 1%. SNPs are the simplest form of DNA variation among individuals occurring throughout the genome at a frequency of about one in 200-300 bp. Recent large-scale studies have identified approximately 15 million single nucleotide polymorphisms in the human genome [51].

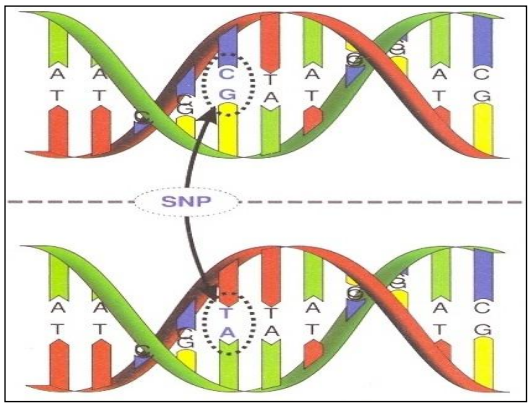
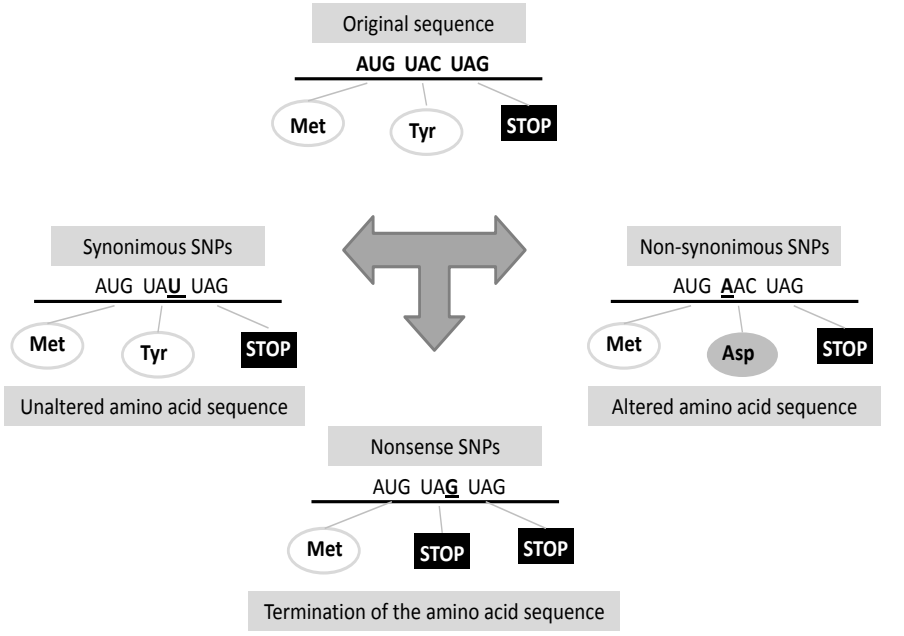
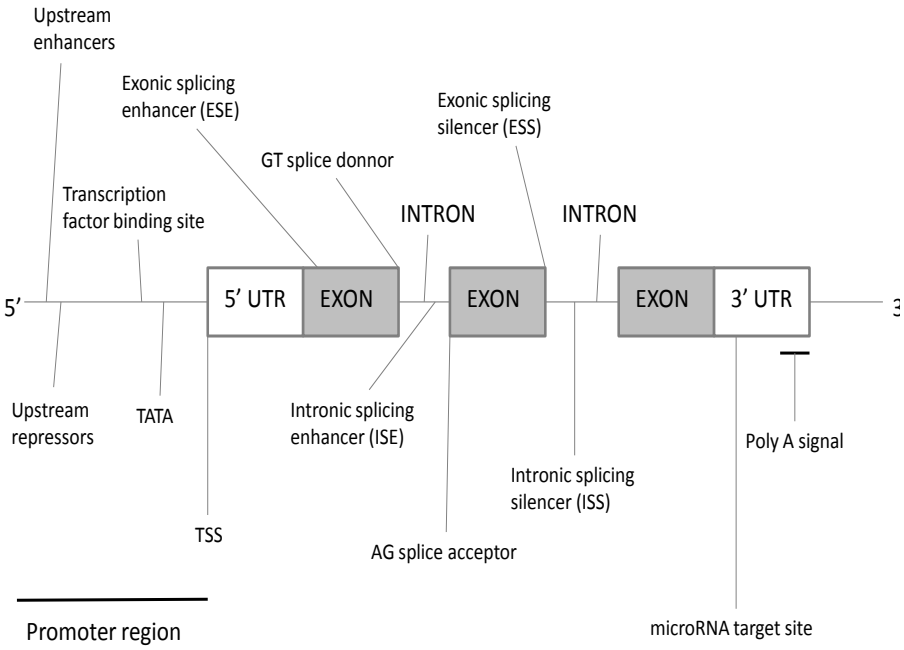


Figure 3: Example of a SNP. Two chromosomes with a C/T SNP.

SNPs can be found across human genome in genes as well as in non-genic regions. Within a gene, SNPs that are located in coding regions are called coding SNPs (cSNPs) (Figure 4A). Even though cSNPs have been the most studied polymorphisms in the past years, SNPs in regulatory regions have also gained importance due to their possible functional effects (Figure 4B).



A)



B)

Figure 4: A) Exonic SNPs with possible functional effect on the amino acid sequence. **B)** SNPs with other possible functional effect on the gene.

These potentially functional effects include alternative splicing, regulation in the promoter region, changes in transcription factor binding sites, or disruption/creation of CpG sites, that could carry changes in the methylation pattern, or miRNAs target sites, involved in the downregulation of gene expression at the post-transcriptional level.

Nevertheless, this type of polymorphism is relatively rare and it is not yet possible to predict whether most noncoding polymorphisms might have functional consequences. Given the high number of SNPs, it is impractical genotyping all existing common variants. SNPs in the same region of DNA form haplotypes that are typically inherited together. The human genome is composed of stretches of high linkage disequilibrium (LD)* (regions with a high level of concomitant inheritance), punctuated by recombination hotspots or points of extremely low LD [52-54]. This means that many SNPs located in the same haplotype block are not inherited independently and show correlated genotypes due to linkage disequilibrium [55,56], which results in redundancy of information. The knowledge of the haplotype structure of the genomic region of interest allows the selection of a reduced number of SNPs which 'tag' the common haplotypes of a region, resulting in a great reduction of cost and time [57,58].

*LD is observed when a particular allele at one locus is likely to co-segregate with a specific allele at neighboring locus on the same chromosome more often than expected by random segregation in a population. LD is a measure of association, correlation or segregation of two separate loci in a population. Various measures have been proposed to characterize LD; the most commonly used are D' and r^2. Both values range between 0 and 1. A value of D' =1 is known as complete LD. Values of D'<1 indicate that the complete ancestral LD has been disrupted. The magnitude of D' depends strongly on sample size and values of D'<1 have no clear interpretation. r^2 = 1 is known as perfect LD between two markers, making the two redundant. r^2 is more useful for dividing closely located SNPs into blocks, but has no direct relationship with recombination.

Therefore, a tagSNP is a representative SNP in a region of the genome with high LD. Nowadays, the selection of tagSNPs is facilitated by the existence of The International HapMap Project, a multi-country effort to identify and catalogue genetic similarities and differences in human beings (Figure 5).

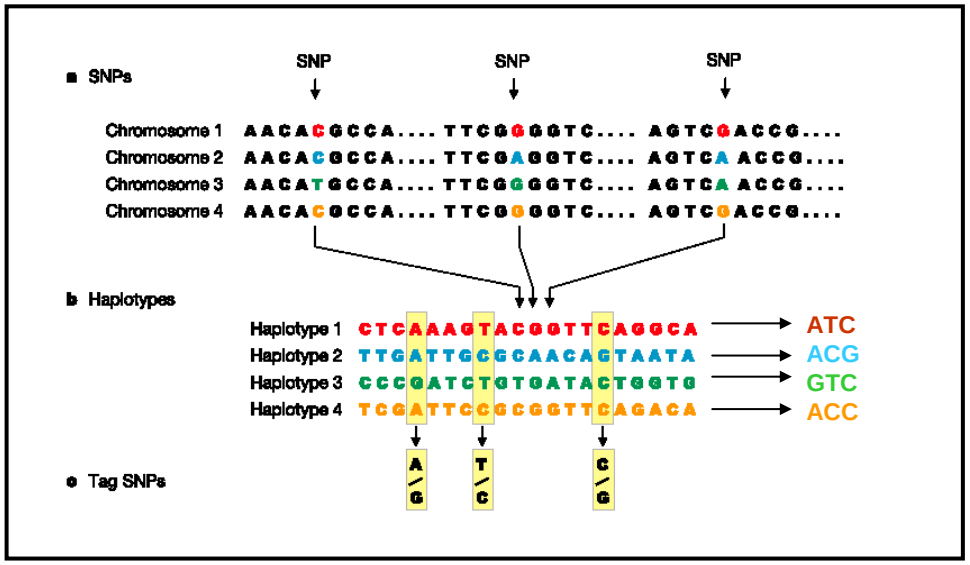


Figure 5: Example of tagSNP **a)** SNPs are identified in DNA samples from multiple individuals. **b)** Adjacent SNPs that are inherited together are compiled into "haplotypes." **c)** Detection of "Tag" SNPs within haplotypes that identify uniquely those compiled haplotypes. By genotyping the three tag SNPs shown in this figure, it can be identified which of the four haplotypes shown here are present in each individual.

The selection of tagSNPs, that maximize the recognition of the common variation across a given gene, together with the evaluation of multiple genes biologically correlated with each other, increases the chances that at least one typed SNP will be associated with disease. Indeed, this strategy is statistically powered to detect such an association.

CLINICAL IMPLEMENTATION OF PHARMACOGENETICS

Pharmacogenomics can play an important role in identifying responders and non-responders to medications, avoiding adverse events, and optimizing drug dose.

After more than half a century of pharmacogenetic research, the clinical use of pharmacogenetic testing remains quite uncommon, despite many examples showing that inherited genomic variation causes substantial interindividual differences in drug effects [59].

The table below lists FDA-approved drugs for oncology with pharmacogenomic information in their labels (http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm) (Table 3). Some, but not all, of the labels include specific actions to be taken based on genetic information. Relevant sections of the label with such information are noted in the last column of the table. Drug labels may contain information that can describe:

- Drug exposure and clinical response variability
- Risk for adverse events
- Genotype-specific dosing
- Mechanisms of drug action
- Polymorphic drug target and disposition genes

Table 3. Pharmacogenomic Biomarkers in Drug Labels

Drug	**Therapeutic Area**	**Biomarker**
Desloratadine and Pseudoephedrine	Allergy	*CYP2D6*
Celecoxib	Analgesics	*CYP2C9*
Codeine/ Tramadol and Acetaminophen	Analgesics	*CYP2D6*
Quinidine	Antiarrhythmics	*CYP2D6*
Terbinafine	Antifungals	*CYP2D6*
Voriconazole	Antifungals	*CYP2C19*
Chloroquine	Antiinfectives	*G6PD*
Rifampin, Isoniazid, and Pyrazinamide	Antiinfectives	*NAT1; NAT2*
Abacavir	Antivirals	*HLA-B*5701*
Boceprevir	Antivirals	*IL28B*
Maraviroc	Antivirals	*CCR5*
Peginterferon alfa-2b/ Telaprevir	Antivirals	*IL28B*
Carvedilol/ Metoprolol/ Propafenone/ Propranolol	Cardiovascular	*CYP2D6*
Clopidogrel/ Prasugrel / Ticagrelor	Cardiovascular	*CYP2C19*
Isosorbide and Hydralazine	Cardiovascular	*NAT1; NAT2*
Pravastatin	Cardiovascular	*ApoE2*
Cevimeline	Dermatology and Dental	*CYP2D6*
Dapsone	Dermatology and Dental	*G6PD*
Fluorouracil	Dermatology and Dental	*DPD*
Tretinoin	Dermatology and Dental	*PML/RARα*
Dexlansoprazole/ Pantoprazole/ Rabeprazole	Gastroenterology	*CYP2C19*
Dexlansoprazole (2)	Gastroenterology	*CYP1A2*
Esomeprazole/ Omeprazole	Gastroenterology	*CYP2C19*
Sodium Phenylacetate and Sodium Benzoate/ Sodium Phenylbutyrate	Gastroenterology	*UCD (NAGS; CPS; ASS; OTC; ASL; ARG)*
Lenalidomide	Hematology	Chromosome 5q
Warfarin (1)	Hematology	*CYP2C9*
Warfarin (2)	Hematology	*VKORC1*
Atorvastatin	Metabolic and Endocrinology	LDL receptor

Table 3. Pharmacogenomic Biomarkers in Drug Labels (Continuation)

Drug	**Therapeutic Area**	**Biomarker**
Carisoprodol	Musculoskeletal	*CYP2C19*
Carbamazepine/ Phenytoin	Neurology	*HLA-B*1502*
Clobazam	Neurology	*CYP2C19*
Dextromethorphan and Quinidine/ Galantamine/ Tetrabenazine	Neurology	*CYP2D6*
Irinotecan	Oncology	*UGT1A1*
Mercaptopurine	Oncology	*TPMT*
Nilotinib	Oncology	*UGT1A1*
Rasburicase	Oncology	*G6PD*
Thioguanine	Oncology	*TPMT*
Aripiprazole/ Atomoxetine/ Chlordiazepoxide and Amitriptyline/ Citalopram/ Clomipramine/ Venlafaxine/ Clozapine/ Desipramine/ Doxepin/ Fluoxetine/ Olanzapine/ Fluvoxamine…	Psychiatry	*CYP2D6*
Citalopram/ Diazepam	Psychiatry	*CYP2C19*
Valproic Acid	Psychiatry	*UCD (NAGS; CPS; ASS; OTC; ASL; ARG)*
Indacaterol	Pulmonary	*UGT1A1*
Ivacaftor	Pulmonary	*CFTR (G551D)*
Drospirenone and Ethinyl Estradiol	Reproductive	*CYP2C19*
Clomiphene	Reproductive and Urologic	Rh genotype
Tolterodine	Reproductive and Urologic	*CYP2D6*
Azathioprine	Rheumatology	*TPMT*
Flurbiprofen	Rheumatology	*CYP2C9*

PHARMACOGENETICS IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

Pharmacogenetic studies may be a useful tool in the context of childhood ALL for several reasons:

- Treatment protocols are standardized and well established.

- Chemotherapy drugs used in treatment have a very narrow therapeutic range. That means that there is little difference between the effective dose and the dose that begins to produce toxicity [45,60]. Consequently, small changes in the function of the genes involved in their pathways can have a great impact in treatment response.

- Genes that influence the response to these drugs are highly variable [48,49,61]. Thus, genetic variants have been described associated with relapse, which would require, therefore, an increase in drug dosage, and variants associated with toxicity that would require a reduction in this dose.

In this context, polymorphisms of the drug transporters, targets, and metabolizing enzymes that could influence the effectiveness and toxic effects of therapy in pediatric ALL have been described.

As methotrexate and 6-mercaptopurine are the backbone of pediatric ALL therapy, there has been a great interest in analyzing polymorphisms in the genes involved in their metabolic and transport pathways. In addition, as ALL patients are treated with complex multidrug regimens, polymorphisms in genes

encoding enzymes affecting the clearance of several drugs have also been studied.

Pharmacogenetics of methotrexate pathway

Methotrexate (MTX) is a folate analogue that, once inside the cell, directly or through its derivatives, inhibits DNA and protein synthesis. MTX is an essential component of therapy in nearly all treatment protocols for childhood ALL. Although the optimal dosage is still under active investigation, high-dose MTX (HDMTX) is commonly given as consolidation therapy, and low-dose oral MTX (LDMTX) is given in continuation therapy in most childhood ALL treatment protocols [49].

MTX enters the cell primarily via active transport mediated by the reduced folate carrier (RFC1) [62,63]. Once inside the cell, MTX is quickly converted into polyglutamated forms (MTXPGs). MTX inhibits dihydrofolate reductase (DHFR), which converts folates (DHF) to their active form tetrahydrofolate (THF), affecting other important enzymes, such as methylenetetrahydrofolate reductase (MTHFR) and serine hydromethyl transferase (SHMT1). On the other hand, the polyglutamated forms of MTX target other folate-dependent enzymes such as thymidylate synthase (TS) directly. As a result, nucleic acid and protein synthesis is inhibited, favoring cell death [64]. Finally, different transporters act pumping MTX out of the organism. These include ABC transporters, such as the multidrug resistance protein (ABCB1) and the breast cancer resistance protein (ABCG2) [65], and organic anion transporters, such as SLCO1B1 [66,67] (Figure 6).

Despite its clinical success, treatment with high-dose MTX often causes toxicity, requiring a dose reduction or cessation of treatment. Therefore, for appropriate use of MTX, it would be useful to identify a predictor of the adverse effects of MTX [68].

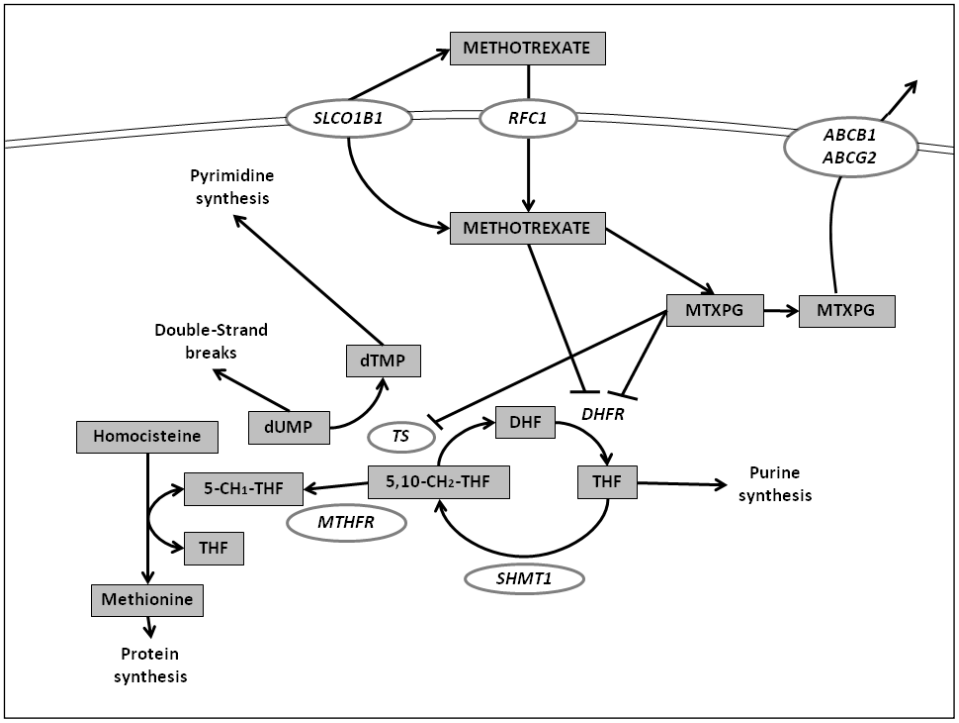


Figure 6. Methotrexate pathway. Genes are marked in italics.

MTX pharmacokinetics exhibit a large interindividual variability that is still incompletely understood in patients with normal renal and liver function and sufficient hydration [49]. In this context, polymorphisms of the MTX transporters, MTX targets, and folate-metabolizing enzymes that could influence the effectiveness and toxic effects of MTX in pediatric ALL have been described. For instance, a polymorphism in *RFC1* (G80A), resulting in a less efficient

transporter, has been associated with outcome [69,70], leucopenia [71], treatment interruption [72], gastrointestinal [73,74], hematologic [70] and hepatic toxicity [68,70]. Polymorphisms in the *MTHFR* (C677T and A1298C) gene, that reduce the enzymatic activity, have been associated with outcome [75,76] and several toxicities [68,71,72,77-82]. Polymorphism C1420T in *SHMT1* gene, that affects the enzymatic activity, has been associated with hepatic toxicity [83]. *TS* 28-bp tandem repeat and 6bp deletion have been linked to differences in *TS* expression and with treatment outcome [82,84-87], leucopenia, thrombocytopenia [71,88] and mucositis [71]. *ABCB1* C3435T and *ABCG2* C421A polymorphisms result in less active transporters and have been associated with outcome [89-92], nervous system toxicity and infections [93-95]. And more recently, the *SLCO1B1* rs4149081 and rs11045879 polymorphisms have been associated with MTX clearance [66].

However, the associations of polymorphisms and toxicity found by several groups are not always confirmed. For example, *MTHFR* C677T polymorphism has been associated with increased toxicity in some populations [68,71,72,77-82] but other authors did not find any association [63,73,74,76,83,88,96-100] or even find a protective effect [101-103]. This lack of replication could be due to differences in treatment protocols among studies, small or non-homogeneous populations or the use of different toxicity criteria. Consequently, studies with patients treated homogeneously and the use of an objectively quantifiable marker of toxicity are needed. In this context, MTX plasma levels at the terminal phase could be an objective marker to analyze MTX-associated toxicity [68].

Pharmacogenetics of 6-mercaptopurine pathway

The thiopurine antimetabolite 6-mercaptopurine (6MP) is an analogue of the purine nucleosides hypoxanthine and guanine which interfere with nucleic acid biosynthesis. MP is a key component in childhood ALL treatment protocols and is used during consolidation treatment, as well as in maintenance therapy [49].

After uptake via nucleoside transporters, 6MP is metabolized into active cytotoxic thioguanine nucleotides (TGNs), with the initial step catalyzed by hypoxanthine phosphoribosyl transferase (HPRT). Cytotoxicity occurs mainly by incorporation of TGNs into DNA or RNA resulting in cell cycle arrest and apoptosis [48,49].

6MP can also be methylated by thiopurine methyltransferase (TPMT) to methylmercaptopurine, an inactive metabolite that cannot be converted to active nucleotides [48,104-106]. The TPMT pathway is the main mechanism of thiopurine's intracellular inactivation in hematopoietic tissues. Thus the balance between TGNs and inactive metabolites in hematopoietic cells is regulated predominantly by TPMT [49].

Large interindividual differences in TPMT activity were recognized as an inherited trait over 25 years ago, and more recently the genetic polymorphisms in the *TPMT* gene that are responsible for this pharmacogenetic trait have been identified and characterized [107]. Although more than 20 less active TPMT variants have been described, three variant alleles, *TPMT*2* (238G>C), *TPMT*3A* (460G>A and 719A>G), and *TPMT*3C* (719A>G), account for more than 95% of the inherited variability in *TPMT* enzyme activity [49,104,108]. These polymorphisms

do not affect the mRNA expression but they render the protein more susceptible to degradation by the proteasome [109], leading to lower drug inactivation (Figure 7). About 90% of the population has two wild-type *TPMT* alleles (*TPMT*1*) and thus "normal" enzyme activity; about 5–10% of individuals inherit one wild-type *TPMT* allele and one non-functional allele and have intermediate activity; and only about one in 300 persons inherits two nonfunctional alleles and is therefore TPMT-deficient [110]. Clinical interest in *TPMT* pharmacogenetics is based on numerous studies showing that *TPMT* genotype or phenotype identifies patients at high risk of hematopoietic toxicity after thiopurine therapy [49,111]. TPMT-deficient patients treated with conventional doses of thiopurines are predisposed to drug-induced complications by accumulation of excessive intracellular concentrations of TGNs [49,112-114]. These patients require a reduction of more than 90% of the conventional dose [49].

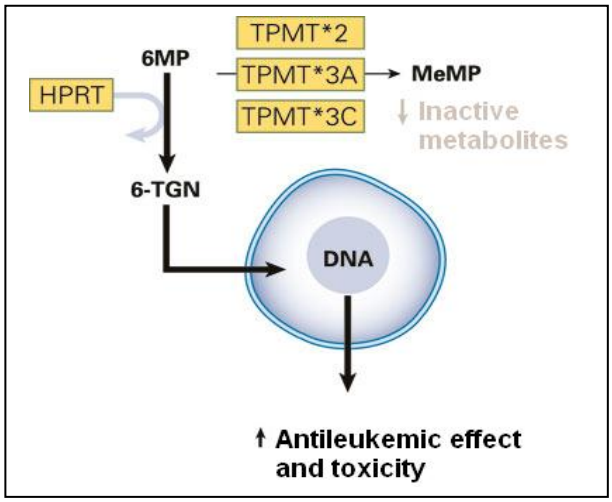


Figure 7. 6-mercaptopurine pathway (adaptation from Lehne et al, 2007 [115])

Whether patients with one functional allele would benefit from dose reduction is less clear [46,49,72,73,75,85,116-130]. Although heterozygous *TPMT* patients treated at conventional doses (75 mg/m2 per day) have been described to be at higher risk of hematopoietic toxicity [114], those treated with lower doses (60mg/m2 per day) did not exhibit a higher rate of hematopoietic toxicity and would therefore not be expected to benefit from dose reduction [46]. In addition, *TPMT* genotype has been associated with a better early response to ALL chemotherapy, measured as minimal residual disease (MRD) following ALL induction and consolidation therapy. This finding was consistent with higher systemic exposure to thiopurines due to lower MP metabolism. Long-term outcome studies are necessary to clarify whether MP dose escalation in the large cohort of *TPMT*-homozygous wild-type patients will yield greater efficacy in protocols that routinely use lower MP doses (50-60 mg/m2/d).

Pharmacogenetics of drug-metabolizing enzymes

ALL patients are treated with complex multidrug regimens. Inherited genetic variation affecting the clearance of several drugs would be expected to be among the polymorphisms most likely to influence the risk of treatment failures [131]. The most important groups of drug-metabolyzing enzymes are phase I enzymes (CYP1A1), which metabolize the functional part of drug molecules, leading to activation or inactivation of the molecule, phase II enzymes (GSTs), which conjugate drugs with endogenous substances so that the drug can be more readily excreted, and free radical metabolyzing enzymes (NQO1).

The family of cytochrome P450 (CYP) is responsible for the metabolism of hormones and drugs. In the gene *CYP1A1*, *CYPA1*2A* allele results in an enzyme with increased activity, so that the removal of drugs may be increased. Thus, this polymorphism has been associated with poorer response to therapy [132] and with increased risk of secondary tumors after treatment [133].

The family of enzymes glutathione-S-transferases (GSTs) is responsible for the inactivation of xenobiotics through conjugation with glutathione. Therefore they are responsible for inactivating a wide range of drugs used in childhood ALL therapy, such as glucocorticosteroids, vincristine, anthracyclines and cyclophosphamide. Among the important pharmacogenetic polymorphisms described in ALL, there are deletions of *GSTM1* and *GSTT1* genes and the A313G substitution in the *GSTP1* gene (*GSTP1*B*).

Homozygous deletion of *GSTM1* gene is present in 50% of the population. A possible role of this polymorphism in outcome has been described in patients with ALL [85,131,134-137] and it has also been related to hepatotoxicity [68,73,82] and infections [95]. The homozygous deletion of the *GSTT1* gene, found in 25% of the population, is associated early response to prednisone and outcome [131,134,136,137]. A possible association between these polymorphisms and the presence of gastrointestinal toxicity has also been proposed [73,82].

On the other hand, the *GSTP1*B* allele codifies a low-activity enzyme. A possible association between this variant and relapse [82,89,136-139] and central nervous system toxicity [73] has been described in patients with ALL.

The enzyme NAD (P) H: quinone oxidoreductase (NQO1) converts benzoquinones in less toxic metabolites, protecting the body against tumor development. It also inactivates drugs such as doxorubicin, which is part of the induction phase of therapy in ALL [140]. The variant *NQO1*2* (C609T) has lower enzyme activity. It has been associated with poorer survival and outcome in patients with ALL [87,132]. It has also been related with an increased risk of secondary tumors after treatment [140,141].

However, the associations are sometimes contradictory and other works do not observe any association. The relevance of these polymorphisms in ALL appears to depend on the therapeutic strategy that is being applied [45]. For this reason, it would be necessary to carry out their analysis in the context of each treatment protocol to evaluate their effect.

MicroRNAs

Most of the pharmacogenetic studies carried until now are focused in coding regions. Nevertheless, these regions correspond only to about 1.5% of the entire genome. Consequently, a major landmark in recent studies is the analysis of regions that do not codify proteins but may have a regulatory function, as microRNAs (miRNAs).

The discovery of miRNA is one of the most exciting scientific breakthroughs in recent history. MiRNAs are a family of endogenous small non-coding RNAs (≈22 nucleotides long) involved in various developmental and physiological processes that downregulate gene expression at the post-transcriptional level [142].

MiRNAs genes are transcribed in the nucleus, synthesizing a double stranded RNA (300-5000bp) called pri-miRNAs. These pri-miRNAs are processed to form the pre-miRNAs, of between 60 and 100 nucleotides. The pre-miRNAs are exported to the cytoplasm and finally, are cleaved to produce two strands of miRNA; a mature miRNA of between 19 and 22 nucleotides and its complementary miRNA. One of the strands of the miRNA binds specifically to an mRNA [143,144] (Figure 8). MiRNAs recognize their target mRNAs by binding to the 3'UTR of the target gene with partial complementarity [145], which leads to an inhibition of translation and facilitated degradation of the target mRNA. Due to this peculiarity, miRNA may influence the expression of many genes involved in fundamental cellular functions such as proliferation, apoptosis and differentiation [146]. So far, over 1000 miRNAs have been described and their number is increasing [147].

MiRNAs deregulation may occur through genetic alterations that can affect the miRNA production, maturation processing and/or interactions with the target mRNA [143]. MiRNA-related SNPs including SNPs in miRNA genes, SNPs in target sites and SNPs in miRNA biogenesis pathway may function as regulatory SNPs [148,149]. Due to the hairpin structure at miRNA processing, the influence of the thermodynamic stability of the strand that will be incorporated into the RISC complex and the base pairing requirements between the miRNA and the mRNA, SNPs in miRNA genes and in the genes involved in the miRNA processing can affect both miRNA biogenesis and function [148]. Similarly, SNPs in the miRNA targets can also affect the function of miRNA. As it is known that a single miRNA can have multiple targets, it is expected that SNPs found in miRNA may have greater biological effects than SNPs in target genes [149].

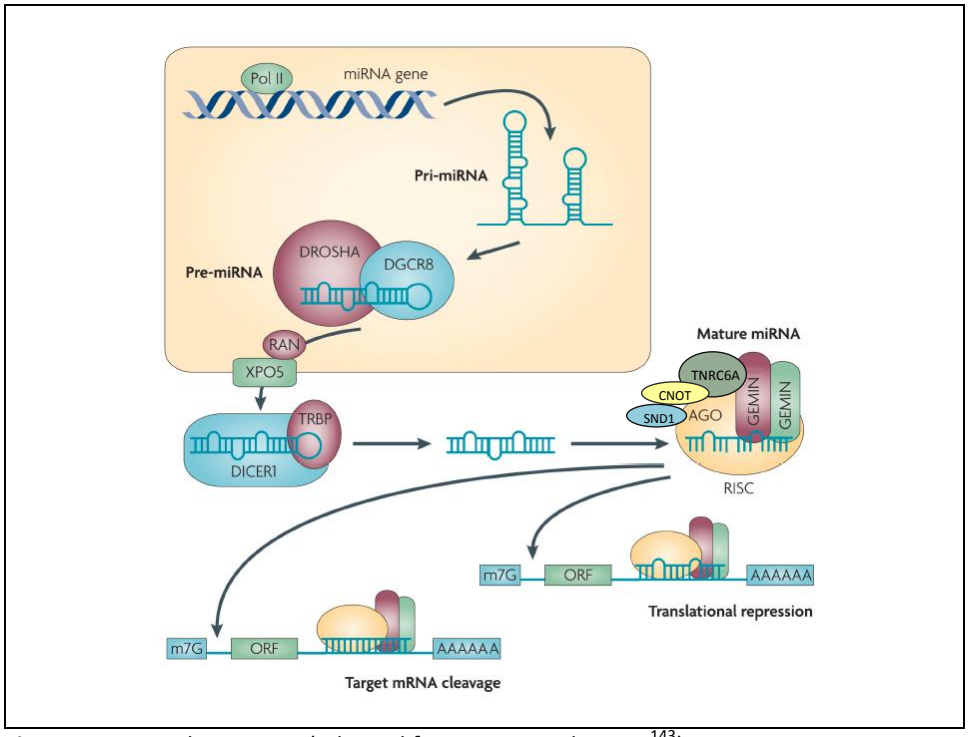


Figure 8. MiRNA biogenesis (adapted from Ryan et al, 2010 [143]).

Lately, it has been shown that the expression of specific miRNA can distinguish different response to therapy in B-ALL. For example, within miRNA associated to poor response, miR-125b, miR99a and miR-100 were related to resistance to vincristine and daunorubicin in pediatric B-ALL. Overall these data suggest that miRNA-related regulatory mechanism may play an important role in the response to treatment [150].

Consequently, miRNA-related SNPs interfering with miRNA function may lead to drug resistance or to drug sensitivity [151]. For instance, a naturally occurring SNP 829C>T near the miR-24 binding site in the 3'UTR of *DHFR* altered miR-24 function and caused increased DHFR expression and MTX resistance [152]. However, there are few studies that show how these SNPs in miRNAs, in target

sites or in the miRNA biogenesis pathway affect patient outcomes following treatment with drug. Understanding the role and function of miRNA-related SNPs has a promising future in pharmacogenomics and individualized medicine and will provide more insights into the complex phenotype of B-ALL.

HYPOTHESIS & OBJECTIVES

HYPOTHESIS

A challenge of cancer treatment is that the combination of acquired (somatic) and inherited (germline) genome variation will influence the toxicity and efficacy of cancer chemotherapy.

If we consider that pediatric Acute Lymphoblastic Leukemia treatment has a narrow therapeutic index and that the administration of the most intensive therapy that is tolerated increases the survival, we should take into account the following:

On the one hand, some patients are undertreated, do not respond well to treatment and must be changed to higher risk groups. This means that risk groups are not completely well defined. Therefore, it would be of interest to characterize those patients who from the beginning should have been considered as higher risk and treated with more intensive therapy.

On the other hand, a high percentage of patients experience toxicity, which can become very serious in some cases, being necessary to stop treatment. Consequently, it would be highly beneficial to recognize patients who are going to be more sensitive to treatment, in order to be able to adjust the dosage.

For these reasons, we propose that ALL survival could be increased and toxicity reduced by more individualized treatment. We hypothesize that the identification of new genetic markers using novel strategies and technologies would allow tumor and individual characterization which would facilitate treatment adjustment in pediatric ALL.

OBJECTIVES

The main goal of the present work was to improve the treatment personalization and adjustment in children with Acute Lymphoblastic Leukemia to make it more safe and effective by the identification of new genetic markers.

For this purpose, we set the following specific aims:

1) Improve the characterization of risk groups and treatment adjustment by the identification of new genetic markers in the tumor.

- Detect new regions of deletion and amplification with oligo arrays.

- Define their usefulness as markers for risk groups' characterization.

2) Predict the treatment toxicity with polymorphisms in key genes.

- Determine if the polymorphisms in the most representative genes of the metabolic pathways of the drugs used in the LAL/SHOP protocol could be used as toxicity predictors in pediatric ALL.

- Determine the involvement of polymorphisms in miRNAs that regulate the genes of the metabolic pathways of the drugs used, in response to pediatric ALL treatment.

-Determine if the polymorphisms in the genes of miRNAs processing have a role in pediatric ALL treatment toxicity.

MATERIAL & METHODS

POPULATION OF THE STUDY

The patients included in the whole study were 161 children all diagnosed with B-ALL from 1995 to 2011 at the Pediatric Oncology Units of 4 Spanish reference hospitals (Hospital Cruces; Hospital Donostia; Hospital Vall d'Hebrón and Hospital La Paz). All patients were homogeneously treated with the LAL-SHOP 94, 99 and 2005 protocols. Informed consent was obtained from all patients or their parents before sample collection.

In the copy number study, we included 23 of the patients diagnosed at the Hospital Cruces, treated with the LAL-SHOP 2005 protocol, for which tumoral and remission material was available. For the candidate genes approach, we included 115 patients, treated in 3 hospitals (Hospital Cruces, Hospital Donostia and Hospital La Paz). A year later, the sample population was increased. Consequently, for the studies of polymorphisms in the MTX transport pathway, we could include 151 patients from the 4 hospitals. In the study of polymorphisms in the microRNAs pathway, 152 patients were included.

Clinical data were collected objectively, blinded to genotypes, from the patients' medical files. Data collected included: risk group, treatment protocol, hepatic toxicity (AST/ALT), hyperbilirubinemia, vomiting, diarrhea, mucositis and renal toxicity (creatinine) and MTX concentrations 72 h and 96 h after infusion. Toxicity was graded according to the Spanish Society of Pediatric Hematology and Oncology (SHOP) standards, adapted from the WHO criteria (grades 0-4). The highest grade of toxicity observed for each patient during the induction and consolidation therapy period was recorded. Monitoring of MTX concentration in

plasma was carried out by a fluorescence polarization immunoassay on a TDx system (Abbott Laboratories, Abbott Park, IL). MTX levels were considered high if the concentration was over 0.2 μM. Other data including age, sex, and risk group were systematically recorded from the clinical records.

DNA ISOLATION

For the copy number analysis, both samples at diagnosis and at remission were collected. Genomic DNA was extracted from lymphocytes isolated with Ficoll-Paque™ PLUS (GE Healthcare) from bone marrow or peripheral blood. Diagnosis samples were assessed to have more than 70% blast cells and remission samples had less than 5% blast cells. DNA was isolated using QIAamp DNA Blood Mini Kit (Qiagen).

For the pharmacogenetic studies, germline genomic DNA was extracted with the phenol-chloroform method (Annex I) from remission peripheral blood, isolated granulocytes or bone marrow slides.

DETECTION OF COPY NUMBER ALTERATIONS

Copy number detection was carried out at the Centre for Applied Medical Research (CIMA) with the Cytogenetics Whole-Genome 2.7M platform

(Affymetrix). This array contains a total number of 2,761,979 copy number probes that enable a high-resolution genome-wide DNA copy number analysis.

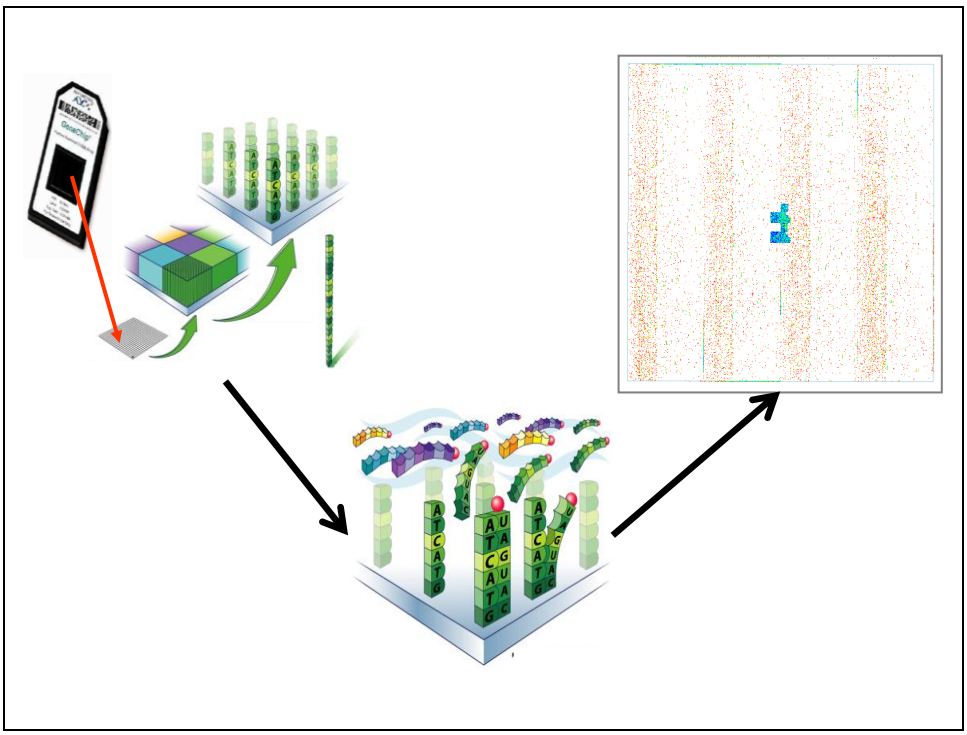


Figure 9. Amplified, fragmented and marked DNA is hybridized into the array that contains probes representing the whole genome. The intensity of each spot is indicative of the number of copies of each region of the genome

We started with 100ng of genomic DNA adjusted to a concentration of approximately 33ng/µl, which was denatured and amplified. Amplified DNA was purified using magnetic beads and its purity and concentration (above 0.55µg/µl) was validated with the Nanodrop spectrophotometer. Subsequently the DNA was enzymatically digested to obtain fragments of 50-100bp. We validated the correct fragmentation by electrophoresis on agarose gel and

proceeded to the hybridization to the microarray. Washing and staining of the Cyto-array was carried out with the GeneChip Fluidics Station 450 (Affymetrix) and subsequent scanning using the GeneChip Scanner 3000 (Affymetrix), which generated the raw data of the Cyto-array. The intensity of each spot is indicative of the number of copies of each region of the genome (Figure 9). During the protocol, specific reagents provided in the commercial Cyto-array kit (Cytogenetics Reagent Kit, Affymetrix) were used in each stage.

GENES AND POLYMORPHISMS SELECTION

We followed different strategies for genes and polymorphisms selection.

CANDIDATE GENES APPROACH: GENES AND POLYMORPHISMS PREVIOUSLY PROPOSED IN THE LITERATURE

We selected 13 genes within the 6-mercaptopurine and MTX pathways and drugs-detoxifying enzymes. In those genes, we selected 18 polymorphisms already studied in association with MTX response by other authors with controversial results and/or with a demonstrated functional effect:

-At drug detoxifying enzymes we selected 5 genes, in which we studied 5 polymorphisms: *GSTM1* and *GSTT1* homozygous deletions, *GSTP1* A313G and *NQO1* C609T, which decrease enzyme activity, and *CYP1A1* T6235C that increases the inducibility of the enzyme.

-In the 6-mercaptopurine pathway, we selected 1 gene (*TPMT*), in which we studied 3 polymorphisms (G238C, G460A and A719G) that reduce the enzymatic activity.

-In the MTX pathway, we selected 7 genes, in which we studied 10 polymorphisms: *RFC1* G80A, *MTHFR* C677T and A1298C, *SHMT1* C1420T and *ABCG2* C421A, which reduce the protein activity, *ABCB1* C3435T, *TS* 5' region 28-bp tandem repeat (3R allele) and 3' region 6-bp deletion (allele -) that change gene expression and *SLCO1B1* rs4149081 and rs11045879, with unknown function, that have been strongly associated with MTX clearance in a genome-wide study (see Introduction).

MTX TRANSPORT PATHWAY

Secondly, we focused on the MTX transport pathway.

A total of 12 candidate genes reported to be involved in methotrexate transport and elimination pathway were selected, based on the information available in the Pharmacogenomics Knowledge database, PharmaGKB (www.pharmgkb.com). These genes encode the following transporter proteins: *ABCB1, ABCC1, ABCC2, ABCC3, ABCC4, ABCG2, SLC19A1, SLC22A6, SLC22A8, SLCO1A2, SLCO1B1* and *SLCO1B3*.

A region ranging from 10-kb upstream of the translation initiation site to 10-kb downstream of the translation stop site of each gene was selected. Candidate

SNPs were identified following 3 strategies, focused mainly to include SNPs with putative functional impact on protein structure and/or gene expression, as well as SNPs maximizing the information of the common variation across a given gene:

(i) TagSNPs defined using Haploview software v.4.2 (http://www.broadinstitute.org/haploview/haploview) with an r2 threshold of 0.8 with other SNPs in individuals with European ancestry (CEU) in HapMap database. The main aim of this selection was to identify a set of SNPs efficiently tagging almost all the known SNPs in the gene. In 11 genes, all tag-SNPs were selected for genotyping. In the *ABCC4* gene, due to its large size, a subset of 71 of the 110 defined tag-SNPs was selected.

(ii) SNPs with potentially functional effects (causing amino acid changes, potentially causing alternative splicing, in the promoter region, in putative transcription factor binding sites, or disrupting CpG sites or miRNAs targets) identified using bioinformatics tools (F-SNP, Fast-SNP, polymirTS, Patrocles).

(iii) SNPs previously reported to be associated with toxicity in the literature.

In all cases, we selected only SNPs with a reported minor allele frequency greater than 10% (MAF≥0.10) in European/Caucasic populations in order to assure informative SNPs. This preliminary list of SNPs was filtered, using as criteria, suitability for the Illumina genotyping platform (selecting from each linkage block those SNPs with an assay score >0.6, associated with a high success rate).

A final number of 384 SNPs relevant to this study was included in an oligonucleotide pool assay for analysis using the Illumina Veracode technology (Illumina Inc., San Diego, CA) (Table 9, Annex I).

MICRORNAS PATHWAY

We have selected a total of 21 genes in the pathway of miRNAs biogenesis and processing after literature review and using Patrocles database (http://www.patrocles.org) (Table 4). We have also selected 42 pre-miRNAs described in the miRbase16 (http://www.mirbase.org) with known polymorphisms.

Table 4. Genes involved in miRNA biogenesis and processing.

miRNA BIOGENESIS PATHWAY GENES		
DROSHA COMPLEX	DGCR8	*DGCR8*
	DROSHA	*DROSHA*
NUCLEAR EXPORT COMPLEX	XPO5	*XPO5*
	RAN	*RAN*
DICER COMPLEX	DICER	*DICER*
	TRBP	*TRBP*
RISC COMPLEX	Argonaute Family	*HIWI*
		EIF2C1
		EIF2C2
	GW182	*TNRC6A*
		TNRC6B
	SND1	*SND1*
	GEMIN Complex	*GEMIN 3*
		GEMIN 4
		GEMIN 5
	CCR-NOT Complex	*CNOT1*
		CNOT2
		CNOT3
		CNOT4
		CNOT5
		CNOT6

Candidate SNPs were chosen according to the following criteria, focused mainly to include SNPs with putative functional impact, as well as maximizing the information:

1 - In the pathway of miRNAs biogenesis and processing, SNPs with potentially functional effects (causing amino acid changes, potentially causing alternative splicing, in the promoter region, in putative transcription factor binding sites, or disrupting miRNAs targets) were identified using bioinformatics tools (F-SNP, Fast-SNP, polymirTS [153,154], Patrocles [155]). We also included SNPs previously described in the literature. We selected only SNPs with a reported minor allele frequency greater than 5% (MAF≥0.05) in European/Caucasic populations.

2 – In the pre-miRNAs, we selected all the known SNPs with a MAF > 0.01 in European/Caucasic populations, using Patrocles, Ensembl and dbSNP databases and literature review.

The preliminary list of SNPs was filtered, using as criteria, suitability for the Taqman Openarray platform.

A final number of 118 SNPs in genes involved in miRNA biogenesis and in pre-miRNAs was included in a Taqman Openarray Plate (Applied Biosystems) (Table 10-11, Annex I).

GENOTYPE ANALYSIS

CANDIDATE GENES APPROACH

The methods used for genotyping were PCR, PCR-RFLP and PCR allele-specific (Table 5). Primers were designed using Primer3 program (http://frodo.wi.mit.edu/primer3). Each PCR was performed with 50 ng DNA. DNA fragments were visualized on a 3% agarose gel with ethidium bromide. An 8% acrylamide gel was used for analyzing *TS* 6-bp deletion. (Figures 10-13).

Table 5: Genotyping method for each of the polymorphisms selected.

Drug detoxifying enzymes				
Polymorphism	Method	Primers	Restriction enzyme	Reference
GSTM1-del (1, 3)	PCR-multiplex	GSTM1-F: GAACTCCCTGAAAAGCTAAAGC GSTM1-R: CTTGGGCTCAAATATACGGTGG	-	[156] [68]
GSTT1-del (1, 3)		GSTT1-F: TTCCTGGGTGAGCCAGTATC GSTT1-R: TTGGCCTTCAGAATGACCTC ALB-F: AAAGCCAGAGCTGGAAGTCA (control) ALB-R: CAGCTTTGGGAAATCTCTGG (control)		DO DO DO DO
GSTP1 A313G (1, 3)	PCR-RFLP (8)	F: AGGTGAGCTCTGAGCACCTG R: GAAGCCCCTTTCTTTGTTCA	BsmAI (NEB) 0.5U, 55º on	DO DO
NQO1 C609T (1, 3)	PCR-RFLP (8)	F: AAGCCCAGACCAACTTCT R: TCTCCTCATCCTGTACCTCT	HinfI (Takara) 0.5U, 37º on	[156] [156]
CYP1A1 T6235C (1, 8)	PCR-RFLP (8)	F: TGTAAAACGACGGCCAGTACAGGGTCCCCAGGTCAT R: GGAAACAGCTATGACCCGGCACTTTGGGAGGCTGAG	MspI (NEB) 2U, 37º on	DO DO
6-mercaptopurine pathway				
Polymorphism	Method	Primers	Restriction enzyme	Reference
TPMT G238C (1, 5)	PCR allele specific	F1: GTATGATTTTATGCAGGTTTG R1: TAAATAGGAACCATCGGACAC F2: GTATGATTTTATGCAGGTTTC R2: TAAATAGGAACCATCGGACAC	-	[110] [110] [110] [110]
TPMT G460A (1, 3)	PCR-RFLP (9)	F: CGACGGCCAGAGGAGGGGACGCTGCTCATCT R: GAAACAGCTATGACCAAGGCCACACAGCTTGA	BseRI (NEB) 3U, 37º on	DO DO
TPMT A719G (1, 3)	PCR-RFLP (8)	F: TAAAACGACGGCCAGTTGGGGAATTGACTGTCTTT R:AACAGCTATGACGTCTACTTGCAATCTGCAAGACACA	AccI (Takara) 0.5U, 37º on	DO DO

Table 5: Genotyping method for each of the polymorphisms selected (Continuation).

Methotrexate pathway				
Polymorphism	Method	Primers	Restriction enzyme	Reference
RFC1 G80A (1,3)	PCR-RFLP (8)	F: CTGCAGACCATCTTCCAAGG R: AGGAGGTAGGGGGTGATGAA	HhaI (Takara) 0.5U, 37º on	DO DO
MTHFR C677T (1, 3)	PCR-RFLP (8)	F: GGAAGGTGCAAGATCAGAGC R: CTCACCTGGATGGGAAAGAT	Hinf I (Takara) 0.5U, 37º on	DO DO
MTHFR A1298C (1, 3)	PCR-RFLP (8)	F: GTAAAACGACGGCCAGGGAGGAGCTGACCAGTGCAG R: GAAACAGCTATGACGCTGCGGTCAGGCCAGGGGCAG	Fnu4HI (NEB) 0.5U, 37º on	DO DO
TYMS 2R/3R (2,4)	PCR	F: CTCCGTTCTGTGCCACACC R: GGAGGATGTGTTGGATCTGC	-	DO DO
TYMS 6bp-del (1, 5)	PCR	F: GGAGCTGAGTAACACCATCG R: CAGAATGAACAAAGCGTGGA	-	DO DO
SHMT1 C1420T (1, 5)	PCR allele specific	F1: GTTGAGAGCTTCGCCTCTT R1: GTCAACAGTTCCCCTTTGGA F2: GCCACCCTGAAAGAGTTCAA R2: GCCAGGCAGAGGGAAGAG	-	DO DO DO DO
SLCO1B1 rs4149081 (1,6)	PCR allele specific	F1: GTGATTCAAGGATAATAACCAACTTG R1: GCCCCAGCTAGTCATTCTGT F2: CTGACTTTGCATGCAGTATGG R2: CCATTTTCTATTATCTCTGATTTTTGAT	-	DO DO DO DO
SLCO1B1 rs11045879 (1,6)	PCR allele specific	F1: TGTTTCTTTGATGATATATATGAAGATGC R1: GAAATTGTCTTTGTTTGCAATATGAC F2: TTAATCACATGCATTTAAATTTCCTC R2: ATCCAGGGTTAATATAACAGAATCAAA	-	DO DO DO DO
ABCB1 C3435T (1, 3)	PCR-RFLP (8)	F: TTCAAAGTGTGCTGGTCCTG R: GCATGTATGTTGGCCTCCTT	MboI (Takara) 0.5U, 37º on	DO DO
ABCG2 C421A (1, 5)	PCR allele specific	F1: CTCTGACGGTGAGAGAAAACTAAC R1: TGCTGATCATGATGCTTTCA F2: CATGGTCTTAGAAAAGACTCATTATCA R2: CGAAGAGCTGCTGAGAAGTT	-	DO DO DO DO

on: Over night
DO: Design and optimization in our laboratory
(1)Master mix: 0.2 mM dNTPs, 1.5 mM MgCl2 (except for SLCO1B1, 2mM MgCl2 and CYP1A1, 1mM), 1X ImmoBuffer, 10 pmoles of each primer and 0.5 U Immolase enzyme (BIOLINE)
(2) Master mix: GC-RICH PCR System (Roche Applied Science), according to the manufacturer's instructions
(3) PCR protocol: 95ºC for 7 min, (95ºC for 30 s, 60ºC for 30 s, 72ºC for 30 s) 35 cycles and 72ºC for 10 min
(4) PCR protocol: 95ºC for 3 min, (95ºC for 30 s, 58ºC for 30 s, 72ºC for 45 s) 35 cycles and 72ºC for 7 min
(5) PCR protocol: 95ºC for 7 min, (95ºC for 30 s, 58ºC for 30 s, 72ºC for 30 s) 35 cycles and 72ºC for 10 min
(6) PCR protocol: 95ºC for 7 min, (95ºC for 30 s, 60ºC for 30 s, 72ºC for 30 s) 30 cycles and 72ºC for 10 min
(7) PCR protocol: 95ºC for 7 min, (95ºC for 30 s, 65ºC for 30 s, 72ºC for 30 s) 35 cycles and 72ºC for 10 min
(8) 15 µl of amplified DNA were used in each digestion
(9) 5 µl of amplified DNA were used in each digestion

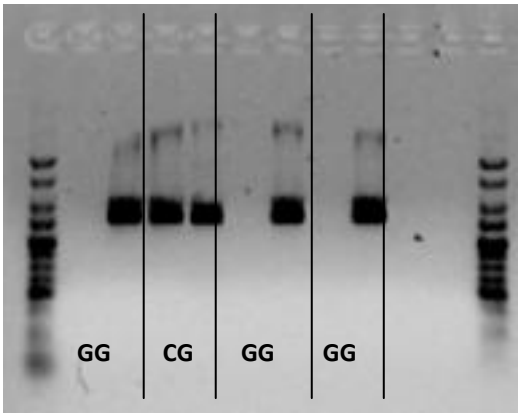


Figure 10. PCR allele specific (2 PCRs per sample). *TPMT* G238C.

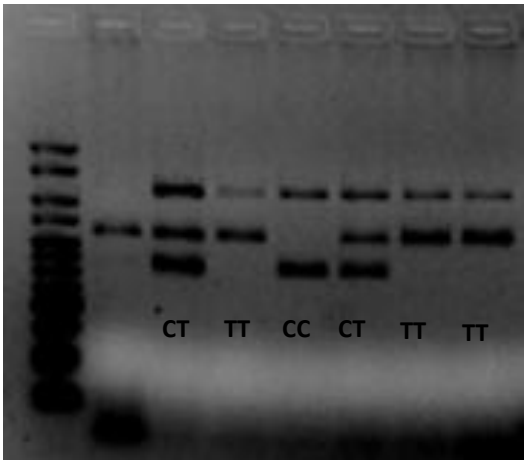


Figure 11. PCR allele specific (1 PCR per sample). *SLCO1B1* rs11045879.

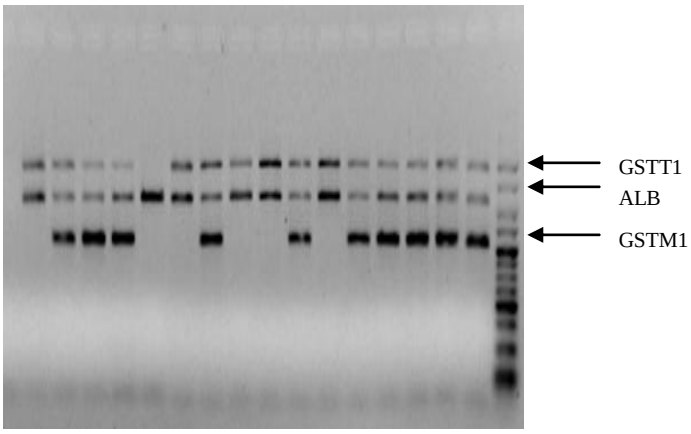


Figure 12. PCR-multiplex. *GSTM1, GSTT1* and *ALB.*

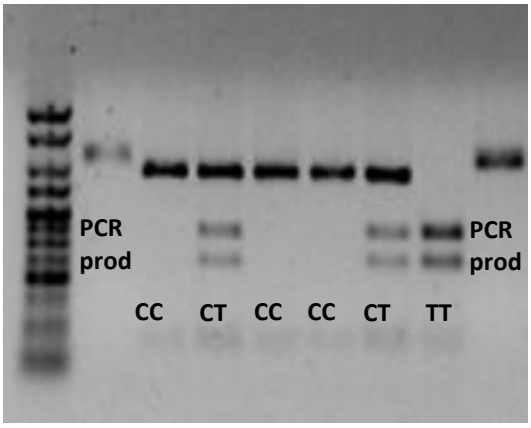


Figure 13. PCR-RFLP. *MTHFR* C677T.

MTX TRANSPORT PATHWAY

Genotyping was performed at the Spanish National Genotyping Center (CeGen) using the GoldenGate Genotyping Assay with Illumina Bead Array System (Illumina Inc., San Diego; USA).

In this approach during the liquid phase, allele specific oligos (ASO) are hybridized to genomic DNA, extended and ligated to a locus specific oligo (LSO). PCR is performed using universal primers. The multiplexed products are hybridized to a universal Sentrix Array for detection and analysis. A schematic view of the principle of the assay is shown in Figure 14.

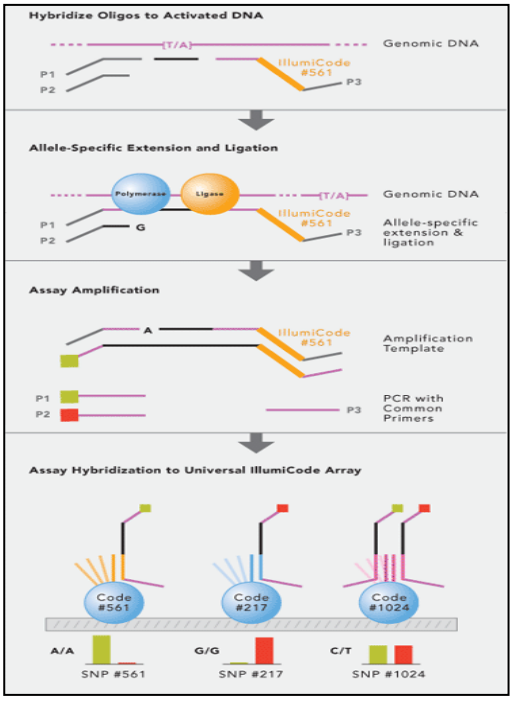


Figure 14. Goldengate assay overview.

Each reaction required a total of 400 ng of DNA. The DNA was re-quantified at the Spanish Genotyping Centre using PicoGreen technique (Invitrogen Corp., Carlsbad, CA) and diluted to a final concentration of 50 ng/µl. With this technique, the concentration of DNA is determined by means of a fluorescent dye that binds to double stranded DNA (PicoGreen®, Molecular Probes), which is then quantified with a fluorometer.

Data were analyzed with GenomeStudio software for genotype clustering and calling. Duplicate samples and CEPH trios (Coriell Cell Repository, Camden, NJ) were genotyped across the plates. SNPs showing Mendelian allele-transmission errors or showing discordant genotypes were excluded from the analysis.

MICRORNAS PATHWAY

Genotyping was performed at the General Research Services (SGIker) of the University of the Basque Country using TaqMan Open Array technology (Applied Biosystems) according to the published Applied Biosystems protocol.

TaqMan OpenArray Genotyping Plates contain the selected TaqMan SNP Genotyping Assays pre-loaded and dried down in the through-holes. Each assay contains: a specific fluorescent-dye labeled probe for each allele of the target SNP (the probes contain different fluorescent reporter dyes in 5' to differentiate each allele), a forward primer, a reverse primer and a nonfluorescent quencher (NFQ) at the 3'end of each probe.

During PCR, each probe anneals specifically to its complementary sequence between the forward and reverse primer sites. The DNA polymerase can cleave only probes that hybridize to their specific SNP allele (match). Cleavage separates the reporter dye from the quencher dye, substantially increasing fluorescence of the reporter dye. Thus, the fluorescence signals generated during PCR amplification indicate the alleles that are present in the sample. A

substantial increase in VIC dye fluorescence indicates homozygosity for allele 1, an increase in FAM dye fluorescence indicates homozygosity for allele 2 and both fluorescence signals indicates heterozygosity (Figure 15).

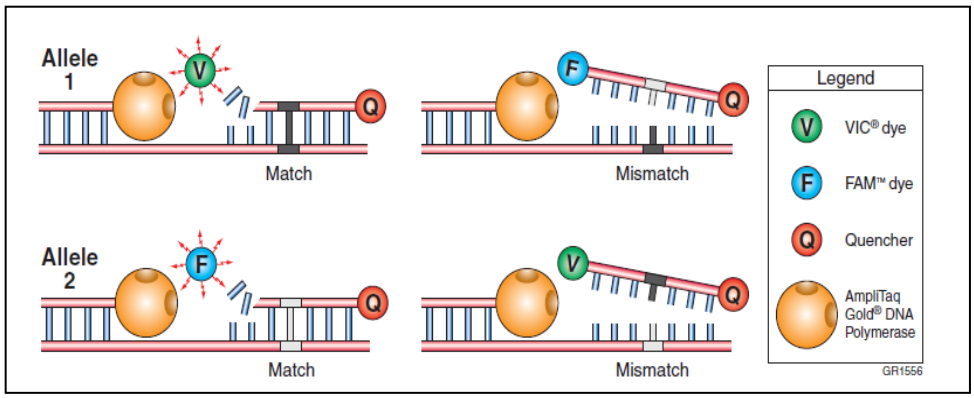


Figure 15. Results from matches and mismatches between target and probe sequences in TaqMan SNP Genotyping Assays [157]

A total of 300 ng of DNA were required from each sample to carry out the analysis.

Data were analyzed with Taqman Genotyper software for genotype clustering and calling (Figure 16). Duplicate samples were genotyped across the plates. SNPs showing discordant genotypes were excluded from the analysis.

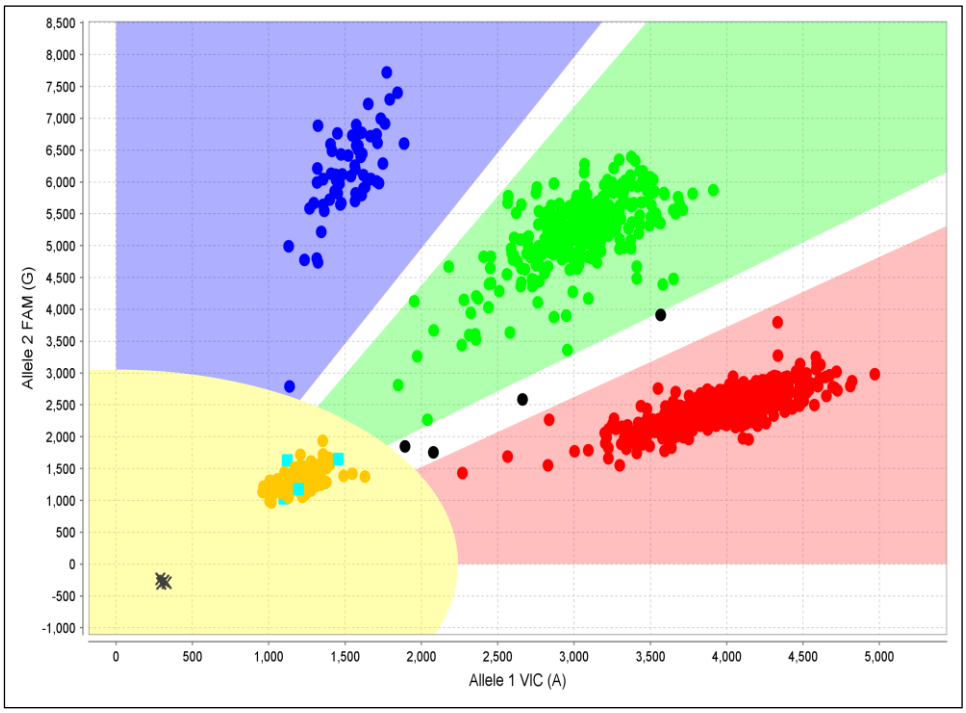


Figure 16. Genotype clustering and calling with Taqman Genotyper software. AA individuals are marked in red, AG in green and GG in dark blue.

DATA ANALYSIS

COPY NUMBER ALTERATIONS

The interpretation of images obtained by scanning the arrays were performed using the Chromosome Analysis Suite (CHAS), Affymetrix annotations and NetAffx version-build-3.1.0, based on version NCBIv37 genome (hg19), under the supervision of Dr. Francesc Solé from the Institut de Recerca Hospital del Mar (IMIM) (Figure 17). Filters were applied for CHAS to report only those gains

or losses that affected at least 50 markers in 100Kbp. When, in some samples, the quality parameters generated by the program were not optimal, we increased the restriction filter to avoid false positives arising from increased background noise in the results (200 markers altered in 200Kbp).

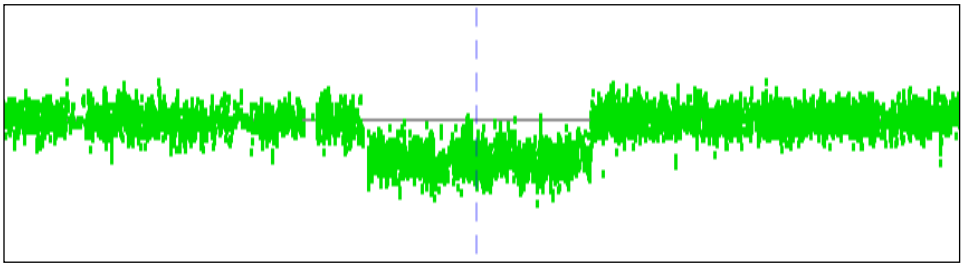


Figure 17. Deletion detected with ChAS.

In addition, we discarded those regions reported in telomeric or centromeric areas with low density of markers in the microarray. We performed a visual check of the changes reported by the program in order to accept or reject them and we also added some variations that did not meet the filters restriction but were clearly identified by visual check.

Abnormalities that remained after the screening described were used for comparison of results obtained in the pathological samples and their matched controls (complete remission). All variations detected in the control subjects were considered polymorphisms of the general population and pathological changes those that were identified only in the diagnosis sample.

CANDIDATE GENES APPROACH

Genotypic frequencies for each SNP were tested for departure from Hardy-Weinberg equilibrium (HWE) using Haploview 4.2 software.

The association between MTX plasma levels, and toxicity parameters was evaluated by the $\chi 2$ or Fisher's exact test. To assess the strength of the association we calculated the odds ratio (OR) ant its 95% confidence intervals followed by the area under the curve (AUC) from a receiver operating characteristic (ROC) approach. This last estimate gives an indication of the probability of discriminating between MTX plasma concentrations knowing the reported toxicity.

The association between outcome and toxicity parameters, and genetic polymorphisms was evaluated by the $\chi 2$ or Fisher's exact test. The effect sizes of the associations were estimated by the OR's from univariate logistic regressions and multivariate logistic regressions to account for the possible confounding effect of sex and age. In all cases the significance level was set at 5%. The p-values obtained in the analyses (univariate and multivariate) of the 10 tested polymorphisms were corrected for multiple comparisons by using the Benjamini & Hochberg [158] false discovery rate (FDR).

Analyses were performed by using Stata v11 and R v2.11 software. Linkage disequilibrium (LD) between the SNPs was analyzed using Haploview 4.2 by calculating pairwise D' and r^2.

MTX TRANSPORT PATHWAY

The association between MTX plasma levels, and genetic polymorphisms was evaluated by the χ2 or Fisher's exact test as well as the Hardy-Weinberg equilibrium. The effect sizes of the associations were estimated by the OR's from univariate logistic regression. The most significant test among dominant and recessive genetic models was used to determine the statistical significance of each SNP [(Liang et al, 2010)]. The results were adjusted for multiple comparisons by the False Discovery Rate (FDR) (ref). We reasoned that gene-based correction was sufficiently conservative because of the a priori hypotheses for these genes. Multivariate logistic regressions were also performed to account for the possible confounding effect of sex, age and MTX dose. In all cases the significance level was set at 5%. Analyses were performed by using R v2.11 software. Haploview v.4.2 was used to determine haplotype block structure and to infer haplotype frequencies between individuals with and without toxicity. For haplotype and correction analysis, *SLC22A6* and *SLC22A8*, that are located in the same region, were considered as a single entity.

META-ANALISIS OF MTHFR POLYMORPHISMS

Search strategy

We performed an exhaustive search to identify studies that examined the association between the C677T and A1298C polymorphisms of *MTHFR* and MTX toxicity in pediatric ALL patients. We used the keywords and subject terms "MTHFR and acute leukemia", and "MTHFR and polymorphism(s) and toxicity"

to search Pubmed (www.ncbi.nlm.nih.gov/pubmed) for articles published through November 2011. All references within the identified studies were then reviewed to possibly identify additional works.

Inclusion and exclusion criteria

The inclusion criteria for meta-analysis required that each trial be an independent association study, that the article supplied enough information on toxicity by genotype, that it studied short term toxic effects and that the population was composed only of pediatric patients (< 18 y). An article was excluded from meta-analysis if the study did not provide enough information (incomplete summary data), was performed on adult patients, the diagnosis was not mainly ALL or was a case study.

Data extraction

For each article included in the study, we gathered ethnicity of study population, number of patients, age and diagnosis, MTX dose, *MTHFR* C677T and A1298C genotype data and toxicity types.

Statistical analysis

Statistical analysis was performed using R software using the Meta library (R version 2.11.0. The R Foundation for Statistical Computing). We used a recessive model, assuming a recessive effect of the minor allele of each *MTHFR* SNP, which was consistent with previous results and allowed inclusion of the maximum number of studies. For the C677T SNP, we compared individuals having the TT homozygous genotype to all others (CC + CT), and for the A1298C SNP we compared CC homozygous individuals to all others (AA + AC). The overall pooled relative risk (RR) and corresponding 95% CI of toxicity to MTX were estimated using Mantel–Haenszel's method with random effect model. The random effects model assumes different underlying effects, considering both within- and between-study variation, offering an advantage as it accommodates diversity between studies and provides a more conservative estimate of the assessed effect.

Heterogeneity of the studies was assessed using the Cochrane Q test with a P-value below 0.05, below which heterogeneity was considered statistically significant. The heterogeneity was also quantified using the I^2 statistic, which is independent of the number of studies in the meta-analysis. This statistic quantifies the effect of heterogeneity, providing a measure of the degree of inconsistency in the study's results. The I^2 statistic has a value between 0 and 100% and describes the percentage of total variation across studies that is due to between-studies heterogeneity rather than chance. A higher I^2 value denotes a greater degree of heterogeneity (customary interpretations of the I^2 value are, 0–25% no heterogeneity, 25–50% moderate heterogeneity, 50–75% large heterogeneity, 75– 100% extreme heterogeneity). Sensitivity analysis leaving

out one study at the time was also performed when possible: outlying studies were identified and excluded and the I^2 estimates for these different sets of studies examined.

MICRORNAS PATHWAY

Haploview 4.2 was used to search for any deviation of Hardy-Weinberg equilibrium in a population of 348 healthy controls. The association between MTX plasma levels, and genetic polymorphisms was evaluated by the χ2 or Fisher's exact test. The effect sizes of the associations were estimated by the OR's from univariate logistic regression. The most significant test among dominant and recessive genetic models was used to determine the statistical significance of each SNP [(Liang et al, 2010)]. The results were adjusted for multiple comparisons by the False Discovery Rate (FDR) (ref). In all cases the significance level was set at 5%. Analyses were performed by using R v2.11 software.

ANNEX I

SAMPLE PROCESSING PROTOCOLS

Lymphocyte and granulocyte isolation

Peripheral blood samples were collected (4 ml) by venous puncture using EDTA as anticoagulant. Granulocyte and lymphocyte cells were separated using a gradient Ficoll-Paque™ Plus (GE Healthcare) following manufacture protocols.

Material

- Fresh EDTA anti-coagulated blood
- Ficoll-Paque™ PLUS (GE Healthcare)
- Balanced salt solution 1 X (PBS) (see Table 8)
- Erythrocyte lysis solution (see Table 8)
- Centrifuge conical tubes 15 ml (Sarstedt)
- Micro tubes 1.5 ml (Sarstedt)
- Pasteur pipettes (3 ml) (Sarstedt)
- Microlitre centrifuge (HERAEUS® Biofuge® pico)

Procedure

1. Add Ficoll-Paque PLUS (4 ml) to the centrifuge tube
2. Carefully layer the blood sample (4 ml) onto the Ficoll-Paque PLUS. It is important not to mix the Ficoll-Paque PLUS and the blood sample when layering the sample
3. Centrifuge at 1500 rpm for 20 min at 20ºC
4. Using a clean Pasteur pipette, transfer the lymphocyte layer (located at the interface) to a clean 1.5 ml micro tube and the

granulocyte layer (mixed with the Ficoll Paque PLUS) to another one

5. Add 1 ml of PBS 1 X to the lymphocytes and suspend the cells by gently drawing them in and out of a Pasteur pipette
6. Centrifuge at 2000 rpm for 15 min at 20ºC to wash the lymphocytes and remove the platelets. Discard the supernatant

The different cell fractions were purified using erythrocyte lysis solutions protocols (incubation on ice for 10 min with erythrocyte lysis solution and posterior centrifugation for 7 min at 2000 rpm).

Lymphocyte pellets were frozen at -80ºC until DNA extraction. When RNA extraction was required, lymphocyte pellets were lysed in 1 ml of TRI Reagent® (Applied Biosystems) and frozen until further use.

Genomic DNA extraction

Genomic DNA was extracted from lymphocytes and granulocytes using a standard phenol-chloroform protocol (Sambrook and Russel, 1956).

Material

- Lymphocyte/granulocyte samples
- PBS 1 X
- Cell lysis solution (see Table 8)
- Proteinase K, prepare 10 mg/ml solution (Sigma-Aldrich®)
- Phenol Ultrapure (USB®)

- Chloroform (Sigma-Aldrich®)
- Isoamyl Alcohol (Sigma-Aldrich®)
- Sodium acetate, prepare 2 M solution (Merck®)
- Glycogen, prepare 20 mg/ml solution (Roche Diagnostics GmbH®)
- Absolute ethanol (Merck®)
- Ethanol 80%
- Micro tubes 1.5 ml (Sarstedt)
- Heated water bath (Selecta®, Precisdig)
- Microlitre centrifuge (HERAEUS® Biofuge® pico)
- Micropipettes (Labsystem Finnpipette®)

Procedure

1. Suspend lymphocytes/granulocytes pellet in 250 µl PBS 1 X
2. Add 500 µl lysis solution and incubate overnight at 37ºC with agitation
3. Add 200 µl of buffer-saturated phenol: chloroform: isoamylalcohol (25:24:1) to the DNA solution
4. Mix well. Solutions can be vortexed for 10 sec
5. Centrifuge in a microfuge for 10 min at 13000 rpm
6. Carefully remove the aqueous layer to a new tube, being careful to avoid the interface. (Steps 3-5 can be repeated until an interface is no longer visible)
7. Precipitate DNA with sodium acetate 2 M (1/10 of volume collected), 1 µl glycogen (20 mg/ml) and 2 volumes of cold ethanol 100%
8. Mix gently and centrifuge for 20 min at 13000 rpm

9. Discard the supernatant and wash the pellet with cold ethanol 80%
10. Centrifuge for 5 min at 13000 rpm and discard supernatant
11. Dry the pellet and resuspend it in 40 µl of distilled H_2O

DNA quantification and quality analysis

DNA concentration and quality were estimated with NanoDrop® ND-1000 Spectrophotometer. The ratio of absorbance at 260 and 280 nm was used to assess the purity of DNA. A ratio of ~1.8 was generally accepted as "pure" for DNA.

DNA samples (100 ng) were then analyzed for integrity and amplifiability using the BIOMED-2 control gene multiplex polymerase chain reaction (PCR) protocol [159]. In this protocol, five pairs of control genes PCR primers were designed to amplify products of exactly 100, 200, 300, 400 and 600 bp (Table 6).

Table 6. Target genes selected to assess the quality of DNA

Gene	Symbol	Exon	GenBank	Size
Human thromboxane synthase gene	*TBXAS1*	exon 9	D34621	100bp
human recombination activating gene	*RAG1*	exon 2	M29474	200bp
Human promyelocytic leukaemia zinc-finger gene	*PLZF*	exon 1	AF060568	300bp
Human AF4 gene	*AF4*	exon 11	Z83687	400bp
Human AF4 gene	*AF4*	exon 3	Z83679	600bp

The sequences of PCR primers are described in the Figure 18.

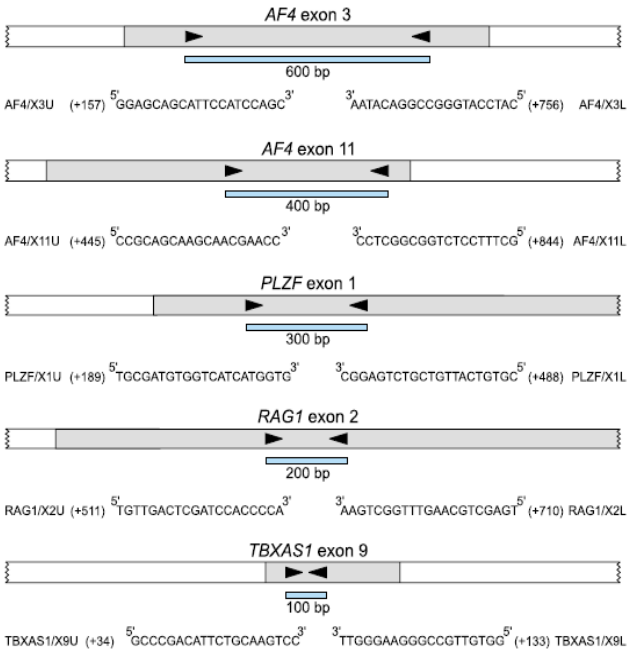


Figure 18: Schematic diagram of five control exons and five primer sets for obtaining PCR products of 600, 400, 300, 200, and 100 bp, respectively. The relative position of the control gene primers is given according to their most 5´ nucleotide downstream of the 50 splice site of the involved control gene exon.

The reaction conditions were developed for a final volume of 25 µl, using 100 ng of DNA (*see* PCR protocol below). The PCR products were size separated by electrophoresis on 2% agarose gels containing 5 µl of ethidium bromide per each 100 ml (*see* electrophoresis conditions below). The results were visualized under ultraviolet light (254 nm) using the Bio-Rad Gel Doc™ 2000 gel documentation system. Hyperladder V weight size marker was selected to identify the approximate size of a molecule run on a gel electrophoresis (Figure 19).

PCR protocol

Reagents (stock)	Final concentration
DNA (100 ng/µl)	100 ng
dNTPs (10 mM)	0.2 mM
$MgCl_2$ (50 mM)	2 mM
Buffer (10 X)	1 x
Primer F (10 µM) (AF4ex3)	5 pmol
Primer F (10 µM) (TBXAS1, RAG1, PLZF, AF4ex11)	2.5 pmol
Primer R (10 µM) (AF4ex3)	5 pmol
Primer R (10 µM) (TBXAS1, RAG1, PLZF, AF4ex11)	2.5 pmol
Enzyme (Immolase 5 U/µl)	0.5 U
H_2Obd	-

Cycling conditions

	Time	Temperature	Nº of cycles
Preactivation	10 min	95ºC	
Denaturation	30 sec	95ºC	
Annealing	30 sec	60ºC	30 cycles
Extension	30 sec	72ºC	
Final extension	10 min	72ºC	

Electrophoresis conditions

Gel: 2% agarose gel

Buffer: TBE 1 X

Loading buffer: 4 µl bromophenol blue

PCR product: 6 µl

Electrophoresis time: 40 min at 90 V

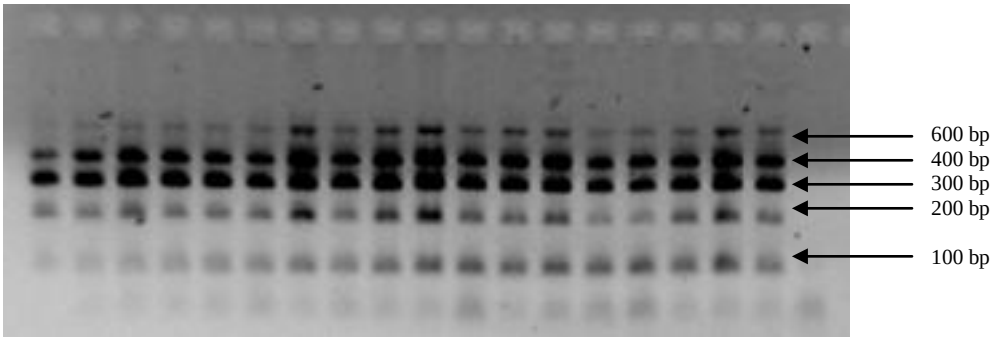


Figure 19. PCR with 5 pairs of primers: AFex 3, AFex 11, PLZF, RAG1 and TBAX1.

Whole genome amplification

When more amount of DNA was required, whole genome amplification (WGA) was done by using the GenomiPhi-mediated amplification system. DNA was briefly heat-denatured and then cooled in sample buffer containing random hexamers that non-specifically bind to the DNA. A master-mix containing DNA polymerase, additional random hexamers, nucleotides, salts and buffers was added, and isothermal amplification proceeded at 30°C for 1.5 hours. After amplification the enzyme was heat inactivated during 10 min incubation at 65°C.

Material

- DNA (10 ng)
- GenomiPhi HY DNA Amplification Kit (GE Healthcare)
- Micro tubes 0.2 ml (Deltalab)
- Thermocycler (Biometra T gradient)
- Microlitre centrifuge (HERAEUS® Biofuge® pico)
- Ice

Procedure

1. Mix 1 µl of template DNA (10 ng) with 9 µl of sample buffer
2. Heat to 95ºC for 3 min and cool to 4ºC on ice
3. Combine 9 µl of reaction buffer with 1 µl of enzyme mix on ice
4. Add it to the cooled sample
5. Incubate the sample at 30ºC for 90 min
6. Heat the sample to 65ºC for 10 min and cool to 4ºC

The list of reagents used and the protocols of preparation of buffers and solutions are described in Tables 7 and 8 respectively.

REAGENTS AND SOLUTIONS

Table 7. List of reagents used

Reagent	Technique	Commercial firm	Reference
Blood collection tubes with EDTA	Blood collection	BD Vacutainer®	368860
Ficoll Paque™ PLUS	Lymphocyte isolation	GE Healthcare	17-1440-02
NaCl	Lymphocyte isolation / DNA extraction	Merck	106404
KCl	Lymphocyte isolation	Sigma	P9541
Na_2HPO_4	Lymphocyte isolation	Panreac	131721
K_2HPO_4	Lymphocyte isolation	Panreac	132333
NH_4Cl	Lymphocyte isolation	Probus	20220
$KHCO_3$	Lymphocyte isolation	Sigma	P9144
Tris base	DNA extraction / Electrophoresis	USB	75825
EDTA	DNA extraction / Electrophoresis	Sigma	E-5134
SDS	DNA extraction	Sigma	L-4390
DTT (Ditiotreitol)	DNA extraction	USB	15397
Proteinase K	DNA extraction	Sigma	P2308
Phenol Ultrapure	DNA extraction	USB	75829
Chloroform	DNA extraction	Sigma	C2432
Isoamylalcohol	DNA extraction	Sigma	I9392
NaAc	DNA extraction	Merck	6268
Glycogen	DNA extraction	Roche	901393
Absolute ethanol	DNA extraction	Merck	1.009.831.000
Primers	PCR	Bonsai Technologies	-
dNTPs	PCR	Bioline	39028
Immolase™ DNA polymerase	PCR	Bioline	21047
Agarose D-1 Low EEO	Electrophoresis	Pronadisa	8016
Agarose 1000 ultrapura	Electrophoresis	Invitrogen	10975-035
Bromophenol blue loading buffer	Electrophoresis	Sigma	B-6896
Glycerol	Electrophoresis	Merck	1.040.921.000
Boric Acid	Electrophoresis	Panreac	131015

Table 7. List of reagents used (Continuation).

Reagent	Technique	Commercial firm	Reference
Hyperladder V	Electrophoresis	Bioline	33031
Ethidium bromide	Electrophoresis	BioRad	161-0433
Acrylamide	Electrophoresis	BioRad	161-144
TEMED	Electrophoresis	BioRad	161-0800
Ammonium persulfate	Electrophoresis	BioRad	161-0700
Loading Buffer 5X	Electrophoresis	BioRad	161-0767

Table 8. Preparation of buffers and solutions.

Solution	Preparation
PBS 10 X	80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 800 ml of distilled H_2O. Adjust pH to 6.8 with HCl. Add H_2O to 1 liter. Sterilize by autoclaving. Prepare 1:10 dilution.
Erythrocyte lysis solution 20X	89.9 g NH_4Cl, 10 g $KHCO_3$, 370 mg EDTA, 9 g NH_4Cl, 1 g $KHCO_3$ in 800 ml of distilled H_2O. Sterilize by autoclaving. Adjust pH to 7.3. Prepare 1:20 dilution.
Cell lysis solution	Tris 10 mM, EDTA 10 mM, NaCl 0.1 M, SDS 2%, DTT 40 mM, Proteinase K 0.2 mg/ml.
SDS solution	10% w/v in distilled H_2O.
Bromophenol blue loading buffer	2 ml EDTA 0'5 M pH=8; 5 ml glycerol 100% v/v; bromophenol blue**.** Make up volume to 10 ml with distilled water.

LIST OF SNP SELECTED

Table 9. Characteristics of the Single Nucleotide Polymorphisms included in the MTX transport pathway study and selection criteria

Gene	SNP ID	Alleles	Chr	Location	Position	Function	Reason for selection
ABCB1	rs10246878	G>A	7	87275641	intron 1	intronic	TAG
ABCB1	rs13233308	C>T	7	87244960	intron 1	intronic	TAG
ABCB1	rs2214102	G>A	7	87229501	intron 2	intronic	TAG
ABCB1	rs3789243	T>C	7	87220886	intron 4	intronic	TAG
ABCB1	rs12535512	C>T	7	87220334	intron 4	intronic	CG
ABCB1	rs13229143	G>C	7	87219481	intron 4	intronic	TAG
ABCB1	rs17327624	G>T	7	87216817	intron 4	intronic	TAG
ABCB1	rs4148733	T>C	7	87213232	intron 5	intronic	TAG
ABCB1	rs1202172	T>G	7	87210974	intron 5	intronic	CG
ABCB1	rs13226726	C>T	7	87206615	intron 5	intronic	TAG
ABCB1	rs1202179	A>G	7	87204279	intron 5	intronic	CG
ABCB1	rs10264990	T>C	7	87202615	intron 5	intronic	TAG
ABCB1	rs10260862	G>C	7	87201482	intron 5	intronic	TAG
ABCB1	rs2520464	A>G	7	87201086	intron 5	intronic	CG
ABCB1	rs4148734	C>T	7	87193597	intron 8	intronic	CG
ABCB1	rs10244266	T>G	7	87188467	intron 9	intronic	CG
ABCB1	rs1922240	T>C	7	87183354	intron 9	intronic	CG
ABCB1	rs2235013	A>G	7	87178626	intron 15	intronic	TAG
ABCB1	rs2235046	G>A	7	87174066	intron 17	intronic	CG
ABCB1	rs6961419	T>C	7	87172136	intron 18	intronic	CG
ABCB1	rs10268314	T>C	7	87169669	intron 19	intronic	CG
ABCB1	rs10274587	G>A	7	87164483	intron 20	intronic	CG
ABCB1	rs4148738	A>G	7	87163049	intron 20	intronic	CG
ABCB1	rs4148743	G>A	7	87151090	intron 22	intronic	CG
ABCB1	rs2235048	C>T	7	87138511	intron 27	intronic	TAG
ABCB1	rs6979885	G>A	7	87137461	intron 27	intronic	TAG
ABCB1	rs3842	A>G	7	87133366	3'UTR	3'UTR	3UTR, CG
ABCB1	rs1055302	G>A	7	87132916	downstream	downstream	DR, CG
ABCB1	rs6946119	T>C	7	87128865	downstream	downstream	TAG
ABCB1	rs7789645	G>C	7	87122603	downstream	downstream	CG
ABCC1	rs8050881	G>A	16	16037261	upstream	upstream	CG
ABCC1	rs4148330	A>G	16	16041768	upstream	upstream	UR, CG
ABCC1	rs504348	C>G	16	16043174	upstream	upstream	UR
ABCC1	rs215101	G>C	16	16052973	intron 1	intronic	CG
ABCC1	rs215099	G>T	16	16054694	intron 1	intronic	CG
ABCC1	rs12923345	T>C	16	16055082	intron 1	intronic	TR, CG
ABCC1	rs215094	A>G	16	16060915	intron 1	intronic	CG
ABCC1	rs215049	G>C	16	16070768	intron 1	intronic	CG
ABCC1	rs6498594	A>C	16	16073437	intron 1	intronic	TR, CG
ABCC1	rs152023	A>G	16	16085236	intron 1	intronic	TR
ABCC1	rs152022	C>G	16	16086666	intron 1	intronic	TR
ABCC1	rs246218	C>T	16	16087565	intron 1	intronic	CG
ABCC1	rs17501331	A>G	16	16089441	intron 1	intronic	TR
ABCC1	rs12934692	T>C	16	16100513	intron 1	intronic	TR, CG

Table 9. Characteristics of the Single Nucleotide Polymorphisms included in the MTX transport pathway study and selection criteria (Continuation I)

Gene	SNP ID	Alleles	Chr	Location	Position	Function	Reason for selection
ABCC1	rs16967145	G>A	16	16106970	intron 3	intronic	TAG
ABCC1	rs1967120	T>C	16	16108894	intron 4	intronic	CG
ABCC1	rs3784862	A>G	16	16110891	intron 5	intronic	BIB[160]
ABCC1	rs246240	A>G	16	16119024	intron 5	intronic	BIB[160]
ABCC1	rs875740	T>G	16	16123048	intron 5	intronic	TAG
ABCC1	rs11642957	T>C	16	16124008	intron 5	intronic	CG
ABCC1	rs3784864	A>G	16	16125325	intron 5	intronic	TAG
ABCC1	rs193538	T>G	16	16127916	intron 6	intronic	CG
ABCC1	rs11075293	G>A	16	16129996	intron 6	intronic	TAG
ABCC1	rs903880	C>A	16	16130514	intron 7	intronic	TAG
ABCC1	rs8054670	T>C	16	16132134	intron 7	intronic	TAG
ABCC1	rs246230	C>T	16	16132880	intron 7	intronic	CG
ABCC1	rs246221	T>C	16	16138322	exon 8	synonymous	CG
ABCC1	rs35592	T>C	16	16141823	intron 9	intronic	CG
ABCC1	rs3765129	C>T	16	16149901	intron 11	intronic	BIB[161]
ABCC1	rs17287570	A>C	16	16155103	intron 12	intronic	TAG
ABCC1	rs35597	G>A	16	16158034	intron 12	intronic	CG
ABCC1	rs35600	C>G	16	16159628	intron 12	intronic	TAG
ABCC1	rs35605	C>T	16	16162019	exon 13	synonymous	SR, BIB[161]
ABCC1	rs35621	C>G	16	16168608	intron 14	intronic	BIB[162], CG
ABCC1	rs35625	T>C	16	16169566	intron 14	intronic	TR
ABCC1	rs4148350	G>T	16	16170477	intron 15	intronic	TAG
ABCC1	rs4148355	A>G	16	16174667	intron 16	intronic	CG
ABCC1	rs10852377	C>T	16	16176824	intron 16	intronic	CG
ABCC1	rs2074086	T>C	16	16181142	intron 18	intronic	TAG
ABCC1	rs2889517	C>T	16	16181956	intron 18	intronic	TAG
ABCC1	rs3888565	G>A	16	16183045	intron 18	intronic	TAG
ABCC1	rs4148359	C>G	16	16187234	intron 19	intronic	CG
ABCC1	rs2269800	A>G	16	16196839	intron 20	intronic	CG
ABCC1	rs16967755	A>G	16	16199255	intron 20	intronic	CG
ABCC1	rs11864374	G>A	16	16201885	intron 21	intronic	CG
ABCC1	rs3784867	C>T	16	16203345	intron 21	intronic	TAG
ABCC1	rs4780591	G>C	16	16204979	intron 21	intronic	TAG
ABCC1	rs3887893	A>G	16	16205501	intron 22	intronic	TAG
ABCC1	rs2299670	A>G	16	16220858	intron 26	intronic	CG
ABCC1	rs212081	C>T	16	16225971	intron 27	intronic	TAG
ABCC1	rs2230671	C>T	16	16228242	exon 28	synonymous	SR, CG, BIB[163]
ABCC1	rs212086	G>A	16	16229735	intron 28	intronic	CG
ABCC1	rs3743527	C>T	16	16235681	3'UTR	3'UTR	3UTR
ABCC1	rs129081	G>C	16	16235939	3'UTR	3'UTR	MIRTS
ABCC1	rs212090	T>A	16	16236004	3'UTR	3'UTR	MIRTS, 3UTR, BIB[164]
ABCC1	rs212093	A>G	16	16237754	downstream	downstream	BIB, CG
ABCC1	rs12448760	G>A	16	16239539	downstream	downstream	CG
ABCC2	rs1885301	A/G	10	101541053	Upstream	Upstream	UR, BIB[165]
ABCC2	rs717620	G>A	10	101542578	5'UTR	5'UTR	5UTR, CG, BIB[161,165-179]
ABCC2	rs2756105	C>T	10	101547042	intron 2	intronic	CG
ABCC2	rs7906080	A>G	10	101547647	intron 2	Intronic	CG
ABCC2	rs4148385	A>C	10	101548177	intron 2	intronic	CG
ABCC2	rs4148386	G>A	10	101548468	intron 2	intronic	CG
ABCC2	rs2145853	G>A	10	101548795	intron 2	Intronic	CG
ABCC2	rs2756109	T>G	10	101558746	intron 7	intronic	TAG

Table 9. Characteristics of the Single Nucleotide Polymorphisms included in the MTX transport pathway study and selection criteria (Continuation II)

Gene	SNP ID	Alleles	Chr	Location	Position	Function	Reason for selection
ABCC2	rs2273697	G>A	10	101563815	exon 10	non-synonymous	NS, SR, CG, BIB[180-185]
ABCC2	rs2073337	A>G	10	101567426	intron 12	intronic	CG
ABCC2	rs4148394	A>C	10	101572343	intron 15	intronic	TAG
ABCC2	rs9794323	T>C	10	101587002	intron 19	intronic	CG
ABCC2	rs4148396	C>T	10	101591944	intron 23	intronic	BIB[183]
ABCC2	rs3740066	A>G	10	101604207	exon 28	synonymous	SR, CG, BIB[165,174,177,178,186,187]
ABCC2	rs3740065	T>C	10	101605693	intron 29	intronic	BIB[188-190], CG
ABCC2	rs12826	A>G	10	101612320	downstream	downstream	CG
ABCC2	rs11190297	G>T	10	101618103	downstream	downstream	TAG
ABCC2	rs12762549	C>G	10	101620771	downstream	downstream	BIB[191]
ABCC2	rs11190298	A>G	10	101620948	downstream	downstream	CG
ABCC3	rs7212045	G>C	17	48706024	upstream	upstream	TAG
ABCC3	rs2412332	C>G	17	48707522	upstream	upstream	CG
ABCC3	rs757421	G>A	17	48707768	upstream	upstream	TAG
ABCC3	rs2189595	A>G	17	48708949	upstream	upstream	TAG
ABCC3	rs8073706	G>A	17	48709941	upstream	upstream	UR
ABCC3	rs12604031	A>G	17	48712705	intron 1	intronic	TR, CG
ABCC3	rs10153257	A>G	17	48713223	intron 1	intronic	TR, CG
ABCC3	rs2412333	G>A	17	48715271	intron 1	intronic	TR
ABCC3	rs739921	C>G	17	48719590	intron 1	intronic	TR, CG
ABCC3	rs1541392	T>G	17	48719889	intron 1	intronic	TR
ABCC3	rs12051822	G>A	17	48723585	intron 1	intronic	TR
ABCC3	rs17562467	C>T	17	48724830	intron 1	intronic	TR, CG
ABCC3	rs4793666	C>G	17	48727253	intron 1	intronic	TR
ABCC3	rs17562516	T>A	17	48729781	intron 1	intronic	TR
ABCC3	rs4148411	G>C	17	48733745	intron 2	intronic	TAG
ABCC3	rs4148412	C>T	17	48733815	intron 2	intronic	TAG
ABCC3	rs739923	G>A	17	48735774	intron 5	intronic	CG
ABCC3	rs733392	G>A	17	48736403	intron 6	intronic	TAG
ABCC3	rs1978153	C>G	17	48737861	intron 7	intronic	CG
ABCC3	rs4148413	C>G	17	48740798	intron 8	intronic	TAG
ABCC3	rs879459	A>G	17	48746135	intron 14	intronic	TAG
ABCC3	rs8075406	T>A	17	48749522	intron 17	intronic	TAG
ABCC3	rs2072365	C>T	17	48752866	intron 20	intronic	CG
ABCC3	rs3785912	G>A	17	48756937	intron 26	intronic	CG
ABCC3	rs2277624	G>A	17	48761105	exon 27	synonymous	SR, CG
ABCC3	rs1558288	G>A	17	48763715	intron 29	intronic	CG
ABCC3	rs3785911	T>G	17	48767431	intron 30	intronic	CG
ABCC3	rs1051640	A>G	17	48768486	exon 31	synonymous	SR, MIRTS
ABCC3	rs17563146	C>T	17	48769329	downstream	downstream	TAG
ABCC3	rs12602161	A>G	17	48769881	downstream	downstream	CG
ABCC3	rs4148418	A>G	17	48770517	downstream	downstream	TAG
ABCC3	rs8196	T>C	17	48770959	downstream	downstream	CG
ABCC4	rs9302061	T>C	13	95966704	upstream	upstream	TAG
ABCC4	rs2993590	T>C	13	95964923	upstream	upstream	CG
ABCC4	rs9524902	T>C	13	95963518	upstream	upstream	CG
ABCC4	rs2992907	T>C	13	95959901	upstream	upstream	CG
ABCC4	rs868853	A>G	13	95955076	upstream	upstream	BIB[192]
ABCC4	rs8001444	C>T	13	95952437	intron 1	intronic	TAG
ABCC4	rs1539068	G>T	13	95950858	intron 1	intronic	TAG

Table 9. Characteristics of the Single Nucleotide Polymorphisms included in the MTX transport pathway study and selection criteria (Continuation III)

Gene	SNP ID	Alleles	Chr	Location	Position	Function	Reason for selection
ABCC4	rs7981095	A>T	13	95945118	intron 1	intronic	TAG
ABCC4	rs7330673	T>G	13	95942492	intron 1	intronic	CG
ABCC4	rs4148421	G>A	13	95932240	intron 1	intronic	TAG
ABCC4	rs4148422	T>C	13	95931992	intron 1	intronic	TAG
ABCC4	rs7317112	A>G	13	95923523	intron 1	intronic	CG
ABCC4	rs870004	G>A	13	95918063	intron 1	intronic	CG
ABCC4	rs4148431	G>A	13	95913123	intron 1	intronic	CG
ABCC4	rs7984157	A>G	13	95911900	intron 1	intronic	CG
ABCC4	rs9516551	C>A	13	95910726	intron 1	intronic	TAG
ABCC4	rs12100301	A>G	13	95909950	intron 1	intronic	TAG
ABCC4	rs12584649	T>C	13	95907085	intron 1	intronic	CG
ABCC4	rs4148436	T>C	13	95899607	intron 2	intronic	CG
ABCC4	rs4148446	G>A	13	95897302	intron 3	intronic	CG
ABCC4	rs4283094	G>C	13	95893787	intron 3	intronic	CG
ABCC4	rs4148454	A>G	13	95889505	intron 3	intronic	CG
ABCC4	rs4148455	G>A	13	95888277	intron 3	intronic	CG
ABCC4	rs9524849	A>G	13	95882596	intron 3	intronic	CG
ABCC4	rs17189481	C>T	13	95882322	intron 4	intronic	CG
ABCC4	rs4773856	G>A	13	95880483	intron 4	intronic	CG
ABCC4	rs4773850	T>G	13	95876543	intron 4	intronic	TAG
ABCC4	rs9302049	T>C	13	95873985	intron 4	intronic	CG
ABCC4	rs899494	G>A	13	95861804	exon 6	synonymous	SR, CG
ABCC4	rs3818494	C>G	13	95858704	intron 8	intronic	CG
ABCC4	rs17268170	C>T	13	95856286	intron 8	intronic	CG
ABCC4	rs1678388	A>G	13	95853780	intron 8	intronic	CG
ABCC4	rs9516530	C>T	13	95848667	intron 8	intronic	CG
ABCC4	rs2274403	A>G	13	95847020	intron 8	intronic	CG
ABCC4	rs1751015	T>C	13	95845662	intron 9	intronic	CG
ABCC4	rs2487566	A>G	13	95845272	intron 9	intronic	CG
ABCC4	rs7319330	C>T	13	95844735	intron 9	intronic	CG
ABCC4	rs17268122	G>T	13	95844494	intron 9	intronic	CG
ABCC4	rs9524821	G>A	13	95843434	intron 9	intronic	CG
ABCC4	rs1678374	T>C	13	95843067	intron 9	intronic	TAG
ABCC4	rs4773843	C>T	13	95839495	intron 10	intronic	TAG
ABCC4	rs2766474	G>A	13	95838523	intron 11	intronic	CG
ABCC4	rs3843689	A>G	13	95838241	intron 11	intronic	TAG
ABCC4	rs1564352	G>T	13	95838046	intron 11	intronic	TAG
ABCC4	rs7330933	G>A	13	95831078	intron 11	intronic	CG
ABCC4	rs2009772	T>C	13	95829588	intron 13	intronic	CG
ABCC4	rs4148494	G>C	13	95829519	intron 13	intronic	CG
ABCC4	rs1729786	G>A	13	95823239	intron 13	intronic	CG
ABCC4	rs11568663	G>A	13	95822761	intron 14	intronic	CG
ABCC4	rs9561797	A>G	13	95820852	intron 14	intronic	CG
ABCC4	rs1729767	T>C	13	95819942	intron 14	intronic	CG
ABCC4	rs7993619	A>C	13	95812745	intron 19	intronic	TAG
ABCC4	rs1678396	T>C	13	95808948	intron 19	intronic	TAG
ABCC4	rs1729788	T>C	13	95808003	intron 19	intronic	CG
ABCC4	rs10508023	G>C	13	95795463	intron 19	intronic	CG
ABCC4	rs1564355	C>T	13	95778166	intron 19	intronic	TAG
ABCC4	rs1751064	G>A	13	95777748	intron 19	intronic	CG
ABCC4	rs1628382	G>A	13	95764061	intron 20	intronic	CG

Table 9. Characteristics of the Single Nucleotide Polymorphisms included in the MTX transport pathway study and selection criteria (Continuation IV)

Gene	SNP ID	Alleles	Chr	Location	Position	Function	Reason for selection
ABCC4	rs2766481	T>C	13	95761583	intron 20	intronic	CG
ABCC4	rs1729775	G>A	13	95757943	intron 20	intronic	CG
ABCC4	rs1751059	C>G	13	95756023	intron 20	intronic	TAG
ABCC4	rs1751050	C>G	13	95731496	intron 22	intronic	TAG
ABCC4	rs1618738	C>T	13	95730800	intron 22	intronic	TAG
ABCC4	rs2619312	T>C	13	95723039	intron 26	intronic	CG
ABCC4	rs1678392	G>A	13	95722180	intron 26	intronic	CG
ABCC4	rs1189457	C>G	13	95719494	intron 26	intronic	TAG
ABCC4	rs9561778	G>T	13	95713715	intron 27	intronic	BIB[193], CG
ABCC4	rs3782946	T>C	13	95711603	intron 27	intronic	CG
ABCC4	rs1750190	G>A	13	95709072	intron 27	intronic	TAG
ABCC4	rs1189445	A>G	13	95707142	intron 27	intronic	CG
ABCC4	rs10219913	T>C	13	95700935	intron 28	intronic	CG
ABCC4	rs10508024	G>A	13	95691692	intron 30	intronic	TAG
ABCC4	rs2182262	C>T	13	95691512	intron 30	intronic	CG
ABCC4	rs17189299	T>C	13	95685794	intron 31	intronic	TAG
ABCC4	rs3742106	A>C	13	95673791	3'UTR	3'UTR	MIRTS, 3UTR
ABCC4	rs9516521	T>C	13	95673122	3' UTR	3' UTR	MIRTS, 3UTR
ABCC4	rs1059751	T>C	13	95672950	3'UTR	3'UTR	3UTR
ABCC4	rs9516519	T>G	13	95672457	3'UTR	3'UTR	MIRTS, 3UTR
ABCC4	rs7321486	T>C	13	95664889	downstream	downstream	CG
ABCG2	rs10011796	C>T	4	89090877	upstream	upstream	TAG
ABCG2	rs3114019	T>C	4	89081441	upstream	upstream	UR, CG
ABCG2	rs2622604	C>T	4	89078924	intron 1	intronic	BIB[194,] TR
ABCG2	rs2622624	A>G	4	89069406	intron 1	intronic	TR, CG
ABCG2	rs2622625	G>A	4	89068737	intron 1	intronic	TR, CG
ABCG2	rs17731799	G>T	4	89068455	intron 1	intronic	TR, CG
ABCG2	rs2725248	T>G	4	89068007	intron 1	intronic	TR, CG
ABCG2	rs2622626	G>T	4	89066715	intron 1	intronic	TR
ABCG2	rs6857600	C>T	4	89066075	intron 1	intronic	TR, CG
ABCG2	rs3114018	C>A	4	89064581	intron 1	intronic	TR
ABCG2	rs2725252	T>G	4	89061910	intron 1	intronic	TR, CG
ABCG2	rs1564481	C>T	4	89061265	intron 1	intronic	TR, CG
ABCG2	rs2869732	A>G	4	89059087	intron 2	intronic	CG
ABCG2	rs17731538	G>A	4	89055379	intron 2	intronic	BIB[160]
ABCG2	rs2231142	C>A	4	89052323	exon 5	non-synonymous	SR, BIB[94,189,195-219]
ABCG2	rs2725256	T>C	4	89050998	intron 5	intronic	CG
ABCG2	Rs2725261	G>A	4	89036353	intron 7	intronic	CG
ABCG2	rs13120400	T>C	4	89033527	intron 9	intronic	BIB[160]
ABCG2	rs2622621	C>G	4	89030920	intron 9	intronic	BIB[220]
ABCG2	rs12505410	T>G	4	89030841	intron 9	intronic	TAG
ABCG2	rs2231148	A>T	4	89028478	intron 9	intronic	TAG
ABCG2	rs2728124	A>T	4	89006160	downstream	downstream	TAG
SLCO1A2	rs10841803	G>A	12	21547875	5'	5'UTR	TAG
SLCO1A2	rs11831407	T>A	12	21543811	intron 1	intronic	TAG
SLCO1A2	rs10770805	T>A	12	21542342	intron 1	intronic	TR
SLCO1A2	rs10770804	A>G	12	21540669	intron 1	intronic	TR
SLCO1A2	rs7964783	A>G	12	21539337	intron 1	intronic	TR, CG
SLCO1A2	rs2417977	T>C	12	21533168	intron 1	intronic	TR
SLCO1A2	rs12319824	G>A	12	21526651	intron 2	intronic	CG
SLCO1A2	rs7137767	A>C	12	21525606	intron 2	intronic	TAG

Table 9. Characteristics of the Single Nucleotide Polymorphisms included in the MTX transport pathway study and selection criteria (Continuation V)

Gene	SNP ID	Alleles	Chr	Location	Position	Function	Reason for selection
SLCO1A2	rs2045938	C>T	12	21520352	intron 2	intronic	TAG
SLCO1A2	Rs2045939	T>C	12	21520243	intron 2	intronic	CG
SLCO1A2	Rs2045940	A>G	12	21520098	intron 2	intronic	CG
SLCO1A2	rs11045994	T>C	12	21507702	intron 2	intronic	CG
SLCO1A2	rs10743413	T>C	12	21507074	intron 2	intronic	TAG
SLCO1A2	rs4762699	C>T	12	21504068	intron 2	intronic	TAG
SLCO1A2	rs11837182	C>T	12	21501956	intron 2	intronic	TAG
SLCO1A2	rs7301895	C>T	12	21497892	intron 2	intronic	TAG
SLCO1A2	rs7954757	A>G	12	21494668	intron 2	intronic	CG
SLCO1A2	rs4762818	G>A	12	21493529	intron 2	intronic	CG
SLCO1A2	rs2306231	T>C	12	21490381	intron 2	intronic	CG
SLCO1A2	rs10841795	A>G	12	21487544	exon 3	non-synonymous	NS, SR, BIB[221]
SLCO1A2	rs4148984	T<C	12	21486196	intron 3	intronic	CG
SLCO1A2	rs4148988	A>G	12	21477990	intron 3	intronic	CG
SLCO1A2	rs10505872	G>A	12	21472254	intron 3	intronic	CG
SLCO1A2	rs7962263	C>T	12	21466117	intron 5	intronic	CG
SLCO1A2	rs11045961	G>A	12	21460603	intron 5	intronic	CG
SLCO1A2	rs11045953	G>A	12	21455051	intron 7	intronic	CG
SLCO1A2	rs16923647	C>T	12	21451395	intron 9	intronic	BIB[66]
SLCO1A2	rs6487215	G>A	12	21444991	intron 13	intronic	CG
SLCO1A2	rs4337089	C>T	12	21427952	intron 14	intronic	CG
SLCO1A2	rs12300594	T>C	12	21426565	intron 15	intronic	CG
SLCO1A2	rs16923597	A>G	12	21423495	intron 15	intronic	CG
SLCO1A2	rs11045919	T>G	12	21422253	3'UTR	3'UTR	MIRTS, CG
SLCO1A2	rs4149008	C>T	12	21421039	3'UTR	3'UTR	MIRTS
SLCO1A2	rs11045918	C>A	12	21420712	3'UTR	3'UTR	MIRTS
SLCO1A2	rs4149009	G>A	12	21420471	3'UTR	3'UTR	MIRTS
SLCO1B1	rs17387842	T>C	12	21274317	upstream	upstream	CG
SLCO1B1	rs11045776	A>G	12	21278192	upstream	upstream	BIB[66]
SLCO1B1	rs17328763	T>C	12	21282570	upstream	upstream	TR, CG, BIB[66]
SLCO1B1	rs2417955	A>T	12	21296475	intron 2	intronic	BIB[66]
SLCO1B1	rs11045787	T>G	12	21300002	intron 2	intronic	CG, BIB[66]
SLCO1B1	rs11513411	G>A	12	21303439	intron 2	intronic	CG
SLCO1B1	rs11045799	T>C	12	21311025	intron 2	intronic	CG
SLCO1B1	rs11045800	T>C	12	21311248	intron 2	intronic	BIB[66]
SLCO1B1	rs16923519	A>G	12	21311718	intron 2	intronic	TAG
SLCO1B1	rs7138177	A>G	12	21312924	intron 2	intronic	TAG
SLCO1B1	rs4149026	A>C	12	21315415	intron 2	intronic	TAG
SLCO1B1	rs10444413	T>C	12	21317668	intron 2	intronic	CG
SLCO1B1	rs4149033	G>A	12	21317810	intron 2	intronic	TAG
SLCO1B1	rs4149034	G>A	12	21317922	intron 2	intronic	TAG
SLCO1B1	rs4149035	C>T	12	21318265	intron 2	intronic	CG
SLCO1B1	rs7973095	C>T	12	21321270	intron 2	intronic	CG, BIB[66]
SLCO1B1	rs10841753	T>C	12	21321370	intron 2	intronic	CG, BIB[66]
SLCO1B1	rs11045812	C>T	12	21321482	intron 2	intronic	CG
SLCO1B1	rs11045813	G>A	12	21322056	intron 2	intronic	CG
SLCO1B1	rs7136445	A>G	12	21324748	intron 2	intronic	CG
SLCO1B1	rs2291073	T>G	12	21325814	intron 3	intronic	TAG
SLCO1B1	rs964614	T>C	12	21329390	intron 4	intronic	TAG
SLCO1B1	rs11045818	G>A	12	21329761	exon 5	synonymous	SR, CG, BIB[66]
SLCO1B1	rs11045819	C>A	12	21329813	exon 5	non-synonymous	NS, SR, PTDR, BIB[66,167]

Table 9. Characteristics of the Single Nucleotide Polymorphisms included in the MTX transport pathway study and selection criteria (Continuation VI)

Gene	SNP ID	Alleles	Chr	Location	Position	Function	Reason for selection
SLCO1B1	rs4149050	T>C	12	21330988	intron 5	intronic	CG
SLCO1B1	rs4149056	C>A	12	21331549	intron 4	intronic	BIB[66]
SLCO1B1	rs2291075	C>T	12	21331625	exon 6	synonymous	SR, CG, BIB[66]
SLCO1B1	rs2291076	C>T	12	21331987	intron 7	intronic	CG, BIB[66]
SLCO1B1	rs11045821	G>A	12	21332423	intron 7	intronic	CG
SLCO1B1	rs12812279	A>G	12	21333040	intron 7	intronic	CG
SLCO1B1	rs4149058	A>G	12	21333214	intron 7	intronic	CG
SLCO1B1	rs11045823	G>A	12	21333745	intron 7	intronic	CG
SLCO1B1	rs991262	G>A	12	21334214	intron 7	intronic	CG, BIB[66]
SLCO1B1	rs2900476	C>T	12	21336063	intron 7	intronic	CG, BIB[66]
SLCO1B1	rs2100996	T>C	12	21338197	intron 7	intronic	CG
SLCO1B1	rs11045834	C>T	12	21341096	intron 7	intronic	CG
SLCO1B1	rs4149061	T>C	12	21350668	intron 8	intronic	CG
SLCO1B1	rs1871395	T>C	12	21352315	intron 8	intronic	CG
SLCO1B1	rs4363657	T>C	12	21368722	intron 11	intronic	BIB[222] (Link et al, 2008
SLCO1B1	rs4149076	T>C	12	21371144	intron 12	intronic	BIB[66]
SLCO1B1	rs11045872	A>G	12	21372344	intron 12	intronic	BIB[66]
SLCO1B1	rs4149081	G>A	12	21378021	intron 14	intronic	BIB[66,223]
SLCO1B1	rs7966613	A>G	12	21379632	intron 14	intronic	CG, BIB[66]
SLCO1B1	rs11045878	A>G	12	21382222	intron 14	intronic	CG, BIB[66]
SLCO1B1	rs11045879	T>C	12	21382619	intron 14	intronic	BIB[66,223]
SLCO1B1	rs11045885	A>G	12	21386018	intron 14	intronic	CG
SLCO1B1	rs12830367	G>T	12	21388905	intron 14	intronic	CG
SLCO1B1	rs12578392	T>C	12	21389970	intron 14	intronic	TAG
SLCO1B1	rs12369881	G>A	12	21391352	intron 14	intronic	CG
SLCO1B1	rs11045891	A>C	12	21392572	3'UTR	3'UTR	CG
SLCO1B1	rs11045893	T>C	12	21392819	downstream	downstream	CG
SLCO1B3	rs1002441	G>T	12	20953580	upstream	upstream	TAG
SLCO1B3	rs10841648	A>C	12	20954557	upstream	upstream	CG
SLCO1B3	rs11045512	T>C	12	20957569	upstream	upstream	TAG
SLCO1B3	rs7962265	G>A	12	20964719	intron 1	intronic	TR, CG
SLCO1B3	Rs1581194	C>A	12	20978417	intron 2	intronic	CG
SLCO1B3	rs10841660	A<G	12	20984349	intron 2	intronic	CG
SLCO1B3	rs10841661	C>T	12	20984832	intron 2	intronic	CG
SLCO1B3	rs4382961	G>A	12	20996314	intron 2	intronic	CG
SLCO1B3	rs975657	A>G	12	20999345	intron 2	intronic	CG
SLCO1B3	rs1304608	A>G	12	21000586	intron 2	intronic	CG
SLCO1B3	rs4149117	G>T	12	21011480	exon 3	non-synonymous	NS, SR, PTDR,BIB[173,216]
SLCO1B3	rs1966648	A>G	12	21013429	intron 3	intronic	CG
SLCO1B3	rs4149121	G>C	12	21015046	intron 4	intronic	CG
SLCO1B3	rs1017385	T>G	12	21015139	intron 4	intronic	CG
SLCO1B3	rs7311358	A>G	12	21015760	exon 6	non-synonymous	NS, SR, BIB[173,216]
SLCO1B3	rs2417940	G>A	12	21017875	intron 6	intronic	BIB[224]
SLCO1B3	rs11045573	C>T	12	21023492	intron 6	Intron	CG
SLCO1B3	rs4149132	C>T	12	21030202	intron 7	intronic	CG
SLCO1B3	rs1549968	G>A	12	21037553	intron 11	intronic	CG
SLCO1B3	rs11045585	A>G	12	21045694	intron 11	intronic	BIB[191]
SLCO1B3	rs2417886	G>A	12	21049997	intron 11	intronic	CG
SLCO1B3	rs7973653	T>A	12	21051769	intron 12	intronic	TAG
SLCO1B3	rs4762803	C>G	12	21055606	intron 13	intronic	CG
SLCO1B3	rs10841697	G>T	12	21056210	intron 13	intronic	TAG

Table 9. Characteristics of the Single Nucleotide Polymorphisms included in the MTX transport pathway study and selection criteria (Continuation VII)

Gene	SNP ID	Alleles	Chr	Location	Position	Function	Reason for selection
SLCO1B3	rs12824715	G>A	12	21056715	intron 13	intronic	CG
SLCO1B3	rs11045598	A>G	12	21071213	downstream	downstream	CG
SLCO1B3	rs2117032	T>C	12	21074122	downstream	downstream	TAG
SLC19A1	rs3788205	C>T	21	46964378	upstream	upstream	TAG
SLC19A1	rs1131596	T>C	21	46957916	5' UTR	5'UTR	CG
SLC19A1	rs1051266	G>A	21	46957794	exon 2	non-synonymous	NS, SR, PTDR, BIB[225-231]
SLC19A1	rs3788200	G>A	21	46956571	intron 2	intronic	CG, BIB[232]
SLC19A1	rs2838958	A>G	21	46948567	intron 5	intronic	BIB[233]
SLC19A1	rs2297291	G>A	21	46945340	intron 6	intronic	CG
SLC19A1	rs3788190	G>A	21	46936958	intron 6	intronic	CG
SLC19A1	rs3788189	G>T	21	46936583	intron 6	intronic	BIB[233]
SLC19A1	rs1888530	C>T	21	46936423	intron 6	intronic	BIB[232]
SLC19A1	rs1051298	C>T	21	46934826	3'UTR	3'UTR	3UTR, BIB[233]
SLC19A1	rs7499	G>A	21	46932328	downstream	downstream	CG
SLC19A1	rs2236484	G>A	21	46931684	downstream	downstream	CG
SLC19A1	rs2838951	C>G	21	46929720	downstream	downstream	CG
SLC19A1	rs1050351	G>A	21	46929467	downstream	downstream	CG
SLC19A1	rs7278425	C>T	21	46926551	downstream	downstream	CG
SLC19A1	rs2838950	C>T	21	46926297	downstream	downstream	CG
SLC19A1	rs3753019	C>T	21	46924785	downstream	downstream	TAG
SLC22A6	rs11231294	T>C	11	62755519	upstream	upstream	UR
SLC22A6	rs4149172	A>G	11	62750858	intron 3	intronic	CG
SLC22A6	rs6591722	T>A	11	62749680	intron 3	intronic	TAG
SLC22A6	rs3017670	G>A	11	62744899	intron 8	intronic	CG
SLC22A6	rs10897310	T>C	11	62741176	downstream	downstream	TAG
SLC22A8	rs10897315	G>A	11	62789131	upstream	upstream	TAG
SLC22A8	rs4963228	C>T	11	62788206	upstream	upstream	CG
SLC22A8	rs3948869	G>C	11	62785998	upstream	upstream	TAG
SLC22A8	rs948980	C>G	11	62783889	upstream	upstream	CG
SLC22A8	rs3809069	T>C	11	62783772	upstream	upstream	UR
SLC22A8	rs4963326	G>A	11	62780577	intron 2	intronic	TAG
SLC22A8	rs2187383	C>A	11	62775898	intron 2	intronic	TAG
SLC22A8	rs4149182	G>C	11	62768113	intron 3	intronic	CG
SLC22A8	rs2276299	A>T	11	62766431	exon 5	synonymous	SR
SLC22A8	rs10792367	G>C	11	62758799	downstream	downstream	TAG

3UTR: 3'UTR regulation; 5UTR: 5'UTR regulation; BIB: Bibliographic; CG: CpG site; DR: Downstream regulation; MIRTS: miRNA target site; NS: Non-synonimous; PTDR: Post-traductional regulation; SR: Splicing regulation; TAG: tagSNP; TCR: Transcriptional regulation; UR: Upstream regulation

Table 10. Characteristics of the Single Nucleotide Polymorphisms in microRNA processing genes and selection criteria

Gene	SNP ID	Alleles	Chr	Location	Function	Reason for selection
GEMIN3	rs197412	T>C	1	112308953	non-synonimous	NS, BIB[234-240]
GEMIN3	rs197414	C>A	1	112309123	non-synonimous	NS, BIB[234,236-240]
GEMIN3	rs197388	T>A	1	112297482	upstream	UR, BIB[234,236-240]
GEMIN3	rs563002	T>C	1	112317135	downstream	BIB[235]
CNOT2	rs10506586	C>A	12	70715490	non-synonimous	NS, SR
TNRC6A	rs6497759	G>A	16	24801737	non-synonimous	NS
CNOT1	rs11644694	G>A	16	58557342	non-synonimous	NS, SR
CNOT1	rs37060	C>T	16	58566304	intronic	SR
CNOT1	rs11866002	C>T	16	58587737	synonimous	SR
GEMIN4	rs1062923	T>C	17	649067	non-synonimous	NS, BIB[236-240]
GEMIN4	rs2740348	G>C	17	649935	non-synonimous	NS, BIB[234,236-240]
GEMIN4	rs34610323	C>T	17	648546	non-synonimous	NS
GEMIN4	rs3744741	C>T	17	649232	non-synonimous	NS, BIB[234,236-240]
GEMIN4	rs7813	C>T	17	648186	non-synonimous	NS, BIB[234-240]
GEMIN4	rs910924	C>T	17	655920	5'UTR	5UTR, BIB[234,236-240]
CNOT3	rs42318	G>A	19	54657069	non-synonimous	NS
TNRC6B	rs2413621	T>C	22	40673999	intronic	SR
TNRC6B	rs9611280	G>A	22	40552119	non-synonimous	NS, SR
TNRC6B	rs4821943	A>G	22	40722745	3'UTR	MIRTS
TNRC6B	rs470113	A>G	22	40729614	3'UTR	MIRTS
TNRC6B	rs139919	T>C	22	40726183	3'UTR	MIRTS
DGCR8	rs35987994	T>C	22	20074006	non-synonimous	NS
DGCR8	rs417309	G>A	22	20098544	3'UTR	3UTR BIB[234,236,237,239,240]
DGCR8	rs3757	G>A	22	20099331	3'UTR	MIRTS, BIB[234-240]
DGCR8	rs1640299	T>G	22	20098359	3'UTR	BIB[234,236-240]
DGCR8	rs9606248	A>G	22	20087539	intronic	BIB[235]
GEMIN5	rs1974777	A>G	5	154291409	non-synonimous	NS
GEMIN5	rs6865950	G>A	5	154275786	non-synonimous	NS
GEMIN5	rs816736	T>C	5	154271948	synonimous	SR
CNOT6	rs6877400	T>C	5	179996111	synonimous	SR
CNOT6	rs11738060	T>A	5	180004154	3'UTR	MIRTS
DROSHA	rs55656741	G>A	5	31515657	non-synonimous	NS, SR
DROSHA	rs10719	C>T	5	31401447	synonimous	SR, BIB[236,237,239,240]
DROSHA	rs6877842	G>C	5	31532638	intronic	BIB[236-240]
DROSHA	rs2287584	T>C	5	31423007	synonimous	SR, BIB[235]
DROSHA	rs4867329	A>C	5	31435627	intronic	BIB[235]
DROSHA	rs7719666	C>T	5	31520778	intronic	BIB[235,241]
DROSHA	rs10035440	T>C	5	31539463	intronic	BIB[235]
DROSHA	rs17408716	A>G	5	31467952	intronic	BIB[235]
DROSHA	rs3792830	T>C	5	31416248	intronic	BIB[235,241]
DROSHA	rs493760	T>C	5	31437040	intronic	BIB[242]
DROSHA	rs7735863	G>A	5	31486540	intronic	BIB[241,242]
DROSHA	rs6884823	G>A	5	31491121	intronic	BIB[241]
DROSHA	rs639174	C>T	5	31433647	intronic	BIB[241]
DROSHA	rs3805500	T>C	5	31462977	intronic	BIB[241]
SMAD5	rs3764941	A>C	5	135469527	non-synonimous	NS, SR
SMAD5	rs3764942	G>A	5	135469500	intronic	SR
XPO5	rs1106841	A>C	6	43496662	synonimous	SR
XPO5	rs34324334	C>T	6	43535018	non-synonimous	NS, SR
XPO5	rs2257082	C>T	6	43492578	synonimous	SR, BIB[241]
XPO5	rs2227301	G>A	6	43485283	downstream	BIB[241]

Table 10. Characteristics of the Single Nucleotide Polymorphisms in microRNA processing genes and selection criteria (Continuation).

Gene	SNP ID	Alleles	Chr	Location	Function	Reason for selection
XPO5	rs7755135	C>T	6	43490809	3'UTR	MIRTS
CNOT4	rs1003226	T>C	7	135046552	3'UTR	SR
CNOT4	rs3812265	C>T	7	135048804	non-synonimous	NS, SR
CNOT4	rs3763425	C>T	7	135195320	upstream	UR
SND1	rs17151639	A>G	7	127637816	non-synonimous	NS
SND1	rs322825	C>T	7	127721507	synonimous	SR
SND1	rs3823994	T>A	7	127669857	intronic	SR
SND1	rs17676986	C>T	7	127636958	intronic	TR
RAN	rs14035	C>T	12	131361241	3'UTR	MIRTS, BIB[234,236-240]
RAN	rs11061209	G>A	12	131364988	downstream	BIB[241]
DICER	rs3742330	A>G	14	95553362	3'UTR	BIB[234,236,237,239,240]
DICER	rs13078	T>A	14	95556747	3'UTR	3UTR, BIB[234,236-240]
DICER	rs1209904	C>T	14	95563712	intronic	BIB[242]
DICER	rs1057035	T>C	14	95554142	3'UTR	MIRTS
TRBP	rs784567	C>T	12	53894465	upstream	BIB[234,236-240]
EIF2C1	rs636832	G>A	1	36363475	intronic	BIB[234,236,237,239,240]
EIF2C1	rs595961	A>G	1	36367780	intronic	BIB[234,236,238-240]
EIF2C2	rs4961280	C>A	1	141647414	upstream	UR, BIB[234,236-240]
EIF2C2	rs2293939	G>A	1	141551407	synonimous	SR
EIF2C2	rs2292778	C>T	1	141568622	synonimous	SR
HIWI	rs1106042	G>A	12	130841638	non-synonimous	NS, SR, BIB[236,237,239,240]

3UTR: 3'UTR regulation; 5UTR: 5'UTR regulation; BIB: Bibliographic; MIRTS: miRNA target site; NS: Non-synonimous; SR: Splicing regulation; UR: Upstream regulation.

Table 11. Characteristics of the Single Nucleotide Polymorphisms in microRNAs

Gene	SNP ID	Alleles	Chr	Location
mir-577	rs34115976	C>G	4	115577997
mir-618	rs2682818	C>A	12	81329536
mir-106b	rs72631827	G>T	7	99691652
mir-1255b-1	rs6841938	G>A	4	36428048
mir-1274a	rs318039	C>T	5	41475766
mir-1307	rs7911488	A>G	10	105154089
mir-154	rs41286570	G>A	14	101526127
mir-16-1	rs72631826	T>C	13	50623143
mir196a2	rs11614913	C>T	12	54385599
mir-220a	rs72631817	T>C	X	122696014
mir-222	rs72631825	G>A	X	45606471
mir-449b	rs10061133	A>G	5	54466544
mir-499	rs3746444	T>C	20	33578251
mir-548a-1	rs12197631	T>G	6	18572056
mir-548h-3	rs9913045	G>A	12	13446924
mir-548h-4	rs73235381	A>G	8	26906402
mir-585	rs62376934	G>A	5	168690612
mir-624	rs11156654	T>A	14	31483955
mir-1178	rs7311975	T>C	12	120151493
mir-1206	rs2114358	T>C	8	129021179
mir-1265	rs11259096	T>C	10	14478618
mir-1269	rs73239138	G>A	4	67142620
mir-1282	rs11269	G>T	15	44085909
mir-1294	rs13186787	A>G	5	153726769
mir-1302-4	rs10173558	T>C	2	208133995
mir-149	rs2292832	C>T	2	241395503
mir-1908	rs174561	T>C	11	61582708
mir-2053	rs10505168	A>G	8	113655752
mir-2110	rs17091403	C>T	10	115933905
mir-216a	rs41291179	A>T	2	56216090
mir-300	rs12894467	C>T	14	101507727
mir-423	rs6505162	A>C	17	28444183
mir-453	rs56103835	T>C	14	101522556
mir-492	rs2289030	C>G	12	95228286
mir-595	rs4909237	C>T	7	158325503
mir-603	rs11014002	C>T	10	24564653
mir-604	rs2368392	C>T	10	29834003
mir-604	rs2368393	T>C	10	29833998
mir-605	rs2043556	A>G	10	53059406
mir-608	rs4919510	C>G	10	102734778
mir-612	rs12803915	G>A	11	65211979
mir-612	rs550894	G>T	11	65211940
mir-656	rs58834075	C>T	14	101533093
mir-943	rs1077020	T>C	4	1988193

RESULTS

GENETIC ALTERATIONS IN THE TUMORAL CELLS AND THEIR IMPLICATION IN PROGNOSIS AND THERAPY

COPY NUMBER ALTERATIONS AS RISK STRATIFICATION AND PROGNOSIS MARKERS IN PEDIATRIC ACUTE LYMPHOBLASTIC

With the aim of improving the stratification of ALL patients, we first sought to detect duplications and deletions characteristic of the tumor. Once identified, we wanted to determine if some of them allow improving the separation into risk groups.

Patients' baseline characteristics

In order to identify deletions and duplications that are characteristic of the tumor, tumoral and normal sample is required from each individual.

The patients included in this study were 23 children all diagnosed with B-ALL at the Hospital Cruces. All patients were homogeneously treated with the LAL-SHOP 2005 protocol.

These were the patients for which tumoral and remission material was available, including patients that were assigned to a risk group and remained in that group until the end of treatment (standard 1-1, high 2-2, very high 3-3) and those who, according to a bad early response to treatment, had to be changed to a higher risk group (standard-high 1-2, high-very high 2-3) (Table 12).

Table 12. Characteristics of the population.

No. of patients, n	23
Mean age at diagnosis ± SD, years	4.8 ± 2.64
Sex, n (%)	
Female	10 (43.5)
Male	13 (56.5)
Risk group, n (%)	
Standard (1-1)	7 (30.5)
Standard-High (1-2)	3 (13.0)
High (2-2)	10 (43.5)
High-Very high (2-3)	2 (8.7)
Very high (3-3)	1 (4.3)

SD Standard Deviation.

Genetic characterization of pediatric Acute Lymphoblastic Leukemia

First of all, we wanted to select copy number pathological changes of the own B-ALL. We analyzed 23 B-ALL patients by Affymetrix Cytogenetics Whole-Genome 2.7M Array. The paired diagnosis and remission samples were available for performing the matched genomic analysis of tumor and normal cells of the same patient. This way, we could differentiate between tumoral aberrations and polymorphisms. All the copy number variations detected in both the diagnosis (tumoral) and remission (normal) samples were considered polymorphisms of the general population and were not further analyzed in this study.

We detected multiple copy number alterations ranging from whole chromosome gains and losses to focal lesions, which in some cases encompassed only a single gene.

In total, 223 aberrations were detected only in the tumor and not in the normal sample, with an average of 9.7 genomic abnormalities per case (Figure 20). A full list of chromosomal aberrations found per patient is included in Table 28 (Annex II). Losses were more numerous than gains (131 losses vs 92 gains).

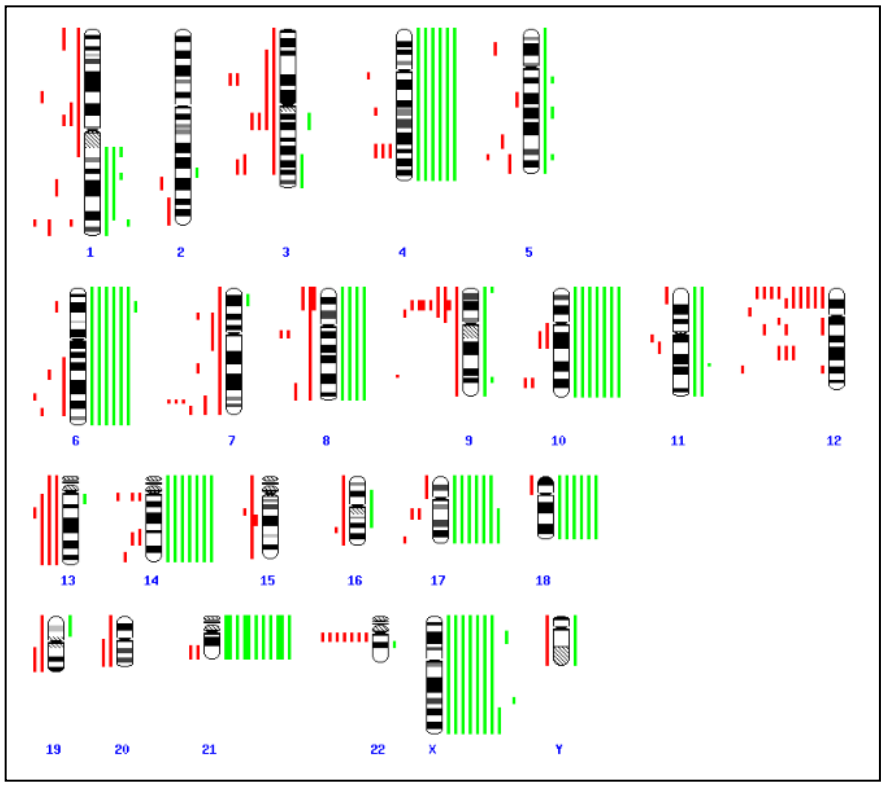


Figure 20. Representation of all the aberrations found in our cohort of 23 B-ALL patients. Green bars represent duplications and red bars represent deletions.

Gain of whole chromosomes was observed in 9 cases: chromosome 4 was gained in 6 cases, 5 in 1, 6 in 6, 8 in 4, 9 in 1, 10 in 7, 11 in 2, 14 in 7, 17 in 6, 18 in 6, 21 in 8, X in 7 and Y in 1. Loss of whole chromosomes was less common and was observed in 2 cases. In one of these cases, only chromosome 13 was lost. In the other case, chromosomes 13, 15, 16, 19, 20, 7, 8, 9 and 4 were lost.

The remaining 151 aberrations included only parts of chromosomes, ranging from 41kbp to 163Mbp. Most of them had a size under 5Mb and were presumably cytogenetically cryptic. The most frequents were the loss of the *ETV6* gene at 12p13.2, present in 9 patients (39.1%) and loss at the *IGL* locus at 22q11.22, in 7 patients (30.4%), loss at the *CDKN2A/B* locus in 4 patients (17.4%) and loss at the *TRB* locus, in 4 patients (17.4%).

In 1 patient, we did not find any tumor-related aberration. Blasts count in this case was greater than 80%, thus excluding the eventual contamination by normal bone marrow cells.

In order to search for patterns, we centered on recurrent abnormalities. Recurrent abnormalities were defined when found in at least two patients. Table 13 summarizes the recurrent chromosomal aberrations.

Table 13. Recurrent abnormalities.

Type	Chr	Min	Max	Start	End	Genes	n
Loss	1	234715202	235072805	q42.3	q42.3	*IRF2BP2, NCRNA00184, LOC100506810, PP2672, RNY4P16*	3
Gain	1	151996653	152192164	q21.3	q21.3	*S100A11, LOC100131107, TCHHL1, TCHH, HDHD1P2, RPTN, LOC100652924, HRNR*	3
Gain	1	175487744	175607795	q25.1	q25.1	*TNR*	3
Loss	3	112063466	112203411	q13.2	q13.2	*CD200, LOC100506591, BTLA*	3
Loss	3	176925938	177351455	q26.32	q26.32	*ASS1P7, LOC100505566*	2
Loss	3	60103639	60372552	p14.2	p14.2	*FHIT*	3
Loss	4	149773707	149848318	q31.23	q31.23	No genes	2
Loss	4	152862748	153021068	q31.3	q31.3	*LOC100505685*	2
Loss/Gain	5	158240867	158320101	q33.3	q33.3	*EBF1*	3
Loss	7	38273812	38395492	p14.1	p14.1	*TRGC2, TRG@, TRGJ2, TRGJP2, TARP, TRGC1, TRGJ1, TRGJP, TRGJP1, TRGV11, TRGVB, TRGV10, TRGV9, TRGVA, TRGV8, TRGV7, TRGV6, LOC100506776, TRGV5P, TRGV5, TRGV4*	2
Loss	7	142308091	142445333	q34	q34	*TRB@, TRBV19, TRBV20-1, TRBV21-1, TRBV22-1, TRBV23-1, TRBV24-1, MTRNR2L6, TRBV25-1, TRBVA, TRBV26, TRBVB, TRBV27, TRBV28*	4
Loss	8	60037057	60242344	q12.1	q12.1	No genes	2
Loss	8	172851	26058609	p23.3	p21.2	322 genes	2
Loss	9	21428463	22483924	p21.3	p21.3	*IFNA1, MIR31HG, IFNWP19, IFNE, MIR31, LOC402359, MTAP, LOC100418937, LOC100533725, C9orf53, CDKN2A, CDKN2B-AS, CDKN2B, UBA52P6, DMRTA1*	4
Loss	10	111758741	111840369	q25.1	q25.2	*LOC100505933, ADD3*	2
Loss	12	11826813	12056722	p13.2	p13.2	*ETV6*	9
Loss	12	46.181.372	46235991	q12	q12	*ARID2*	3
Loss	12	92267405	92531075	q21.33	q21.33	No genes	3
Loss	12	99881976	100343680	q23.1	q23.1	*ANKS1B, FAM71C*	2
Loss	14	73222960	73355261	q24.2	q24.2	*DPF3*	2
Loss	14	22737500	23002382	q11.2	q11.2	*PIP4K2A, TRNAP22P*	3
Loss	17	45181435	45419478	q21.32	q21.32	*CDC27, LOC100506228, RPS2P47, MYL4, ITGB3, LOC100506252, C17orf57*	2
Loss	22	22454109	22518006	q11.22	q11.22	*IGL@, LOC91219, IGLV4-60*	7

Some of those recurrent aberrations were present in patients from different risk groups and might be associated with the leukemic process. These aberrations included the loss at 1q42.3 that included the *IRF2BP2* gene among others; the loss at 3q13.2, which includes *CD200* and *BTLA* genes; loss at 3q26.32; loss at 3p14.2, affecting *FHIT* gene; loss at 7q34, including T cell receptor cluster; loss at 8q12.1; loss at 8p; loss at 9p21.3, including *CDKN2A* and *CDKN2B*.; loss of the *ETV6* gene at 12p13.2; loss at 14q11.2, including *PIP4K2A* gene; loss at 17q21.32, including *CDC27* gene among others; and loss at 22q11.22, that includes the immunoglobulin lambda locus. The losses at 4q31.23, 4q31.3 and 12q21.33 in regions that included no gene were only found in patients with the TEL-AML1 translocation, independent of their risk group.

Improvement of genetic characterization of risk groups

In order to identify potential new markers to differentiate among prognostic risk groups, we analyzed the alterations found in each risk group (Figure 21). Some of the recurrent alterations were exclusive of a risk group and could be associated with prognosis and be of help for a better risk group classification.

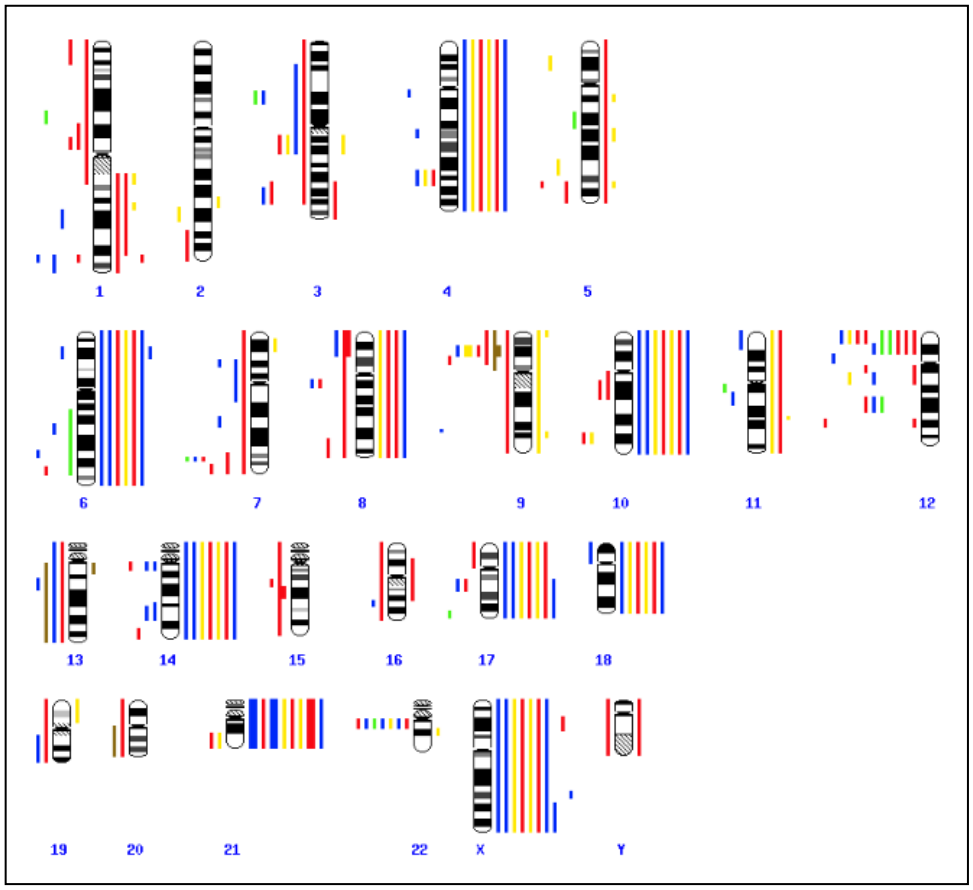


Figure 21. Representation of the aberrations found in each risk group. Bars located on the right of each chromosome represent duplications and bars on the left represent deletions. Blue bars represent aberrations found in 1-1 risk group; yellow bars stand for 1-2 risk group; red bars stand for 2-2 risk group; green is for 2-3 risk group; and brown is for 3-3 risk group.

All the alterations found in standard risk patients (risk 1-1) can be seen in Figure 22. Most of the aberrations were found in single cases or were common to different risk groups. From all the aberrations found, we detected 2 recurrent aberrations that were present only in patients assigned to the standard risk group. These aberrations were the loss at 7p14.1, which includes the *TRG* locus, and the loss at 14q24.2, that affects the *DPF3* gene.

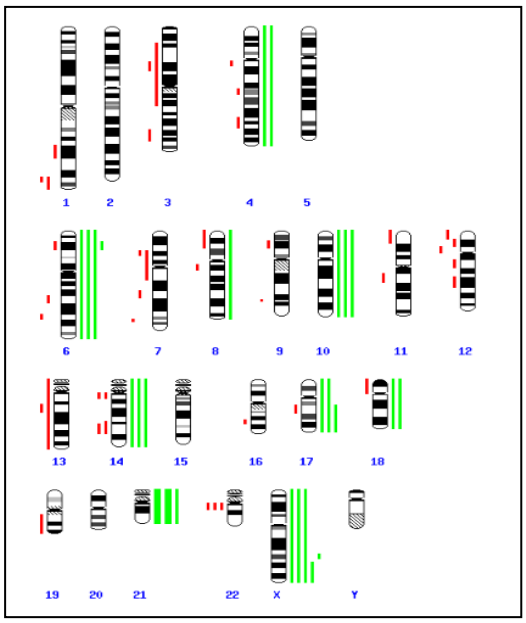


Figure 22. Alterations found in standard risk group patients (risk 1-1). Green bars represent duplications and red bars represent deletions.

All the alterations found in high risk patients (risk 2-2) can be seen in Figure 23. Of those, loss at 12q23.1, affecting *ANKS1B* and *FAM71C* genes was a recurrent aberration that was only observed in this group of patients and could be associated with a more aggressive disease. Another remarkable phenomenon in

this risk group is the greater incidence of alterations in chromosomes 1 and 12 than in other risk groups. In addition, we only found alterations of the Y chromosome in this group.

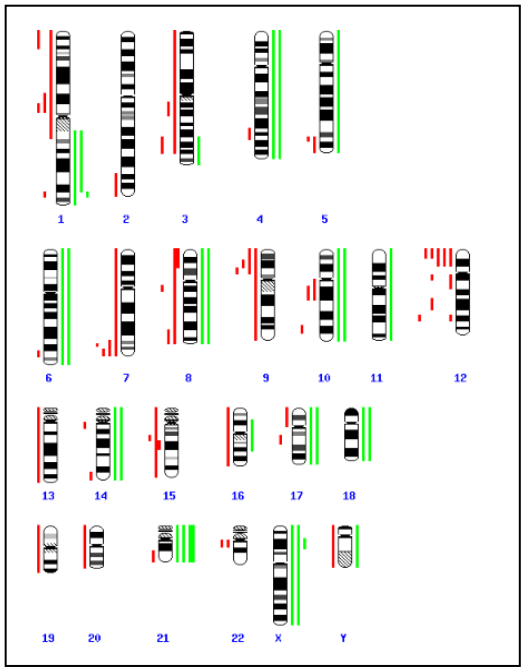


Figure 23. Alterations found in high risk group patients (risk 2-2). Green bars represent duplications and red bars represent deletions.

The alterations found in the very high risk group (risk 3-3) are represented in Figure 24. We could only screen a patient that belonged to this group. This patient presented 6 aberrations in chromosomes 9, 13 and 20 that were not recurrent.

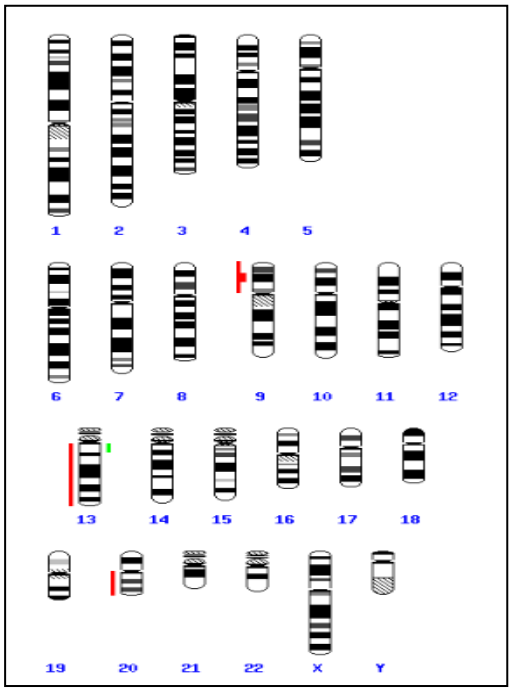


Figure 24. Alterations found in very high risk group patients (risk 3-3). Green bars represent duplications and red bars represent deletions.

Genetic variation that could allow better discrimination of risk groups

In order to search for new markers that could allow better risk group definition, It was of special interest for us to screen the patients that were first assigned to a risk group and lately had to be moved to a higher risk group due to a bad early response to treatment.

We detected genetic markers that could allow the differentiation between standard risk patients that stay in this group (risk 1-1) and those that must be moved to high risk (risk 1-2). These aberrations included the gain at 1q21.3,

including *S100A11* gene among others; the gain at 1q25.1 that includes only the *TNR* gene; the loss or gain at 5q33.3 affecting *EBF1* gene; loss at 10q25.1-q25.2, including *ADD3* gene; and loss at 12q12, which contains *ARID2* gene. These recurrent aberrations were common to patients that were assigned to the high risk group (risk 2-2) from the beginning. These aberrations could be new cytogenetic markers that could be used to better assign the patients to their corresponding risk group.

When we searched for markers to differentiate the high-risk patients who remain at this risk (2-2) from patients switching to very high risk (2-3), we did not find any exclusive recurrent alterations. Risk 2-3 patients only presented recurrent aberrations that were common to different risk groups.

GENETIC VARIANTS IN THE GERMINAL LINE AND THEIR IMPLICATION IN TREATMENT TOXICITY

CANDIDATE GENES APPROACH

Considering that in the well-established LAL/SHOP protocol, 6-mercaptopurine and methotrexate are the backbone of therapy, we have selected 13 genes within three different pathways: 6-mercaptopurine and methotrexate metabolism and drug-detoxifying enzymes. In those genes, we have selected 18 polymorphisms already studied in association with MTX response by other authors with controversial results and/or with a demonstrated functional effect. We have analyzed their association with response and toxicity during therapy with the LAL/SHOP protocol in a Spanish pediatric B-ALL population.

Characteristics of the study population

For the study of candidate genes and polymorphisms, we have analyzed 115 B-ALL patients, whose characteristics are reported in Table 14.

Clinical data about therapy-related toxicity were available for 102 patients. Among all patients who developed toxicity (n=52, 51%), the prevalence of different types of toxicity were as follows: hepatic (n=30, 29.4%), vomits (n=22, 21.6%), mucositis (n=11, 10.8%), renal (n=9, 8.8%), diarrhea (n=7, 6.9%), and hyperbilirubinemia (n=5, 4.9%). Clinical data about MTX plasma concentration

were available for 111 patients 72 h after infusion and for 108 patients after 96 h. High MTX plasma levels (>2 µM) were reported in 35 patients (31.5%) 72 h after MTX infusion and 25 (23.1%) continued with high MTX levels after 96 h.

Table 14. Characteristics of the population.

No. of patients, n	115
Mean age at diagnosis ± SD, years	5.49 ± 3.49
Sex, n (%)	
Female	53 (46.1)
Male	62 (53.9)
Risk group, n (%)	
Standard	44 (38.3)
High	52 (45.2)
Very high	19 (16.5)
Treatment protocol, n (%)	
LAL-SHOP 99	45 (39.1)
LAL-SHOP 2005	70 (60.9)
MTX dose in consolidation, n (%)	
$3g/m^2$	51 (44.3)
$5g/m^2$	64 (55.7)
Toxicity during consolidation therapy, n (%)	
Global toxicity	52 (51.0)
Hepatic	30 (29.4)
Vomits	22 (21.6)
Diarrhea	7 (6.9)
Mucositis	11 (10.8)
Hyperbilirrubinemia	5 (4.9)
Renal	9 (8.8)
MTX concentration in plasma, n (%)	
Higher than 0.2uM at 72h	35 (31.5)
Higher than 0.2uM at 96h	25 (23.1)

SD: Standard Deviation

Genotyping results

The 18 polymorphisms were genotyped with an average rate of success of 98.80%. All the genotypes analyzed were in Hardy-Weinberg equilibrium.

Toxicity analysis

Drugs detoxifying enzymes

We did not find any significant association between the 5 polymorphisms analyzed in drugs detoxifying enzymes and any of the toxicity parameters studied in both the induction and consolidation phases (data not shown).

6-mercaptopurine pathway

We did not find any patient with the *TPMT*-deficient homozygous genotype. Only 6 individuals (5.36%) were heterozygous and 106 (94.64%) were *TPMT*1* homozygous, with two normal alleles.

We did not find any significant association between *TPMT* polymorphisms and any of the toxicity parameters studied in the consolidation phase (data not shown).

Methotrexate pathway

In order to analyze MTX toxicity, we selected methotrexate clearance as an objective and quantifiable toxicity marker. In order to confirm the suitability of MTX plasma levels as a toxicity marker, we analyzed the association between

different toxicity parameters and plasma concentration of MTX 72 h (Table 15) and 96 h after MTX treatment. For analyses, toxicity grades were used to dichotomize toxicities as "present" versus "absent," with grade 2 to 4 considered as present as defined in Table 15.

When we analyzed the toxicity in patients with high MTX plasma levels *versus* toxicity of those with low MTX plasma levels, there was a significantly higher frequency of patients with global toxicity in the group of individuals with high MTX concentration (72 h, p=0.004). The frequency of renal toxicity, by itself, was significantly increased in patients with a high MTX concentration at 72 h (p=0.005). Similar results were obtained at 96h. The frequency of vomiting in the total population was also significantly higher in the group of patients with a high MTX concentration, but only at 72 h after infusion (p=0.020).

Our results show that MTX plasma concentration is a good toxicity marker in our population. Indeed it has a strong association with the parameter "global toxicity", here we considered grouped any kind of toxicity. With these results, we decided to use it as marker of toxicity in the following analyses, as it is an objectively quantifiable variable.

Results

Table 15. MTX clearance and toxicity.

		MTX concentration in plasma at 72 hr				
Toxicity	Status	<0.2 mM, n (%)	>0.2 mM, n (%)	OR (95% CI)	P value	AUC Roc (95% CI)
Global toxicity	No toxicity	41 (60.3)	10 (29.4)			
	Toxicity	27 (39.7)	24 (70.6)	3.64 (1.51–8.82)	0.004*	0.65 (0.56–0.75)
Hepatic	Grade 0–1	51 (75.0)	21 (61.8)			
	Grades 2–4	17 (25.0)	13 (38.2)	1.86 (0.77–4.49)	0.169	0.57 (0.47–0.66)
Vomits	Grade 0–1	58 (85.3)	22 (64.7)			
	Grades 2–4	10 (14.7)	12 (35.3)	3.16 (1.20–8.36)	0.020*	0.60 (0.51–0.70)
Diarrhea	Grade 0–1	65 (95.6)	30 (88.2)			
	Grades 2–4	3 (4.4)	4 (11.8)	2.89 (0.61–13.72)	0.182	0.54 (0.48–0.60)
Mucositis	Grade 0–1	60 (88.2)	31 (91.2)			
	Grades 2–4	8 (11.8)	3 (8.8)	0.73 (0.18–2.93)	0.653	0.49 (0.42–0.55)
Hyperbilirubinemia	No toxicity	66 (97.1)	31 (91.2)			
	Toxicity	2 (2.9)	3 (8.8)	3.19 (0.51–20.10)	0.216	0.53 (0.48–0.58)
Renal	No toxicity	67 (98.5)	26 (76.5)			
	Toxicity	1 (1.5)	8 (23.5)	20.62 (2.46–173.06)	0.005*	0.61 (0.54–0.68)

*p<0.05; Grade 0-1 is considered as no toxicity and grade 2-4 is considered as toxicity.

We investigated if 10 genetic polymorphisms in MTX pathway genes may influence the clearance of MTX. For each polymorphism, we considered two genotypic groups, one of risk of higher toxicity and other of normal expected toxicity, according to the function and previous reports, as described in the introduction (Table 16).

In the gene *MTHFR*, individuals with the genotype 1298CC had lower frequency of toxicity than expected, without reaching the significance level. When we analyzed the correlation of polymorphisms in *SHMT1, TS, ABCB1, ABCG2* and *RFC1* genes with MTX plasma concentration at 72 or 96h, we did not find any significant association.

Table 16. Genetic polymorphisms and methotrexate clearance.

Gene	Polymorphism	Genotype	MTX concentration in plasma at 72h		Univariate analysis			Multivariate analysis		
			< 0.2 μM, n (%)	>0.2 μM, n (%)	OR (95% CI)	p	p (corrected)	OR (95% CI)	p	p (corrected)
MTHFR	C677T	CC/CT	58 (65.9)	30 (34.1)	1.00			1.00		
		TT	18 (78.3)	5 (21.7)	0.54 (0.18-1.59)	0.244	0.415	0.53 (0.17-1.64)	0.256	0.427
MTHFR	A1298C	AA/AC	65 (65.7)	34 (34.3)	1.00			1.00		
		CC	10 (90.9)	1 (9.1)	0.19 (0.02-1.56)	0.060	0.300	0.21 (0.03-1.69)	0.078	0.260
SHMT1	C1420T	CC	40 (71.4)	16 (28.6)	1.00			1.00		
		CT/TT	36 (65.5)	19 (34.5)	1.32 (0.59-2.95)	0.498	0.622	1.15 (0.49-2.67)	0.748	0.749
TS	28bp	2R3R/3R3R	62 (70.5)	26 (29.5)	1.00			1.00		
		2R2R	12 (57.1)	9 (42.9)	1.79 (0.67-4.76)	0.249	0.415	1.31 (0.47-3.69)	0.608	0.749
TS	6bp-del	++	34 (72.3)	13 (27.7)	1.00			1.00		
		+-/--	42 (65.6)	22 (34.4)	1.37 (0.60-3.11)	0.450	0.622	1.67 (0.70-4.00)	0.245	0.427
ABCB1	C3435T	CC/CT	60 (72.3)	23 (27.7)	1.00			1.00		
		TT	16 (57.1)	12 (42.9)	1.96 (0.80-4.76)	0.142	0.355	2.19 (0.86-5.61)	0.104	0.260
ABCG2	C421A	CC	64 (69.6)	28 (30.4)	1.00			1.00		
		CA/AA	12 (63.2)	7 (36.8)	1.33 (0.47-3.74)	0.588	0.636	1.19 (0.41-3.46)	0.749	0.749
RFC1	G80A	AA	20 (64.5)	11 (35.5)	1.00			1.00		
		AG/GG	54 (69.2)	24 (30.8)	0.81 (0.34-1.95)	0.636	0.636	0.84 (0.33-2.11)	0.709	0.749
SLCO1B1	rs4149081	GG/GA	76 (69.7)	33 (30.3)	1.00			1.00		
		AA	0 (0)	2 (100)	N.E.	0.097	0.323	N.E.	0.057	0.260
SLCO1B1	rs11045879	TT/TC	76 (70.4)	32 (29.6)	1.00			1.00		
		CC	0 (0)	3 (100)	N.E.	0.030*	0.300	N.E.	0.008*	0.080

*N.E. Not Estimable; *p<0.05; Genotypes MTHFR 677 TT, MTHFR 1298 CC, SHMT1 CC, TS 2R2R, TS +-/-- ABCB1 TT, ABCG2 CA/AA, RFC1 AA, SLCO1B1 AA and SLCO1B1 CC were considered of higher risk of toxicity. Genotypes MTHFR 677 CC/CT, MTHFR 1208 AA/AC, SHMT1 CT/TT, TS 2R3R/3R3R, TS ++, ABCB1 CC/CT, ABCG2 CC, RFC1 AG/GG, SLCO1B1 GG/GA and SLCO1B1 TT/TC were considered of low risk of toxicity.*

We found a statistically significant association between MTX plasma concentration and the *SLCO1B1* rs11045879 CC homozygous risk genotype. All the patients with the CC genotype had high MTX plasma concentrations 72 h after MTX infusion (p= 0.030) (Table 16). In the multivariate analysis, SLCO1B1 rs11045879 remained associated with MTX plasma levels (p=0.008) (Table 16). When we corrected for multiple comparisons, we obtained a p-value near the significance level (p= 0.08). In the rs4149081 polymorphism, of the same gene, *SLCO1B1*, the AA genotype was always associated with high MTX plasma concentrations at 72 h, although this association did not reach statistical significance (p= 0.097; p=0.057). As shown in Figure 25, in the group of patients with the rs11045879 TT/TC and rs4149081 GG/GA genotypes only a third of them had high MTX concentrations in plasma, while all the individuals with the rs11045879 GG and rs4149081 AA had high MTX plasma levels. It is worth to note that all the individuals homozygous for the risk allele rs4149081 AA had also the rs11045879 GG risk genotype. This was expected due to the high degree of linkage disequilibrium between both SNPs (r^2=0.807), which are located in the same linkage block. None of the other polymorphisms analyzed were in high linkage disequilibrium (pairwise D' was lower than 0.70).

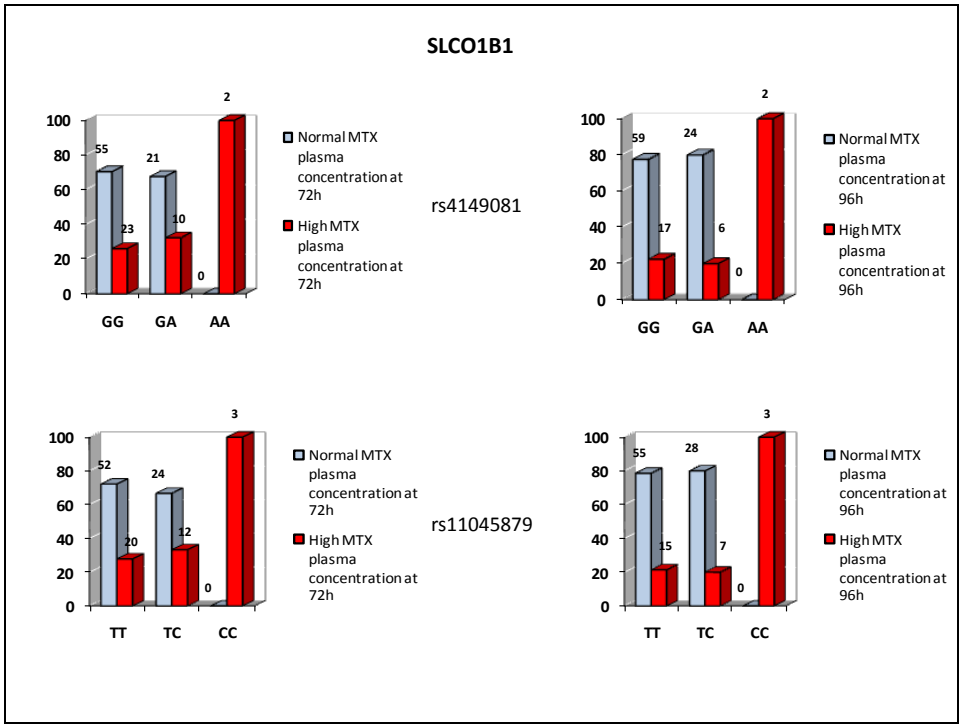


Figure 25. Frequency of ALL patients with high and normal MTX plasma concentration at 72 (A, C) and 96 hours (B, D) after MTX infusion according to genotypes of the *SLCO1B1* polymorphisms rs4149081 and rs11045879.

In summary, in this candidate gene studies, we have obtained two important results:

On the one hand, we have confirmed the association between *SLCO1B1* rs11045879 polymorphism and MTX toxicity. As SLCO1B1 is a hepatic transporter involved in MTX elimination, other polymorphisms in transporter genes from the same pathway could also have a role in MTX toxicity.

Another important result of this study is that *MTHFR* C677T and A1298C polymorphisms do not increase the risk of MTX toxicity.

POLYMORPHISMS OF THE MTX TRANSPORT PATHWAY AND MTX TOXICITY

In order to search for markers that predict MTX toxicity in pediatric B-ALL patients, we have performed an exhaustive selection of SNPs in the 12 most important genes involved in MTX transport and elimination to deeply cover the genetic variability of each gene. The study has been carried out in a large and homogeneous population of 151 Spanish pediatric B-ALL patients, all of them homogeneously treated according to the standardized LAL/SHOP protocol, and we have used MTX plasma concentration as an objective and quantifiable toxicity marker.

Patients' baseline characteristics

In this study, we have analyzed 151 B-ALL patients, whose characteristics are reported in Table 17. Clinical data about MTX plasma concentration 72 h after infusion were available for 143 patients. There were 51 patients (35.7%) that had high MTX plasma levels (>2 μM). Clinical data about other therapy-related toxicity were available for 130 patients. Among all patients who developed any of these toxicities (n=66, 41.7%), the prevalence of different types of toxicity were as follows: hepatic (n=36, 28.1%), vomits (n=28, 21.9%), mucositis (n=12, 9.4%), renal (n=12, 9.4%), hyperbilirubinemia (n=12, 9.4%) and diarrhea (n=7, 5.5%).

Table 17. Characteristics of the study population.

No. of patients, n	151
Mean age at diagnosis ± SD, years	5.32 ± 3.47
Sex, n (%)	
Female	63 (41.7)
Male	88 (58.3)
Risk group, n (%)	
Standard	54 (38.6)
High	61 (43.6)
Very high	25 (17.8)
Treatment protocol, n (%)	
LAL-SHOP 94/99	58 (38.4)
LAL-SHOP 2005	93 (61.6)
MTX dose in consolidation, n (%)	
$3g/m^2$	66 (43.7)
$5g/m^2$	85 (56.3)
Toxicity during consolidation therapy, n (%)	
Any toxicity	66 (50.8)
Hepatic	36 (28.1)
Vomits	28 (21.9)
Diarrhea	7 (5.5)
Mucositis	12 (9.4)
Hyperbilirubinemia	12 (9.4)
Renal	12 (9.4)
MTX concentration in plasma	
Higher than 0.2µM at 72h	51 (35.7)

SD: standard deviation.

Genotyping Results

A successful genotyping was obtained in 137 DNA samples (90.7%). In the genotyping process, 41 SNPs out of 384 failed (no PCR amplification, insufficient intensity for cluster separation, or poor or no cluster definition). These SNPs were excluded from the study (Table 29, Annex II). The other 343 SNPs were genotyped satisfactorily (89.3%). The average genotyping rate for all SNPs was 96.7%.

Analysis of the association between polymorphisms and toxicity

In order to investigate if genetic variation in the MTX transport and elimination pathway may influence MTX toxicity, we tested the association between the 343 genotyped polymorphisms in 12 genes and MTX plasma concentration 72 hr after intravenous infusion.

Significant association with MTX clearance ($p<0.05$) was found for 21 polymorphisms from 7 genes: 6 SNPs in *ABCC4* , 4 SNPs in *ABCC2*, 3 SNPs in *SLC22A6*, 3 SNPs in *SLC19A1*, 2 SNPs in *ABCG2*, 1 SNP in *ABCC1* and 2 SNPs in *SLCO1B1*, including rs11045879, which had been previously described by our group. Most SNPs remained associated with MTX toxicity when we accounted for the possible confounding effect of sex, age and MTX dose (Table 18).

After FDR correction, rs9516519 in *ABCC4* and rs3740065 in *ABCC2* continued being significantly associated with MTX clearance (corrected P-value<0.05). Nucleotide T in rs9516519 (*ABCC4*), and nucleotide C in rs3740065 (*ABCC2*) were associated with an increased risk of MTX toxicity.

Although MTX clearance was the most objective and quantifiable toxicity parameter, directly linked to MTX, we also analyzed the other toxicity parameters but we did not find any clear associations (data not shown).

Analysis of the association between haplotypes and toxicity

To test the association between haplotypes and MTX clearance, we first determined the linkage disequilibrium (LD) block structure for each gene (block definition was based on Gabriel et al., 2002). *ABCB1* was defined by 5 blocks which showed 21 haplotypes with frequencies higher than 1%; *ABCC1* was defined by 7 blocks which showed 27 haplotypes; *ABCC2* was defined by 3 blocks which showed 12 haplotypes (Figure 26); *ABCC3* was defined by 6 blocks which showed 20 haplotypes; *ABCC4* was defined by 18 blocks which showed 61 haplotypes (Figure 27); *ABCG2* was defined by 3 blocks which showed 13 haplotypes; *SLC19A1* 2 blocks which showed 10 haplotypes; *SLC22A6-SLC22A8* cluster was defined by 3 blocks which showed 18 haplotypes; *SLCO1A2* 6 blocks which showed 24 haplotypes; *SLCO1B1* 6 blocks which showed 28 haplotypes; *SLCO1B3* was defined by 4 blocks which showed 15 haplotypes.

Significant results of the association analyses comparing the frequency of each haplotype between normal MTX clearers (<2μM) and slow clearers (>2μM) are shown in Table 19. Significant associations were found for 15 haplotypes (4 in *ABCC2*, 6 in *ABCC4*, 2 in *SLC22A6-SLC22A8*, 1 in *SLCO1B1*, 1 in *SLCO1A2* and 1 in *ABCG2*). After p correction, haplotype GCGGG in *ABCC2* remained statistically significant (p= 0.0360). This haplotype was associated with increased MTX toxicity (slow MTX clearance) and included polymorphisms rs3740066, rs3740065 and rs12826, which were associated with toxicity in the single analysis.

Table 18. Genetic polymorphisms and methotrexate clearance.

Gene	Polymorphism	Genotype	MTX concentration in plasma at 72h		OR (95% CI)	p	p adjusted for age sex and dose	p after FDR correction
			< 0.2 µM, n(%)	>0.2 µM, n(%)				
ABCC4	rs9516519	TT	52 (55.3)	42 (44.7)	1.00			
		GT/GG	30 (88.2)	4 (11.8)	0.17 (0.05-0.51)	0.00026	0.00021	0.01878
ABCC2	rs3740065	TT	69 (71.1)	28 (28.9)	1.00			
		TC/CC	12 (40)	18 (60)	3.7 (1.58-8.67)	0.00226	0.00358	0.03395
SLC22A6	rs11231294	TT	35 (53.8)	30 (46.2)	1.00			
		TC/CC	47 (75.8)	15 (24.2)	0.37 (0.17-0.80)	0.00917	0.02783	N.S.
SLC22A6	rs4149172	AA	33 (34.0)	64 (66.0)	1.00			
		AG/GG	48 (57.8)	35 (42.2)	0.38 (0.18-0.81)	0.01036	0.02762	N.S.
ABCC4	rs2619312	TT	49 (57.0)	37 (43.0)	1.00			
		TC/CC	34 (79.1)	9 (20.9)	0.35 (0.15-0.82)	0.011367	0.00445	N.S.
SLC19A1	rs1051266	AA/AG	53 (59.6)	36 (40.4)	1.00			
		GG	28 (82.4)	6 (17.6)	0.32 (0.12-0.84)	0.01328	0.03916	N.S.
ABCC4	rs1678392	GG	51 (57.3)	38 (42.7)	1.00			
		GA/AA	31 (79.5)	8 (20.5)	0.35 (0.14-0.84)	0.01330	0.00375	N.S.
ABCG2	rs2725252	GG/GT	58 (60.4)	38 (39.6)	1.00			
		TT	22 (84.6)	4 (15.4)	0.28 (0.09-0.87)	0.015306	N.S.	N.S.
ABCG2	rs2622621	CC	54 (73.0)	20 (27.0)	1.00			
		CG/GG	27 (51.9)	25 (48.1)	2.50 (1.18-5.28)	0.01540	N.S.	N.S.
SLC19A1	rs3788200	GG	28 (82.4)	6 (17.6)	1.00			
		GA/AA	53 (60.2)	35 (39.8)	3.08 (1.16-8.21)	0.01609	0.04449	N.S.
ABCC2	rs3740066	GG	21 (50.0)	21 (50.0)	1.00			
		GA/AA	58 (71.6)	23 (28.4)	0.40 (0.18-0.86)	0.01864	0.00903	N.S.
ABCC2	rs12826	GG	21 (50.0)	21 (50.0)	1.00			
		GA/AA	58 (71.6)	23 (28.4)	0.40 (0.18-0.86)	0.01864	0.00996	N.S.
ABCC2	rs717620	GG	39 (54.9)	32 (45.1)	1.00			
		GA/AA	41 (74.5)	14 (25.5)	0.42 (0.19-0.90)	0.02197	N.S.	N.S.
ABCC4	rs7317112	AA/AG	77 (67.5)	37 (32.5)	1.00			
		GG	5 (35.7)	9 (64.3)	3.75 (1.17-11.97)	0.02215	N.S.	N.S.
SLC19A1	rs1131596	CC/CT	47 (59.5)	32 (40.1)	1.00			
		TT	20 (83.3)	4 (16.7)	0.29 (0.09-0.94)	0.02486	0.04668	N.S.
ABCC4	rs9302061	TT/TC	44 (62.0)	27 (38.0)	1.00			
		CC	11 (91.7)	1 (8.3)	0.15 (0.02-1.21)	0.02660	0.00284	N.S.

Table 18. Genetic polymorphisms and methotrexate clearance (Continuation)

Gene	Polymorphism	Genotype	MTX concentration in plasma at 72h		OR (95% CI)	p	p adjusted for age sex and dose	p after FDR correction
			< 0.2 µM, n(%)	>0.2 µM, n(%)				
SLC22A6	rs10897310	TT	25 (52.1)	23 (47.9)	1.00			
		TC/CC	51 (70.8)	21 (29.2)	0.45 (0.21-0.96)	0.03733	N.S.	N.S.
ABCC4	rs10219913	TT	67 (69.1)	30 (30.9)	1.00			
		TC/CC	15 (48.4)	16 (51.6)	2.38 (1.04-5.44)	0.03933	0.03885	N.S.
SLCO1B1	rs4149035	CC	26 (54.2)	22 (45.8)	1.00			
		CT/TT	57 (72.2)	22 (27.8)	0.46 (0.22-0.97)	0.03995	N.S.	N.S.
SLCO1B1	rs11045879	TT/TC	83 (65.9)	43 (34.1)	1.00			
		CC	0 (0.0)	3 (100)	NE	0.04343	0.01129	N.S.
ABCC1	rs2230671	GG	39 (55.7)	31 (44.3)	1.00			
		GA/AA	38 (73.1)	14 (26.9)	0.46 (0.21-1.00)	0.04738	N.S.	N.S.

N.E. Not Estimable. N.S. non significant (p>0.05)

Table 19. Haplotypes and MTX clearance

Gene	SNPs	Haplotype	< 0.2 µM (freq)	>0.2 µM (freq)	p-Value
ABCC2	rs1885301; rs717620; rs2756105; rs4148385; rs2145853	AATAA	0.299	0.163	0.0181
	rs1885301; rs717620; rs2756105; rs4148385; rs2145853	AGTAA	0.206	0.337	0.0235
	rs3740066; rs3740065; rs12826; rs12762549; rs11190298	ATAGG	0.475	0.318	0.0171
	rs3740066; rs3740065; rs12826; rs12762549; rs11190298	GCGGG	0.076	0.193	0.0063*
ABCC4	rs1059751; rs3742106	TA	0.170	0.076	0.0349
	rs10219913; rs1189445	TG	0.319	0.201	0.0428
	rs10219913; rs1189445	CG	0.092	0.207	0.0097
	rs1189457; rs1678392; rs2619312	GAC	0.198	0.087	0.0193
	rs9524849; rs4148455; rs4148454; rs4283094; rs4148446; rs4148436	AGAGGT	0.065	0.007	0.0298
	rs870004; rs7317112	GG	0.118	0.240	0.0109
SLC22A6/SLC22A8	rs10897310; rs3017670; rs6591722; rs4149172; rs11231294	CGTGC	0.319	0.191	0.0268
	rs10792367; rs2276299; rs4149182; rs2187383	CAGC	0.013	0.072	0.0125
SLCO1A2	rs11045994; rs2045940; rs2045939; rs2045938	TGCT	0.000	0.034	0.0189

Table 19. Haplotypes and MTX clearance (Continuation).

Gene	SNPs	Haplotype	< 0.2 µM (freq)	>0.2 µM (freq)	p-Value
SLCO1B1	rs11045813; rs2291073; rs964614; rs11045818; rs11045819; rs4149050; rs4149056; rs2291075; rs2291076; rs11045821; rs12812279; rs4149058; rs11045823; rs2900476; rs2100996	GGTGCCTTCGAGGTT	0.000	0.034	0.0174
ABCG2	rs2622621; rs13120400; rs2725261	GTA	0.210	0.322	0.0464

* Statistically significant (p<0.05) after FDR correction.

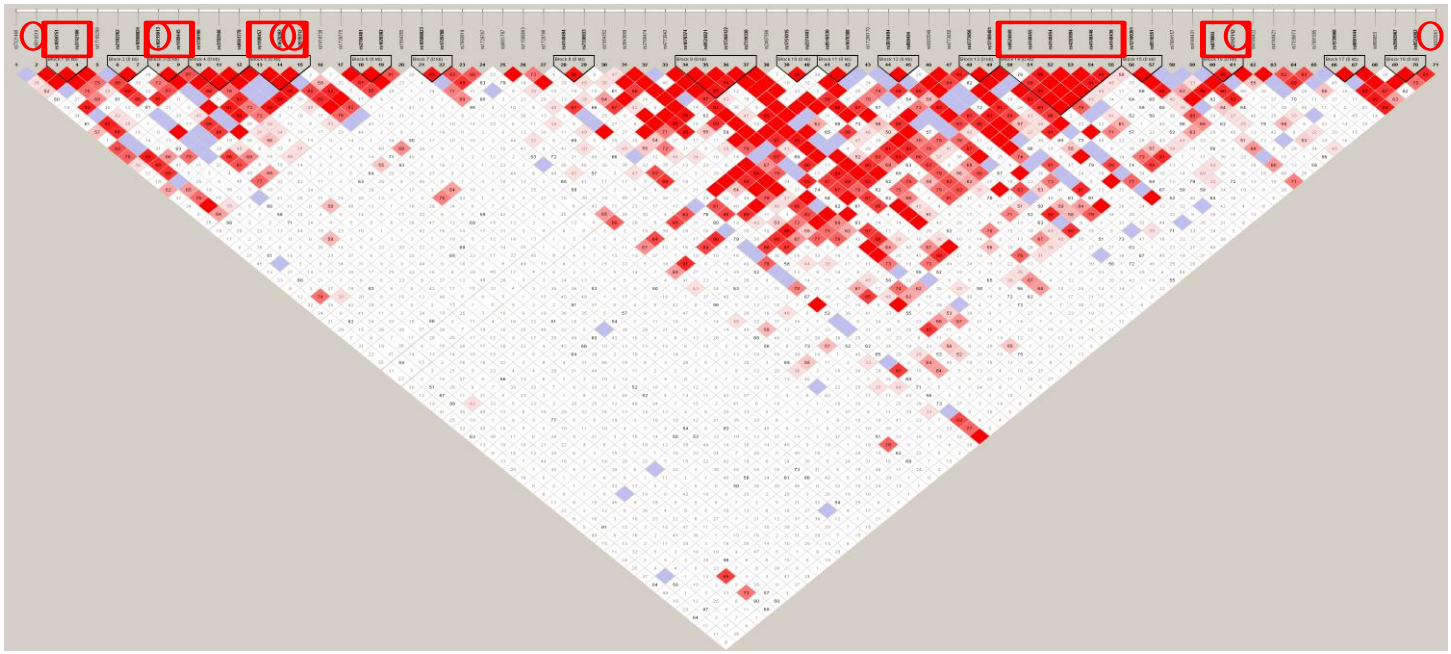


Figure 26. Gene Map and LD Plot of *ABCC4* and flanking regions.

Scheme is based on D0 and logarithm of the odds of linkage (LOD) score values: white D' < 1 and LOD < 2, blue D' = 1 and LOD < 2, bright red D' = 1 and LOD ≥ 2, shades of red: D' < 1 and LOD ≥ 2. Numbers in squares are D' values. Block definition is based on the Gabriel et al. method. SNPs significantly associated with MTX clearance are encircled.

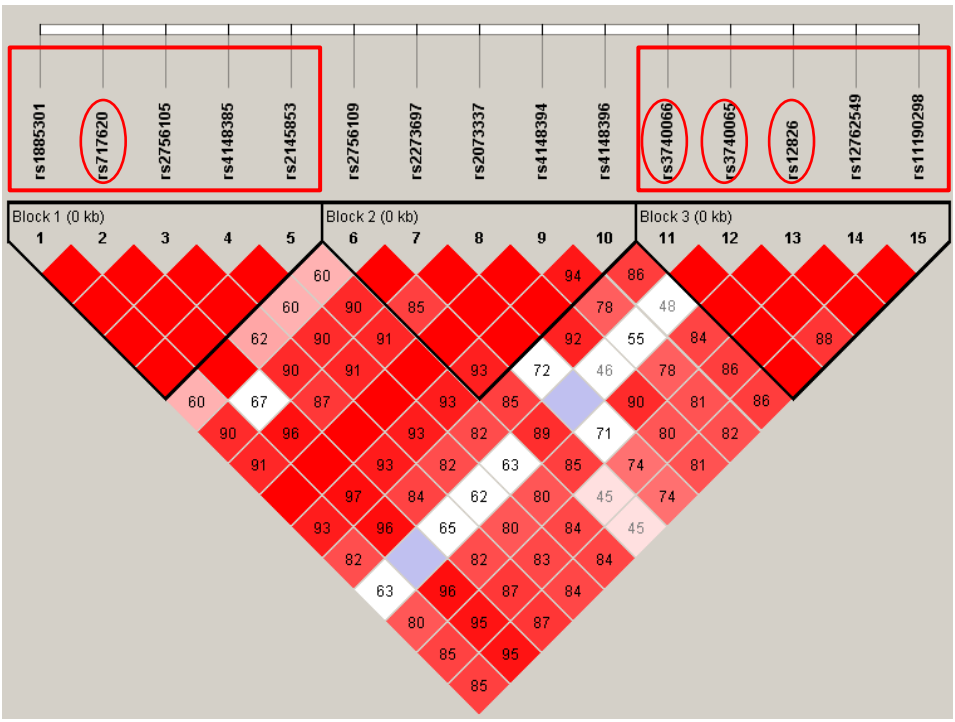


Figure 27. Gene Map and LD Plot of *ABCC2* and flanking regions.

Scheme is based on D0 and logarithm of the odds of linkage (LOD) score values: white D' < 1 and LOD < 2, blue D' = 1 and LOD < 2, bright red D' = 1 and LOD ≥ 2, shades of red: D' < 1 and LOD ≥ 2. Numbers in squares are D' values. Block definition is based on the Gabriel et al. method. SNPs significantly associated with MTX clearance are encircled.

SYSTEMATIC REVIEW AND META-ANALYSIS OF *MTHFR* POLYMORPHISMS IN METHOTREXATE TOXICITY PREDICTION

In our candidate genes and polymorphisms approach we did not find any association between *MTHFR* C677T and A1298C polymorphisms and MTX toxicity. A large body of published studies has investigated the potential role of *MTHFR* C677T and A1298C polymorphisms in toxicity and response to MTX in pediatric ALL, with conflicting results. Possible reasons for these discrepancies are differences in treatment protocols among studies, small or non-homogeneous populations, ethnic differences, and the use of different criteria defining toxicity.

For these reasons, we decided to perform a critical review of the published articles on the relationship between genetic variants of *MTHFR* and the toxicity of MTX in pediatric ALL. Then, we undertook a meta-analysis on all eligible studies, separating them by toxicity criteria, to determine the role of the *MTHFR* C677T and A1298C polymorphisms on MTX toxicity in this pediatric ALL patients.

Meta-Analysis Database

For the meta-analysis, we performed an exhaustive search using the keywords and subject terms “MTHFR and acute leukemia”, and “MTHFR and polymorphism(s) and toxicity”. The original search provided 264 records. After eliminating duplications, 238 records remained. Of these, 117 were discarded after reviewing the abstracts because they clearly did not meet the required criteria for inclusion. The full texts of the remaining 121 studies were examined

in detail. Of these, we identified 24 studies which investigated *MTHFR* SNPs and MTX related toxicity in pediatric ALL patients for meta-analysis (Figure 28). All 24 studied the C677T polymorphism (Table 20) and 16 of these also studied the A1298C polymorphism (Table 21).

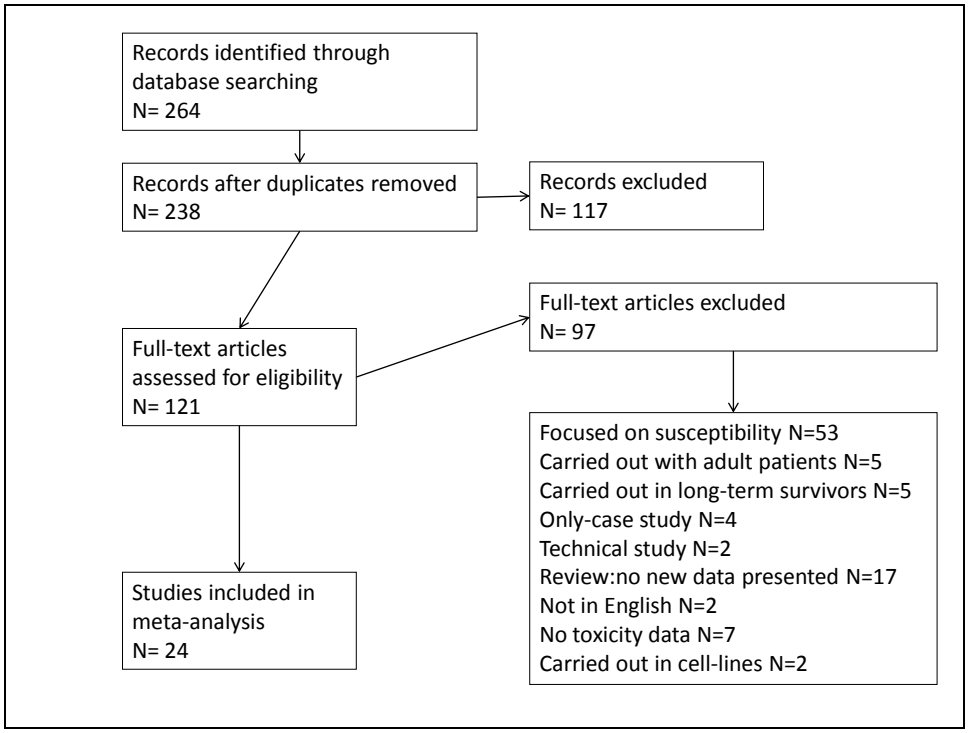


Figure 28. Flow diagram of study selection.

In general, the 24 studies could be categorized according to the level of association between *MTHFR* SNPs and MTX toxicity: those that found no association, those that found an association between *MTHFR* SNP and a significant increase in toxicity, and those that found an association between *MTHFR* SNP and a significant decrease in toxicity. No ethnicity was overrepresented in any of the 3 groups of studies. Additionally, toxicity was not associated with MTX dosage, as both high and low dose MTX were found in all

Table 20. List of 24 studies that analyzed association between the *MTHFR* C677T polymorphism and MTX toxicity in pediatric ALL, grouped according to the level of association between the SNP and MTX toxicity

MTHFR C677T				
Patient population	**MTX dose**	**Population**	**Association with toxicity**	**Reference**
15 ALL or LBL	high	Japanese	NA	Shimasaki et al, 2006[74]
24 ALL or LBL	low	Japanese	NA	Horinouchi et al, 2010 [100]
35 ALL	high	Cretan	NA	Karathanasis et al, 2011[96]
46 ALL	high	Greek	NA	Chatzidakis et al, 2006[97]
53 ALL	high	Various	NA	Kishi et al, 2003[98]
76 ALL	high	Thai	NA	Pakakasama et al, 2007[99]
81 ALL	high	European	NA	Huang et al, 2008[83]
115 ALL	high	Spanish	NA	Lopez-Lopez et al, 2011[223]
167 ALL	High	European	NA	Erculj et al, 2012[88]
240 ALL	high	North American	NA	Kishi et al, 2007[73]
201 ALL	low	French-Canadian	NA	Krajinovic et al, 2004[63]
520 ALL	low	Various	NA	Aplenc et al, 2005[76]
37 ALL or NHL	high	Turkish	-T	Kantar et al, 2009[101]
88 ALL	high	European	-T	van Kooten et al, 2008[102]
186 ALL	low	European	-T	Costea et al, 2006[103]
20 ALL or LBL	low	Japanese	+T	Shimasaki et al, 2008[72]
26 ALL or ML	high	Japanese	+T	Imanishi et al, 2007[68]
40 ALL	high	Egyptian	+T	Tantawy et al, 2010[78]
40 ALL	High	Egyptian	+T	EL-Khodary et al, 2011[77]
64 ALL or ML	high	European	+T	Faganel Kotnik et al, 2011[71]
141 ALL	High	Spanish	+T	Salazar et al, 2011[79]
151 ALL	high	European	+T	D'Angelo et al, 2011[80]
181 ALL	high	Chinese	+T	Liu et al, 2011[81]
557 ALL	High	Various	+T	Sepe et al, 2012[82]

High MTX dose = 1.5 – 5 g / m^2; Low MTX dose = 15 – 30 mg / m^2
NA, no association between the SNP and toxicity
+T, SNP is associated with increased toxicity (light shading)
–T, SNP is associated with decreased toxicity (dark shading)

Table 21. Association of *MTHFR* A1298C polymorphism and toxicity in pediatric acute lymphoblastic leukemia

MTHFR A1298C				
Patient population	**MTX dose**	**Population**	**Association with toxicity**	**Reference**
40 LLAs	**high**	**Egyptian**	**NA**	**Tantawy et al, 2010[78]**
115 LLA	**high**	**Spanish**	**NA**	**Lopez-Lopez et al, 2011[223]**
151 ALL	**high**	**European**	**NA**	**D'Angelo et al, 2011[80]**
167 ALL	**high**	**European**	**NA**	**Erculj et al, 2012[88]**
186 ALL	**low**	**European**	**NA**	**Costea et al, 2006[103]**
201 LLA	**low**	**French-Canadian**	**NA**	**Krajinovic et al, 2004[63]**
240 LLA	**high**	**North American**	**NA**	**Kishi et al, 2007[73]**
520 LLA	**low**	**Various**	**NA**	**Aplenc et al, 2005[76]**
64 ALL or ML	**high**	**European**	**-T**	**Faganel Kotnik et al, 2011[71]**
76 LLA	**high**	**Thai**	**-T**	**Pakakasama et al, 2007[99]**
81 LLA	**high**	**European**	**-T**	**Huang et al, 2008[83]**
88 LLA	**high**	**European**	**-T**	**van Kooten et al, 2008[102]**
181 ALL	**high**	**Chinese**	**-T**	**Liu et al, 2011[81]**
35 LLA	**high**	**Cretan**	**+T**	**Karathanasis et al, 2011[96]**
37 LLA or NHL	**high**	**Turkish**	**+T**	**Kantar et al, 2009[101]**
141 ALL	**high**	**Spanish**	**+T**	**Salazar et al, 2011[79]**

High MTX dose = 1.5 – 5 g / m^2; Low MTX dose = 15 – 30 mg / m^2
NA, no association between the SNP and toxicity
+T, SNP is associated with increased toxicity (light shading)
–T, SNP is associated with decreased toxicity (dark shading)

Table 22. Types of toxicities analyzed and the findings in each study of the associations between the *MTHFR* C677T polymorphism and MTX toxicity.

***MTHFR* C677T**									
Reference	**Anemia**	**Leucopenia**	**Neutropenia**	**Trombocytopenia**	**Myelosuppression**	**MTX plasma levels**	**Mucositis**	**Hepatic toxicity**	**Other**
Shimasaki et al, 2006[74]					NA		NA	NA	NA
Horinouchi et al, 2010[100]								NA	
Karathanasis et al, 2011[96]	NA	NA		NA			NA	NA	
Chatzidakis et al, 2006[97]	NA	NA						NA	
Kishi et al, 2003[98]									NA
Pakakasama et al, 2007[99]					NA		NA		NA
Huang et al, 2008[83]	NA	NA		NA		NA	NA	NA	NA
Lopez-Lopez et al, 2011[223]						NA			
Erculj et al, 2012[88]	NA	NA		NA			NA	NA	NA
Kishi et al, 2007[73]									NA
Krajinovic et al, 2004[63]									NA
Aplenc et al, 2005[76]							NA	NA	NA
Kantar et al, 2009[101]	NA	NA		-T		NA		NA	NA
van Kooten et al, 2008[102]		NA	-T	NA				NA	NA
Costea et al, 2006[103]		-T	NA	NA				NA	
Shimasaki et al, 2008[72]									+T
Imanishi et al, 2007[68]						+T		NA	
Tantawy et al, 2010[78]	+T	+T		+T				+T	+T/NA
EL-Khodary et al, 2011[77]			+T				+T	+T	+T
Faganel Kotnik et al, 2011[71]		NA		NA			+T		NA
Salazar et al, 2011[79]	NA	NA		+T			NA	NA	+T
D'Angelo et al, 2011[80]					NA				+T
Liu et al, 2011[81]	NA		NA	+T	NA	NA	NA	NA	NA
Sepe et al, 2012[82]								+T	NA

NA, no association between the SNP and toxicity. +T, SNP is associated with increased toxicity. –T, SNP is associated with decreased toxicity

Results

Table 23. Types of toxicities analyzed and the findings in each study of the associations between the *MTHFR* A1298C polymorphism and MTX toxicity.

	***MTHFR* A1298C**								
Reference	**Anemia**	**Leucopenia**	**Neutropenia**	**Trombocytopenia**	**Myelosuppression**	**MTX plasma levels**	**Mucositis**	**Hepatic toxicity**	**Other**
Tantawy et al, 2010[78]			NA				NA	NA	NA
Lopez-Lopez et al, 2011[223]						NA			
D'Angelo et al, 2011[80]					NA				NA
Erculj et al, 2012[88]	NA	NA		NA			NA	NA	NA
Costea et al, 2006[103]		NA	NA	NA				NA	
Krajinovic et al, 2004[63]									NA
Kishi et al, 2007[73]									NA
Aplenc et al, 2005[76]							NA	NA	NA
Faganel Kotnik et al, 2011[71]		-T		NA			NA		NA
Pakakasama et al, 2007[99]					-T		NA		NA
Huang et al, 2008[83]	NA	NA				NA	NA	NA	-T/NA
van Kooten et al, 2008[102]		NA	NA	-T				NA	NA
Liu et al, 2011[81]	NA		NA	NA	NA	NA	NA	NA	-T
Karathanasis et al, 2011[96]	NA	NA		NA			NA	+T	
Kantar et al, 2009[101]	+T	NA		+T		+T		+T	+T
Salazar et al, 2011[79]	NA	NA		+T			NA	NA	+T

NA, no association between the SNP and toxicity. +T, SNP is associated with increased toxicity. –T, SNP is associated with decreased toxicity

three study groupings (Tables 20-21). Because different studies analyzed toxicity according to different criteria, we performed in-depth analysis for each toxicity criterion (Tables 22-23).

***MTHFR* C677T polymorphism and toxicity in pediatric ALL**

In the 24 published studies used in this analysis, 12 did not find a significant association between the *MTHFR* 677T low functional allele and MTX toxicity [63,73,74,76,83,88,96-100,223]. Three studies found an association between the 677T allele and a decrease in toxicity [101-103]. Nine studies found an association between this allele and increased toxicity [68,71,72,77-82] (Table 20). Below we analyze the findings from the 24 studies for each toxicity criterion and report results from meta-analysis if enough data was provided to make it possible.

Treatment interruption:

Three studies analyzed MTX treatment interruption. An association between the 677T allele and an increase in interruption was reported by Shimasaki et al [72], however this study was carried out with a small and heterogeneous population (20 ALL or lymphoblastic lymphoma (LBL)) and only one patient with the TT genotype was reported. Two larger studies of 201 and 88 ALL patients did not find any association between 677T and MTX treatment interruption [63,102]. The three articles did not provide enough information to carry out a meta-analysis.

MTX plasma levels:

MTX plasma levels were studied in five works. Imanishi et al studied 26 children with ALL or malignant lymphoma (ML) [68] and concluded that patients with the

677TT homozygous genotype had higher MTX plasma levels 48 h after infusion. The other 4 studies found no association between the C677T SNP and MTX plasma levels 48 h or 72 h after infusion [81,83,101,223]. Only 2 studies provided enough data, from a total of 137 patients, to be included in the meta-analysis [68,223]. We found no statistical association between C677T and MTX plasma levels (Figure 29).

MTX clearance:

Two studies analyzed MTX clearance and reported conflicting results. One study of 64 children with LLA or ML [71] found an association between the 677TT homozygous genotype and a decrease in MTX clearance. The larger study of 240 pediatric ALL patients did not find any association between the C677T SNP and MTX clearance [73]. We could not carry out a meta-analysis for this parameter.

Diarrhea:

Four studies analyzed diarrhea. An association between the 677TT homozygous genotype and higher risk of diarrhea was found in a single study of 40 pediatric ALL patients [78]. Three additional studies carried out with 240, 520, and 557 pediatric ALL patients did not find this correlation [73,76,82]. Accordingly, the 677TT genotype cannot be considered a good predictor of severe diarrhea in response to MTX treatment for ALL. Only one of the 4 articles provided genotype information, so we were unable to confirm this with a meta-analysis.

Mucositis:

Mucositis was surveyed in 10 studies. Two studies of 64 and 40 children with ALL found an association between 677TT genotype and higher risk of mucositis [71,78]. The other 8 studies, most of which were larger and studied various ethnic

populations, did not find this association [74,76,79,81,83,88,96,99]. This lack of consistent results across these studies does not support an effect of the 677TT genotype in the risk of mucositis in response to MTX treatment for ALL. We performed meta-analysis on 4 studies [71,78,81,96] with data from a total of 484 observations. No association with mucositis was observed (Figure 29). As the heterogeneity among studies was high, a sensitivity analysis was undertaken and this identified the study by Tantawy et al. as an outlier. Removing this data from the meta-analysis reduced the heterogeneity, yet the pooled RR remained non significant.

Hepatic toxicity:

We compiled 16 studies that analyzed hepatic toxicity. Three of them found an association between the 677TT genotype and increased hepatic toxicity [77,78,82]. However, two of these studies do not have a very high statistical power, and the other 13 studies that analyzed this parameter found no association between 677TT genotype and hepatic toxicity [68,72,76,79,81,83,88,96,97,100-103], therefore we conclude that the 677TT genotype does not appear to be a good predictor of hepatic toxicity in response to MTX treatment for ALL. Of these 16 studies, 6 presented enough data to allow meta-analysis [68,78,81,82,96,100] with data from a total of 757 patients. No association between C677T genotypes and hepatic toxicity was observed (Figure 29). Since there was a great heterogeneity between studies, a sensitivity analysis was undertaken and this identified the study by Tantawy et al. as an outlier. Removing this data from the meta-analysis reduced the heterogeneity yet the pooled RR remained non significant.

Hyperbilirubinemia:

Hyperbilirubinemia was studied in four reports. One study of 37 patients [(23)] found that individuals with 677CT or 677TT genotypes had less

hyperbilirubinemia. Three larger studies of 240, 520 and 557 patients did not find this association [73,76,82]. None of these articles provided enough information to perform a meta-analysis. We conclude that the 677TT genotype does not appear to be a good predictor of hyperbilirubinemia in response to MTX treatment for ALL.

Neutropenia:

From the 4 papers that analyzed neutropenia, only one reported an association between the 677TT genotype and higher risk of neutropenia [78]. Two larger studies did not find this association [81,103]. A fourth study reported the opposite effect, finding an association between the 677TT genotype and a lower risk of neutropenia [102] (Table 22). Of these 4 studies, 2 provided enough data to be included in the meta-analysis [78,81] with data from 200 patients. No association between C677T SNP and neutropenia was observed (Figure 29).

Thrombocytopenia:

A total of 10 studies [71,77,79,81,83,88,96,101-103] analyzed thrombocytopenia. An association between the 677CT and 677TT genotypes and an increased risk of thrombocytopenia was reported in 2 studies [77,81], but was only statistically significant for the 677CT genotype. The apparent disadvantage of the heterozygous genotype is difficult to explain from a functional point of view. Furthermore, another study reported a correlation between the 677CT and TT genotypes with decreased risk of thrombocytopenia [101]. In another study that looked at both C677T and A1298C, an association between the combined 677T and 1298C alleles and increased thrombocytopenia was found [79]. An additional 6 studies did not find any association between C677T SNP and thrombocytopenia [71,81,88,96,102,103]. In conclusion, the available data do not

support a clear association between the 677T allele and a higher risk of thrombocytopenia in response to MTX treatment for ALL. In the meta-analysis, 3 studies were included [71,81,96] with data from a total of 381 observations. No association between the C677T SNP and thrombocytopenia was observed (Figure 29).

Anemia:

From the 7 reports that studied anemia [77,79,81,88,96,97,101], a single study [77] found an association between C677T and increased anemia. In the meta-analysis, we excluded 5 studies due to lack of data, leaving 2 studies with data from 192 patients [81,96]. We observed no association with anemia (Figure 29).

Leucopenia:

We found 9 reports that studied leucopenia. One [77] found an association between C677T and increased leucopenia. One study reported the opposite, finding an association between 677T and decreased leucopenia [103]. 7 studies did not find any association [71,83,88,96,97,101,102]. From these 9 studies, 2 provided genotype data from 221 observations [71,96]. No association between C677T and leucopenia in response to MTX treatment in ALL was observed (Figure 29).

Renal toxicity:

Renal toxicity was reported in 3 studies. One [77] found an association between the 677T allele and increased renal toxicity. In a combined study of C677T and A1298C, association with increased renal toxicity was also found [79]. Another larger study did not find any association between the 677T allele and increased renal toxicity [88]. Consequently, the published data do not support a clear association between the 677T allele and renal toxicity in response to MTX

treatment in ALL. We could not carry out a meta-analysis to confirm it, due to lack of data.

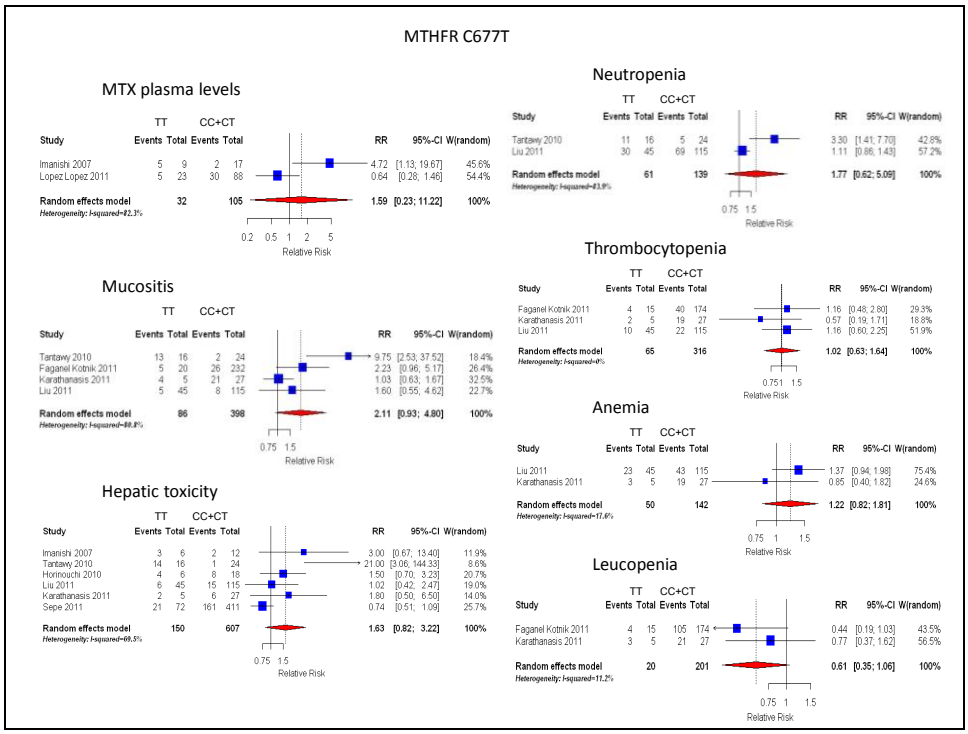


Figure 29. Results of meta-analysis of association between the *MTHFR* C677T SNP and MTX toxicities in treatment of ALL. No associations were confirmed between genotype and toxicity.

Finally, in our review of the literature, we re-analyzed, when possible, the data provided in the articles. In one case in which the authors reported an association between the 677TT genotype and an increase in global toxicity [80], we detected a statistical error and drew the opposite conclusion to what the authors proposed (Table 24).

Table 24. Methotrexate toxicity and MTHFR C677T polymorphism.

	MTHFR C677T	r / N	OR (95% CI)	*P*-value
MTX 2 g				
Global toxicity	CC	14 / 21	1.00	
	CT	22 / 38	0.69 (0.23 to 2.09)	0.509
	TT	14 / 19	1.40 (0.36 to 5.49)	0.629
Haematological toxicity	CC	8 / 21	1.00	
	CT	10 / 38	0.58 (0.19 to 1.81)	0.349
	TT	3 / 19	0.30 (0.07 to 1.39)	0.124
Non-haematological toxicity	CC	6 / 21	1.00	
	CT	12 / 38	1.15 (0.36 to 3.71)	0.810
	TT	11 / 19	3.44 (0.92 to 12.79)	0.065
MTX 5 g				
Global toxicity	CC	20 / 27	1.00	
	CT	29 / 33	2.54 (0.65 to 9.83)	0.177
	TT	4 / 13	0.16 (0.04 to 0.67)	0.012*
Haematological toxicity	CC	9 / 27	1.00	
	CT	13 / 33	1.30 (0.45 to 3.76)	0.628
	TT	1 / 13	0.17 (0.02 to 1.49)	0.109
Non-haematological toxicity	CC	11 / 27	1.00	
	CT	16 / 33	1.37 (0.49 to 3.82)	0.549
	TT	3 / 13	0.44 (0.10 to 1.96)	0.279

* $p < 0.05$.
r= number of subjects presenting toxicity.
N= Total number of subjects.

***MTHFR* A1298C polymorphism and toxicity in pediatric ALL**

In the 16 studies that analyzed this polymorphism (Table 21), 8 studies [63,73,76,78,80,88,103,223] found no association between A1298C and any toxic effect. In 5 studies, the authors reported a protective effect of the 1298C allele against various types of MTX toxicity [71,81,83,99,102]. We found three studies in which this allele was associated with higher MTX toxicity [79,96,101].

We could not perform a meta-analysis for transfusions, skin toxicity, MTX plasma levels, or febrile neutropenia due to lack of data. We did perform meta-

analyses for leucopenia, myelosuppression, thrombocytopenia, hepatic toxicity, and anemia and only observed a slight protective effect of the 1298CC genotype for leucopenia in a meta-analysis study with data from only two reports (Figure 30).

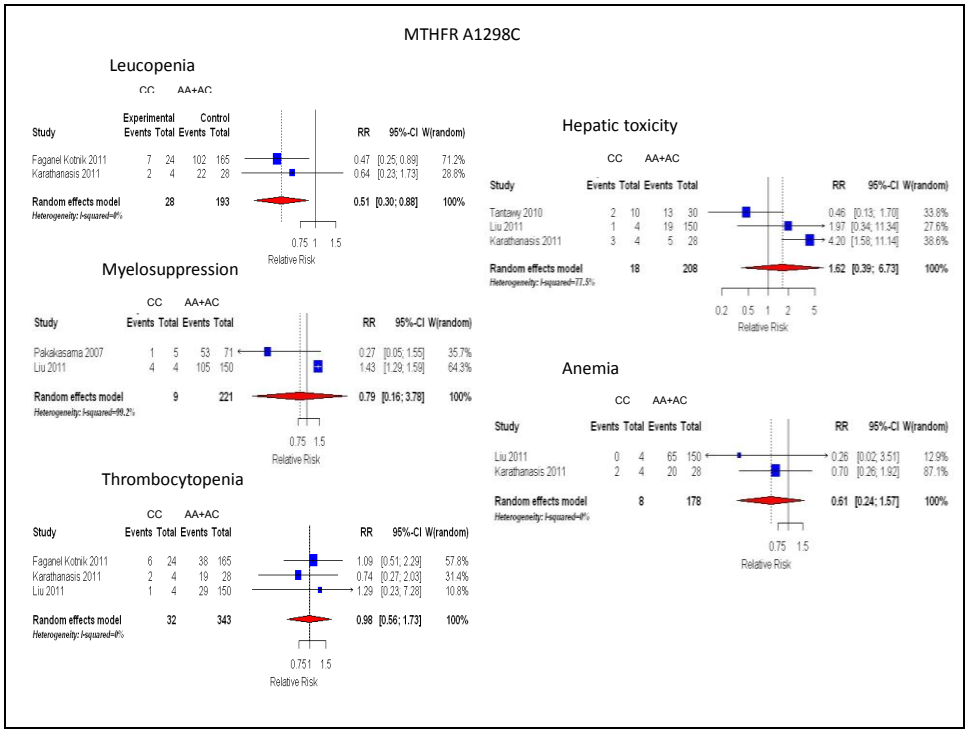


Figure 30. Results of meta-analysis of association between the *MTHFR* A1298C polymorphism and MTX toxicities in treatment of ALL. We observed a slight protective effect of the 1298CC genotype with leucopenia using data from only two reports.

POLYMORPHISMS IN MICRORNAS AND MICRORNAS BIOGENESIS MACHINERY IN DRUG RESPONSE IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

MiRNAs, by regulating the expression of pharmacogenomic-related genes, can play a pivotal role in drug toxicity, having potential clinical implications for personalized medicine, and genetic polymorphisms can affect their function. Taking this into consideration, we selected 118 polymorphisms in pre-miRNAs and in genes of their biosynthesis pathway and analyzed their role in toxicity.

Patients' baseline characteristics

In this study, we have analyzed 152 B-ALL patients, whose characteristics are reported in Table 25. Clinical data about MTX plasma concentration 72 h after infusion were available for 141 patients. There were 51 patients (36.17%) that had high MTX plasma levels (>2 μM). Clinical data about other therapy-related toxicity in induction were available for 137 patients and in consolidation for 130 patients.

Table 25. Characteristics of the study population.

No. of patients, n	152
Mean age at diagnosis ± SD, years	5.1456 ± 3.41
Sex, n (%)	
Female	65 (42.76)
Male	87 (57.23)
Risk group, n (%)	
Standard	56 (40.57)
High	56 (40.57)
Very high	26 (18.84)
Treatment protocol, n (%)	
LAL-SHOP 94/99	65 (43.05)
LAL-SHOP 2005	86 (56.95)
MTX dose in consolidation, n (%)	
$3g/m^2$	73 (48.34)
$5g/m^2$	78 (51.66)
Toxicity during induction therapy, n (%)	
Any toxicity	79 (57.66)
Hepatic	45 (32.84)
Vomits	36 (26.28)
Diarrhea	16 (11.67)
Mucositis	29 (21.17)
Hyperbilirubinemia	21 (15.32)
Renal	5 (3.65)
Toxicity during consolidation therapy, n (%)	
Any toxicity	71 (54.61)
Hepatic	39 (30)
Vomits	31 (23.85)
Diarrhea	9 (6.92)
Mucositis	14 (10.77)
Hyperbilirubinemia	11 (8.46)
Renal	13 (10)
MTX concentration in plasma, n (%)	
Higher than 0.2uM at 72h	51 (36.17)

Genotyping Results

A successful genotyping was obtained in 145 DNA samples (96.02%). In the genotyping process, 12 SNPs out of 118 failed (no PCR amplification, insufficient intensity for cluster separation, or poor or no cluster definition) and 106 were genotyped satisfactorily (89.83%). The average genotyping rate for all SNPs was 97.81%. Of those 106 SNPs, 14 were not in HWE in a population of 348 healthy controls and were not considered for further analysis. In total, 26 SNPs were

excluded from the association study (Table 30, Annex II). The other 92 SNPs were used in the association studies.

Analysis of the association with toxicity

In order to investigate if genetic variation may influence MTX toxicity, we tested the association between the 92 polymorphisms successfully genotyped and in HWE and different toxicity parameters in the induction and consolidation phases.

Toxicity in induction

We found 30 statistically significant associations between polymorphisms in miRNA biosynthesis genes and toxicity during the induction phase of treatment (Table 26). Of them, 16 were located in processing genes and 14 in pre-miRNAs. The most significant associations were between *XPO5* rs34324334 and hyperbilirubinemia, *TNRC6A* rs6497759 and mir-300 rs12894467 and hepatic toxicity, *DROSHA* rs10035440 and hyperbilirubinemia, *CNOT1* rs11866002 and mucositis, *GEMIN3* rs197388 and renal toxicity, *GEMIN4* rs3744741 and hepatic toxicity and *EIF2C1* rs595961 and vomits. After FDR correction, none of these associations remained statistically significant.

Table 26. Significant associations between polymorphisms and toxicity parameters during the induction phase.

Gene	SNP	Toxicity	Genotype	No tox n(%)	Tox n(%)	OR (95% CI)	p
XPO5	rs34324334	Hyperbiliru binemia	CC CT	101 (88.6) 7 (50.0)	13 (11.4) 7 (50.0)	1.00 7.77 (2.35-25.7)	0.0011
	rs7755135	Mucositis	GG AG/AA	68 (73.9) 33 (89.2)	24 (26.1) 4 (10.8)	1.00 2.68 (1.14-6.32)	0.0251
TNRC6A	rs6497759	Hepatic toxicity	GG AG/AA	63 (76.8) 23 (48.9)	19 (23.2) 24 (51.1)	1.00 3.46 (1.60-7.46)	0.0013
DROSHA	rs10035440	Hyperbiliru binemia	TT CT/CC	43 (72.9) 65 (92.9)	16 (27.1) 5 (7.1)	1.00 0.23 (0.08-0.68)	0.0041
	rs6877842	Diarrhea	GG GC/CC	73 (92.4) 39 (79.6)	6 (7.6) 10 (20.4)	1.00 3.12 (1.05-9.23)	0.0359
	rs2287584	Vomits	TT CT/CC	41 (65.1) 54 (80.6)	22 (34.9) 13 (19.4)	1.00 0.45 (0.20-1.00)	0.0455
CNOT1	rs11866002	Mucositis	CC/CT TT	98 (81.7) 4 (40.0)	22 (18.3) 6 (60.0)	1.00 6.68 (1.74-25.70)	0.0056
	rs37060	Hepatic toxicity	GG AG/AA	47 (77.1) 40 (57.1)	14 (22.9) 30 (42.9)	1.00 2.52 (1.18-5.39)	0.0152
		Hyperbiliru binemia	GG AG/AA	56 (91.8) 54 (77.1)	5 (8.2) 16 (22.9)	1.00 3.32 (1.14-9.69)	0.0193
GEMIN 3	rs197388	Renal toxicity	AA/AT TT	121 (97.6) 3 (60.0)	3 (2.4) 2 (40.0)	1.00 26.89 (3.21-225)	0.0068
GEMIN4	rs3744741	Hepatic toxicity	CC CT/TT	70 (73.7) 17 (48.6)	25 (26.3) 18 (51.4)	1.00 2.96 (1.33-6.63)	0.0080
EIF2C1	rs595961	Vomits	AA AG/GG	67 (79.8) 24 (57.1)	17 (20.2) 18 (42.9)	1.00 2.96 (1.31-6.65)	0.0086
CNOT4	rs3812265	Mucositis	CC CT/TT	61 (72.6) 39 (90.7)	23 (27.4) 4 (9.3)	1.00 0.27 (0.09-0.85)	0.0129
TRBP	rs784567	Vomits	GG/AG AA	66 (68.8) 27 (90.0)	30 (31.3) 3 (10.0)	1.00 0.24 (0.07-0.87)	0.0131
DGCR8	rs417309	Renal toxicity	GG/AG AA	125 (96.9) 0 (0.0)	4 (3.1) 1 (100)	1.00 NE (NE-NE)	0.0346
SND1	rs322825	Mucositis	CC CT/TT	39 (69.6) 62 (84.9)	17 (30.4) 11 (15.1)	1.00 0.41 (0.17-0.96)	0.0394
mir-300	rs12894467	Hepatic toxicity	CC/CT TT	82 (71.9) 5 (33.3)	32 (28.1) 10 (66.8)	1.00 5.12 (1.63-16.16)	0.0038
		Hyperbiliru binemia	CC/CT TT	99 (86.8) 9 (60.0)	15 (13.2) 6 (40.0)	1.00 4.40 (1.37-14.13)	0.0174
mir-449b	rs10061133	Renal toxicity	AA GA/GG	111 (98.2) 15 (83.3)	2 (1.8) 3 (16.7)	1.00 11.10 (1.71-71.9)	0.0132
mir-423	rs6505162	Diarrhea	CC AC/AA	25 (75.8) 88 (91.7)	8 (24.2) 8 (8.3)	1.00 0.28 (0.10-0.83)	0.0240
mir-1307	rs7911488	Diarrhea	AA/AG GG	100 (90.1) 10 (66.7)	11 (9.9) 5 (33.3)	1.00 4.55 (1.31-15.72)	0.0241
		Mucositis	AA AG/GG	44 (88.0) 55 (72.4)	6 (12.0) 21 (27.6)	1.00 2.80 (1.04-7.54)	0.0311
mir-618	rs2682818	Hyperbiliru binemia	CC/AC AA	110 (85.4) 0 (0.0)	19 (14.7) 2 (100.0)	1.00 NE (NE- NE)	0.0247
mir-146a	rs2910164	Diarrhea	GG CG/CC	70 (93.3) 45 (80.4)	5 (6.7) 11 (19.6)	1.00 3.42 (1.11-10.50)	0.0251
		Mucositis	GG CG/CC	64 (85.3) 39 (69.6)	11 (14.7) 17 (30.4)	1.00 2.54 (1.08-5.97)	0.0309

Table 26. Significant associations between polymorphisms and toxicity parameters during the induction phase (Continuation).

Gene	SNP	Toxicity	Genotype	No tox n(%)	Tox n(%)	OR (95% CI)	p
mir-577	rs34115976	Hyperbiliru binemia	CC CG/GG	67 (78.8) 41 (93.2)	18 (21.2) 3 (6.8)	1.00 0.27 (0.08-0.98)	0.0261
mir-492	rs2289030	Vomits	GG CG	90 (76.3) 6 (46.2)	28 (23.7) 7 (53.9)	1.00 3.75(1.16-12.08)	0.0282
mir-27a	rs895819	Hyperbiliru binemia	TT CT/CC	46 (92.0) 61 (78.2)	4 (8.0) 17 (21.8)	1.00 3.20 (1.01-10.17)	0.0320
mir-196a-2	rs11614913	Diarrhea	CC/CT TT	101 (90.2) 12 (70.6)	11 (9.8) 5 (29.4)	1.00 3.83 (1.14-12.89)	0.0407
mir-656	rs58834075	Hyperbiliru binemia	GG GA	105 (86.1) 3 (50.0)	17 (13.9) 3 (50.0)	1.00 6.18(1.15-33.15)	0.0426

Toxicity in consolidation

We found 31 statistically significant associations between SNPs in the miRNA biosynthesis genes and premiRNAs and toxicity during the consolidation phase of treatment (Table 27). Of them, 23 were located in processing genes and 8 in pre-miRNAs. The most significant associations were between *DROSHA* rs639174, rs2287584 and rs4867329 and vomits and rs3805500, in the same gene, and *TNRC6B* rs9611280 and hepatic toxicity.

After FDR correction, the association between rs639174 in *DROSHA* and vomits remained statistically significant (p = 0.031).

Table 27. Significant associations between polymorphisms and toxicity parameters during the consolidation phase.

Gene	SNP	Toxicity	Genotype	Absence n (%)	Presence n (%)	OR (95% CI)	p
DROSHA	rs639174	Vomits	CC CT/TT	47 (92.2) 43 (65.2)	4 (7.8) 23 (34.9)	1.00 6.28 (2.01-19.64)	0.0003
	rs2287584	Vomits	TT CT/CC	54 (88.5) 41 (66.1)	7 (11.5) 21 (33.9)	1.00 3.95 (1.53-10.18)	0.0026
	rs4867329	Vomits	AA/AC CC	74 (72.6) 21 (95.5)	28 (27.5) 1 (4.6)	1.00 0.13 (0.02-0.98)	0.0088
	rs3805500	Hepatic toxicity	AA/AG GG	70 (68.0) 17 (94.4)	33 (32.0) 1 (5.6)	1.00 0.12 (0.02-0.98)	0.0091
		Vomits	AA AG/GG	41 (89.1) 53 (70.7)	5 (10.9) 22 (29.3)	1.00 3.40 (1.19-9.76)	0.0137
	rs10719	Vomits	GG AG/AA	56 (83.6) 35 (66.0)	11 (16.4) 18 (34.0)	1.00 2.62 (1.11-6.19)	0.0259
		MTX clearance	GG/AG AA	77 (63.6) 9 (100)	44 (36.4) 0 (0.0)	1.00 NE (NE-NE)	0.0279
	rs7735863	Hepatic toxicity	GG AG/AA	58 (66.7) 30 (85.7)	29 (33.3) 5 (14.3)	1.00 0.33 (0.12-0.95)	0.0266
	rs6877842	Hepatic toxicity	GG CG/CC	59 (77.6) 27 (60.0)	17 (22.4) 18 (40.0)	1.00 2.31 (1.04-5.17)	0.0405
	rs10035440	Diarrhea	TT/CT CC	104 (94.6) 9 (75.0)	6 (5.5) 3 (25.0)	1.00 5.78 (1.23-27.06)	0.0411
TNRC6B	rs9611280	Hepatic toxicity	GG AG	70 (67.3) 19 (95.0)	34 (32.7) 1 (5.0)	1.00 0.11 (0.01-0.84)	0.0042
	rs139919	Mucositis	TT CT/CC	73 (86.9) 36 (100)	11 (13.1) 0 (0.0)	1.00 NE (NE-NE)	0.0327
XPO5	rs34324334	Hepatic toxicity	CC CT	73 (68.2) 14 (100)	34 (31.8) 0 (0.0)	1.00 NE (NE-NE)	0.0101
DICER	rs1209904	Diarrhea	CC CT/TT	58 (98.3) 56 (87.5)	1 (1.7) 8 (12.5)	1.00 8.29 (1.00-68.39)	0.0141
	rs13078	Diarrhea	TT AT/AA	68 (97.1) 45 (86.5)	2 (2.9) 7 (13.8)	1.00 5.29 (1.05-26.62)	0.0255
		MTX clearance	TT AT/AA	57 (72.2) 29 (54.7)	22 (27.9) 24 (45.3)	1.00 2.14 (1.03-4.45)	0.0400
SND1	rs3823994	MTX clearance	AA/AT TT	84 (68.9) 4 (33.3)	38 (31.2) 8 (66.7)	1.00 4.42 (1.25-15.58)	0.0165
CNOT4	rs3812265	Mucositis	CC CT/TT	74 (94.9) 34 (81.0)	4 (5.1) 8 (19.1)	1.00 4.35 (1.23-15.5)	0.0183
GEMIN4	rs1062923	MTX clearance	AA AG/GG	73 (71.6) 15 (48.4)	29 (28.4) 16 (51.6)	1.00 2.69 (1.18-6.13)	0.0191
		Vomits	AA AG/GG	74 (81.3) 20 (62.5)	17 (18.7) 12 (37.5)	1.00 2.61 (1.07-6.35)	0.0366
GEMIN3	rs563002	Hyperbilirubinemia	TT CT/CC	73 (96.1) 37 (84.1)	3 (3.9) 7 (15.9)	1.00 4.60 (1.12-18.84)	0.0252
EIF2C1	rs595961	Mucositis	AA AG/GG	70 (87.5) 39 (100)	10 (12.5) 0 (0.0)	1.00 NE (NE-NE)	0.0294
CNOT1	rs11866002	Hepatic toxicity	CC CT/TT	38 (63.3) 50 (79.4)	22 (36.7) 13 (20.6)	1.00 0.45 (0.20-1.00)	0.0480
mir-2053	rs10505168	Mucositis	TT CT/CC	43 (82.7) 68 (95.8)	9 (17.3) 3 (4.2)	1.00 0.21 (0.05-0.82)	0.0154

Table 27. Significant associations between polymorphisms and toxicity parameters during the consolidation phase (Continuation).

Gene	SNP	Toxicity	Genotype	Absence n (%)	Presence n (%)	OR (95% CI)	p
mir-453	rs56103835	Vomits	AA	66 (83.5)	13 (16.5)	1.00	0.0141
			GA/GG	28 (63.6)	16 (36.4)	2.90 (1.23-6.82)	
		MTX clearance	AA	62 (72.1)	24 (27.9)	1.00	0.0297
			GA/GG	25 (53.2)	22 (46.8)	2.27 (1.08-4.77)	
mir-1206	rs2114358	Mucositis	AA/AG	96 (92.3)	8 (7.7)	1.00	0.0254
			GG	15 (75.0)	5 (25.0)	4.57 (1.28-16.28)	
		Diarrhea	AA/AG	98 (95.2)	5 (4.9)	1.00	0.0365
			GG	16 (80.0)	4 (20.0)	4.90 (1.49-20.21)	
mir-604	rs2368393	Renal toxicity	AA	65 (95.6)	3 (4.4)	1.00	0.0271
			AG/GG	47 (83.9)	9 (16.1)	4.15 (1.07-16.15)	
mir-1294	rs13186787	Hyperbilirubinemia	AA	110 (93.2)	8 (6.8)	1.00	0.0424
			AG	3 (60.0)	2 (40.0)	9.17 (1.33-63.01)	
mir-2110	rs17091403	Vomits	CC	84 (80.0)	21 (20.0)	1.00	0.0471
			CT	11 (57.9)	8 (42.1)	2.91 (1.04-8.14)	

ANNEX II

TABLES

Table 28. List of aberrations found in each patient.

Patient	CN State	Type	Chrom	Min	Max	Size	Start	End
LLA 1	1.0	Loss	1	107141883	108650358	1.508.475	p21.1	p13.3
	1.0	Loss	1	229507580	235866556	6.358.976	q42.13	q42.3
	1.0	Loss	5	157944613	158520217	575.604	q33.3	q33.3
	1.0	Loss	6	150814768	151073488	258.720	q25.1	q25.1
	1.0	Loss	8	128902762	129650650	747.888	q24.21	q24.21
	1.0	Loss	12	9616668	14613836	4.997.168	p13.31	p13.1
	1.0	Loss	12	46127063	46235991	108.928	q12	q12
	1.0	Loss	12	92212378	92531608	319.230	q21.33	q21.33
	1.0	Loss	14	95379498	95776681	397.183	q32.13	q32.13
	1.0	Loss	15	41251914	43101366	1.849.452	q15.1	q15.2
	1.0	Loss	22	22382520	22599075	216.555	q11.22	q11.22
	3.0	Gain	X	33978797	34640543	661.746	p21.1	p21.1
LLA2	1.0	Loss	9	5512283	41759593	36.247.310	p24.1	p12
	0.0	Loss	9	21284630	23559324	2.274.694	p21.3	p21.3
	3.0	Gain	13	28645708	28849843	204.135	q12.2	q12.2
	1.0	Loss	13	31047541	115103119	84.055.578	q12.3	q34
	1.0	Loss	20	35149977	47671494	12.521.517	q11.23	q13.13
	1.0	Loss	20	48913398	62917655	14.004.257	q13.13	q13.33
LLA15	1.0	Loss	1	225696605	249212628	23.516.023	q42.12	q44
	1.0	Loss	7	29236372	64925676	35.689.304	p14.3	q11.21
	1.0	Loss	8	172851	26058609	25.885.758	p23.3	p21.2
	1.0	Loss	12	11503086	14129569	2.626.483	p13.2	p13.1
	1.0	Loss	13	-	-	-	pter	qter
	3.0	Gain	X	123144507	155186537	32.042.030	q25	q28
LLA23	1.0	Loss	3	60103639	60372552	268.913	p14.2	p14.2
	1.0	Loss	6	87975431	151014895	63.039.464	q14.3	q25.1
	1.0	Loss	11	62810764	63213006	402.242	q12.3	q12.3
	1.0	Loss	12	10999595	16273060	5.273.465	p13.2	p12.3
	1.0	Loss	12	92140299	92531075	390.776	q21.33	q21.33
	1.0	Loss	17	75166748	76094319	927.571	q25.2	q25.3
LLA26	1.0	Loss	1	234715202	235072805	357.603	q42.3	q42.3
	1.0	Loss	3	60067283	60560026	492.743	p14.2	p14.2
	1.0	Loss	3	176925938	177351455	425.517	q26.32	q26.32
	1.0	Loss	4	56620183	56795604	175.421	q12	q12
	1.0	Loss	6	109175532	109324248	148.716	q21	q21
	1.0	loss	7	38273812	38395492	121.680	p14.1	p14.1
	1.0	loss	7	106580672	106671931	91.259	q22.3	q22.3
	1.0	Loss	9	21301749	26440331	5.138.582	p21.3	p21.2
	1.0	Loss	11	3390050	4586729	1.196.679	p15.4	p15.4
	1.0	Loss	12	31882513	32413968	531.455	p11.21	p11.21
	1.0	Loss	13	41550544	41592097	41.553	q14.11	q14.11
	1.0	Loss	13	49710170	51684822	1.974.652	q14.2	q14.3
	1.0	Loss	14	22393801	23002382	608.581	q11.2	q11.2
	1.0	Loss	14	66687438	76753362	10.065.924	q23.3	q24.3
	1.0	Loss	14	77313186	78810604	1.497.418	q24.3	q24.3
	1.0	Loss	16	67618885	67824490	205.605	q22.1	q22.1

Table 28. List of aberrations found in each patient (Continuation I).

Patient	CN State	Type	Chrom	Min	Max	Size	Start	End
LLA26	1.0	Loss	19	36874541	38533388	1.658.847	q13.12	q13.13
	1.0	Loss	22	22386244	22549168	162.924	q11.22	q11.22
LLA32	1.0	Loss	1	92547699	92657558	109.859	p22.1	p22.1
	1.0	loss	5	88189373	89429886	1.240.513	q14.3	q14.3
	1.0	loss	7	142308091	142467867	159.776	q34	q34
	1.0	Loss	12	10310373	19235772	8.925.399	p13.31	p12.3
	1.0	Loss	22	22392472	22602286	209.814	q11.22	q11.22
LLA34	3.0	Gain	1	145388014	249212628	103.824.614	q21.1	q44
	1.0	loss	12	99881976	100343680	461.704	q23.1	q23.1
LLA35	1.0	Loss	1	194589855	194697754	107.899	q31.3	q31.3
	1.0	Loss	3	43158348	49335240	6.176.892	p22.1	p21.31
	1.0	Loss	3	60065318	60678655	613.337	p14.2	p14.2
	1.0	Loss	3	63602582	106882550	43.279.968	p14.2	q13.12
	1.0	Loss	3	112063466	112216445	152.979	q13.2	q13.2
	1.0	Loss	4	103604234	103743580	139.346	q24	q24
	1.0	Loss	4	149773707	149904178	130.471	q31.23	q31.23
	1.0	Loss	4	152661995	153273320	611.325	q31.3	q31.3
	3.0	Gain	6	27708071	27808081	100.010	p22.1	p22.1
	1.0	Loss	6	132272492	132460947	188.455	q23.2	q23.2
	1.0	Loss	7	142009932	142445333	435.401	q34	q34
	1.0	Loss	8	60037057	60242344	205.287	q12.1	q12.1
	1.0	Loss	9	115251331	115417148	165.817	q32	q32
	1.0	Loss	11	68905335	69440036	534.701	q13.3	q13.3
	1.0	Loss	12	25398882	25538043	139.161	p12.1	p12.1
	1.0	Loss	12	48407719	48509640	101.921	q13.11	q13.11
	1.0	Loss	12	92267405	92533525	266.120	q21.33	q21.33
	1.0	Loss	14	22181092	23010320	829.228	q11.2	q11.2
	1.0	Loss	14	24258980	24513885	254.905	q11.2	q11.2
	1.0	Loss	14	73222960	73355261	132.301	q24.2	q24.2
	3.0	Gain	17	41426626	45180652	3.754.026	q21.31	q21.32
	1.0	Loss	17	45181435	45419478	238.043	q21.32	q21.32
	3.0	Gain	17	45420261	80726260	35.305.999	q21.32	q25.3
	1.0	Loss	18	272909	570814	297.905	p11.32	p11.32
	3.0	Gain	X	113888820	115007722	1.118.902	q23	q23
	1.0	Gain	Y	6915517	7434526	519.009	p11.2	p11.2
LLA61	3.0	Gain	4	-	-	-	pter	qter
	3.0	Gain	6	-	-	-	pter	qter
	3.0	Gain	10	-	-	-	pter	qter
	3.0	Gain	14	-	-	-	pter	qter
	3.0	Gain	17	-	-	-	pter	qter
	3.0	Gain	18	-	-	-	pter	qter
	4.0	Gain	21	-	-	-	pter	qter
	3.0	Gain	X	-	-	-	pter	qter
LLA58	-	-	-	-	-	-	-	-
LLA69	1.0	Loss	1	712576	150101236	149.388.660	p36.33	q21.2
	4.0	Gain	1	231596450	231828319	231.869	q42.2	q42.2
	1.0	Loss	2	209782263	242482696	32.700.433	q34	q37.3
	1.0	Loss	3	81668	162994210	162.912.542	p26.3	q26.1
	1.0	Loss	7	-	-	-	pter	qter
	1.0	Loss	8	172851	27380090	27.207.239	p23.3	p21.2

Table 28. List of aberrations found in each patient (Continuation II).

Patient	CN State	Type	Chrom	Min	Max	Size	Start	End
LLA69	1.0	Loss	8	-	-	-	pter	qter
	1.0	Loss	9	-	-	-	pter	qter
	1.0	Loss	13	-	-	-	pter	qter
	1.0	Loss	15	-	-	-	pter	qter
	0.0	loss	15	57200825	57400892	200.067	q21.3	q21.3
	1.0	Loss	16	-	-	-	pter	qter
	1.0	Loss	19	-	-	-	pter	qter
	1.0	Loss	20	-	-	-	pter	qter
	3.0	Gain	21	-	-	-	pter	qter
	1.0	Loss	Y	-	-	-	pter	qter
LLA78	1.0	Loss	3	112063466	112186457	122.991	q13.2	q13.2
	1.0	Loss	9	37032513	37277759	245.246	p13.2	p13.2
	1.0	Loss	12	9902498	16016902	6.114.404	p13.31	p12.3
	1.0	loss	14	22737500	23009854	272.354	q11.2	q11.2
LLA80	3.0	Gain	6	-	-	-	pter	qter
	2.0	loss	6	26117304	26330383	213.079	p22.2	p22.2
	3.0	Gain	10	-	-	-	pter	qter
	3.0	Gain	14	-	-	-	pter	qter
	3.0	Gain	17	-	-	-	pter	qter
	4.0	Gain	21	-	-	-	pter	qter
	1.0	Loss	22	22454109	22590428	136.319	q11.22	q11.22
	3.0	Gain	X	-	-	-	pter	qter
LLA85	1.0	Loss	7	142001805	142453916	452.111	q34	q34
	1.0	loss	10	111758741	111840369	81.628	q25.1	q25.1
	1.0	Loss	21	39763017	39810401	47.384	q22.2	q22.2
LLA95	1.0	Loss	5	157298744	158320101	1.021.357	q33.3	q33.3
	1.0	Loss	5	167212457	169456037	2.243.580	q34	q35.1
	1.0	Loss	7	137738379	148445034	10.706.655	q33	q36.1
	1.0	loss	10	68037703	68161439	123.736	q21.3	q21.3
	1.0	Loss	12	8853346	15745442	6.892.096	p13.31	p12.3
LLA99	1.0	Loss	3	112070473	112203411	132.938	q13.2	q13.2
	3.0	Gain	4	-	-	-	pter	qter
	3.0	Gain	8	-	-	-	pter	qter
	3.0	Gain	9	-	-	-	pter	qter
	3.0	Gain	10	-	-	-	pter	qter
	3.0	Gain	11	-	-	-	pter	qter
	3.0	Gain	14	-	-	-	pter	qter
	3.0	Gain	17	-	-	-	pter	qter
	3.0	Gain	18	-	-	-	pter	qter
	3.0	Gain	21	-	-	-	pter	qter
	2.0	Loss	21	32417732	32883549	465.817	q22.11	q22.11
	3.0	Gain	X	-	-	-	pter	qter
LLA101	1.0	Loss	4	149341755	149848318	506.563	q31.23	q31.23
	1.0	Loss	5	142792649	143113895	321.246	q31.3	q31.3
	1.0	Loss	10	111632024	111941434	309.410	q25.1	q25.2
	1.0	Loss	12	10775652	13780334	3.004.682	p13.2	p13.1
	1.0	Loss	12	46181372	47338794	1.157.422	q12	q13.11
	1.0	Loss	22	22386244	22593041	206.797	q11.22	q11.22
LLA103	3.0	Gain	1	145398010	204922780	59.524.770	q21.1	q32.1
	3.0	Gain	1	216606664	216895800	289.136	q41	q41

Table 28. List of aberrations found in each patient (Continuation III).

Patient	CN State	Type	Chrom	Min	Max	Size	Start	End
LLA103	3.0	Gain	1	217228377	217560254	331.877	q41	q41
	3.0	Gain	1	217612193	217754833	142.640	q41	q41
	3.0	Gain	1	218272370	218810685	538.315	q41	q41
	3.0	Gain	4	-	-	-	pter	qter
	3.0	Gain	5	-	-	-	pter	qter
	3.0	Gain	6	-	-	-	pter	qter
	3.0	Gain	8	-	-	-	pter	qter
	3.0	Gain	10	-	-	-	pter	qter
	3.0	Gain	11	-	-	-	pter	qter
	1.0	Loss	12	11826813	12056722	229.909	p13.2	p13.2
	3.0	Gain	14	-	-	-	pter	qter
	3.0	Gain	16	21527622	61409802	39.882.180	p12.2	q21
	3.0	Gain	17	-	-	-	pter	qter
	3.0	Gain	18	-	-	-	pter	qter
	4.0	Gain	21	-	-	-	pter	qter
	2.0	Gain	X	-	-	-	pter	qter
LLA109	3.0	Gain	1	151996653	152192164	195.511	q21.3	q21.3
	3.0	Gain	1	175487744	175607795	120.051	q25.1	q25.1
	3.0	Gain	2	179424459	179546030	121.571	q31.2	q31.2
	1.0	Loss	2	190501522	190602439	100.917	q32.2	q32.2
	3.0	Gain	3	111234582	111341501	106.919	q13.13	q13.2
	3.0	Gain	4	-	-	-	pter	qter
	1.0	Loss	5	21192776	21439841	247.065	p14.3	p14.3
	3.0	Gain	5	60884252	61107889	223.637	q12.1	q12.1
	3.0	Gain	5	107128784	107229451	100.667	q21.3	q21.3
	3.0	Gain	5	158240867	158362976	122.109	q33.3	q33.3
	3.0	Gain	6	-	-	-	pter	qter
	3.0	Gain	7	8436626	8543085	106.459	p21.3	p21.3
	3.0	Gain	9	8263437	8367269	103.832	p24.1	p24.1
	0.0	Loss	9	21428463	22483924	1.055.461	p21.3	p21.3
	3.0	Gain	9	127926063	128041949	115.886	q33.3	q33.3
	3.0	Gain	10	-	-	-	pter	qter
	3.0	Gain	11	94493296	94601293	107.997	q21	q21
	3.0	Gain	14	-	-	-	pter	qter
	3.0	Gain	17	-	-	-	pter	qter
	3.0	Gain	18	-	-	-	pter	qter
LLA107	3.0	Gain	19	18338457	18479647	141.190	p13.11	p13.11
	3.0	Gain	21	-	-	-	pter	qter
	3.0	Gain	22	27381388	27494460	113.072	q12.1	q12.1
	3.0	Gain	X	-	-	-	pter	qter
	3.0	Gain	3	152295700	197870805	45.575.105	q25.33	q26.31
	1.0	Loss	8	60035097	60244524	209.427	q12.1	q12.1
	1.0	Loss	9	209111	36943396	36.734.285	p24.3	p13.2
	1.0	Loss	17	64214	20115378	20.051.164	p13.3	p13.1
LLA119	3.0	Gain	4	-	-	-	pter	qter
	3.0	Gain	6	-	-	-	pter	qter
	3.0	Gain	8	-	-	-	pter	qter
	1.0	Loss	9	21105602	22763714	1.658.112	p21.3	p21.3
	3.0	Gain	10	-	-	-	pter	qter
	3.0	Gain	14	-	-	-	pter	qter

Table 28. List of aberrations found in each patient (Continuation III).

Patient	CN State	Type	Chrom	Min	Max	Size	Start	End
LLA119	3.0	Gain	17	-	-	-	pter	qter
	2.0	loss	17	39371073	48801533	9.430.460	q21.2	q21.33
	3.0	Gain	18	-	-	-	pter	qter
	4.0	Gain	21	-	-	-	pter	qter
	2.0	Gain	X	-	-	-	pter	qter
	2.0	Gain	Y	-	-	-	pter	qter
LLA133	3.0	Gain	4	-	-	-	pter	qter
	3.0	Gain	6	-	-	-	pter	qter
	3.0	Gain	8	-	-	-	pter	qter
	3.0	Gain	10	-	-	-	pter	qter
	3.0	Gain	14	-	-	-	pter	qter
	3.0	Gain	18	-	-	-	pter	qter
	3.0	Gain	21	-	-	-	pter	qter
	1.0	Loss	22	22395113	22518006	122.893	q11.22	q11.22
	3.0	Gain	X	-	-	-	pter	qter
LLA147	1.0	loss	1	29645850	29792290	146.440	p35.3	p35.3
	1.0	loss	1	115865352	116130012	264.660	p13.2	p13.1
	1.0	Loss	3	153971601	155642789	1.671.188	q25.2	q25.31
	1.0	Loss	3	175950018	178147264	2.197.246	q26.32	q26.32
	1.0	Loss	4	152862748	153021068	158.320	q31.3	q31.3
	1.0	Loss	7	149629657	150665665	1.036.008	q36.1	q36.1
	1.0	Loss	10	52117579	63326624	11.209.045	q11.23	q21.2
	1.0	Loss	12	1595206	3396447	1.801.241	p13.33	p13.32
	1.0	Loss	12	9019848	19657112	10.637.264	p13.31	p12.3
	1.0	Loss	12	41183159	41562567	379.408	q12	q12
	1.0	Loss	12	42946669	51474483	8.527.814	q12	q13.12
	1.0	Loss	12	97430486	108401784	10.971.298	q23.1	q23.3
	1.0	Loss	22	22382520	22518122	135.602	q11.22	q11.22

Table 29. List of SNPs excluded from the MTX transport pathway study due to genotyping failure

SNP ID	Gene	Position	Alleles
rs193538	*ABCC1*	intron 6	T>G
rs35625	*ABCC1*	intron 14	T>C
rs4780591	*ABCC1*	intron 21	G>C
rs2299670	*ABCC1*	intron 26	A>G
rs129081	*ABCC1*	3'UTR	G>C
rs4382961	*SLCO1B3*	intron 2	G>A
rs7311358	*SLCO1B3*	exon 6	A>G
rs2417940	*SLCO1B3*	intron 6	G>A
rs11513411	*SLCO1B1*	intron 2	G>A
rs4149034	*SLCO1B1*	intron 2	G>A
rs7136445	*SLCO1B1*	intron 2	A>G
rs4149061	*SLCO1B1*	intron 8	T>C
rs11045878	*SLCO1B1*	intron 14	A>G
rs11045885	*SLCO1B1*	intron 14	A>G
rs11045891	*SLCO1B1*	3'	A>C
rs11045918	*SLCO1A2*	3'UTR	C>A
rs7301895	*SLCO1A2*	intron 2	C>T
rs7964783	*SLCO1A2*	intron 1	A>G
rs10770804	*SLCO1A2*	intron 1	A>G
rs3753019	*SLC19A1*	downstream	C>T
rs1888530	*SLC19A1*	intron 5	C>T
rs3788189	*SLC19A1*	intron 5	C>T
rs4148412	*ABCC3*	intron 2	C>T
rs4148413	*ABCC3*	intron 8	C>G
rs8075406	*ABCC3*	intron 17	T>A
rs10260862	*ABCB1*	intron 5	G>C
rs2214102	*ABCB1*	intron 2	G>A
rs2869732	*ABCG2*	intron 2	A>G
rs2622625	*ABCG2*	intron 1	G>A
rs9516521	*ABCC4*	3' UTR	T>C
rs1751050	*ABCC4*	intron 22	C>G
rs1751059	*ABCC4*	intron 20	C>G
rs1751064	*ABCC4*	intron 19	G>A
rs1678396	*ABCC4*	intron 19	T>C
rs2009772	*ABCC4*	intron 13	T>C
rs12584649	*ABCC4*	intron 1	T>C
rs2993590	*ABCC4*	upstream	T>C
Rs7906080	*ABCC2*	intron 2	A>G
rs4148386	*ABCC2*	intron 2	G>A
rs9794323	*ABCC2*	intron 19	T>C
rs11190297	*ABCC2*	downstream	G>T

Table 30. SNPs excluded from the miRNAs pathway association study.

SNP ID	Gene	Alleles	Reason for exclusion
rs11738060	*CNOT6*	T>A	Genotyping failure
rs2368392	mir-604	C>T	Genotyping failure
rs318039	mir-1274a	C>T	Genotyping failure
rs34610323	*GEMIN4*	C>T	Genotyping failure
rs72631826	mir-16-1	T>C	Genotyping failure
rs493760	*RNASEN*	T>C	Genotyping failure
rs73239138	mir-1269	G>A	Genotyping failure
rs72631825	mir-222	G>A	Genotyping failure
rs12197631	mir-548a-1	T>G	Genotyping failure
rs11014002	mir-603	C>T	Genotyping failure
rs11061209	*RAN*	G>A	Genotyping failure
rs1003226	*CNOT4*	T>C	Genotyping failure
rs11156654	mir-624	T>A	No HWE
rs174561	mir-1908	T>C	No HWE
rs2292832	mir-149	C>T	No HWE
rs2413621	*TNRC6B*	T>C	No HWE
rs3742330	*DICER1*	A>G	No HWE
rs3757	*DGCR8*	G>A	No HWE
rs42318	*CNOT3*	G>A	No HWE
rs470113	*TNRC6B*	A>G	No HWE
rs4919510	*mir-608*	C>G	No HWE
rs55656741	*RNASEN*	G>A	No HWE
rs7719666	*RNASEN*	C>T	No HWE
rs7813	*GEMIN4*	C>T	No HWE
rs816736	*GEMIN5*	T>C	No HWE
rs910924	*GEMIN4*	C>T	No HWE

DISCUSSION

DISCUSSION

In this study our main goal was to achieve a more personalized therapy. For this, we aimed to identify new genetic markers in order to recognize individuals who tolerate the treatment better and more aggressive tumors that require more intensive treatment. This will enable to make the treatment of children with Acute Lymphoblastic Leukemia more safe and effective. To that end, we conducted on the one hand, a pharmacogenetic study of germline polymorphisms to determine their role in treatment toxicity and, on the other hand, a cytogenetic analysis of copy number aberrations in the tumor cells for a better characterization of risk groups and treatment adjustment.

GENETIC ALTERATIONS IN THE TUMORAL CELLS AND THEIR IMPLICATION IN PROGNOSIS

First of all, in this study we wanted to identify novel deletions and duplications, cryptic for the traditional cytogenetic techniques, present in the tumoral cells that could allow a better risk-group classification. With this aim, we analyzed DNA samples from 23 patients diagnosed with B-ALL from the different risk groups with Affymetrix Cytogenetics Whole-Genome 2.7M Array. We detected a high number of genomic abnormalities per case, including recurrent aberrations that could contribute to the differentiation between standard risk and high risk groups. We also detected alterations (1q21.3 and 1q25.1 gain, 5q33.3 gain or loss, 10q25.1-q25.2 and 12q12 loss) that could allow a better risk group characterization as they distinguish standard-risk patients who remain in this

group from those who were changed to high-risk and, consequently, should have been treated as high-risk from the beginning. We did not find any recurrent alteration to differentiate the high-risk patients who remain in this risk group from patients switching to very-high-risk.

In order to reach those results, it was necessary to differentiate between tumoral aberrations and polymorphisms, for which we had the paired diagnosis and remission samples. All the copy number variations detected in both the diagnosis (tumoral) and remission (normal) samples were considered polymorphisms of the general population and were not further analyzed in this study. This is an advantage in comparison with other works in which they used DNA from controls as reference [243-249], as we can be completely sure about which copy number alterations have taken place in the tumoral cells.

In total, in this study, we have detected 223 alterations, with an average of 9.7 genomic abnormalities per case. Losses outnumbered gains. This number is higher than in other previous studies [39,250]. This can be due to the fact that the array platform we have used has an increased resolution and number of markers (2.7 million markers across the genome) compared with other platforms (usually presenting less than 1 million markers), which gives our study a higher power to detect copy number aberrations.

Among the recurrent aberrations we have detected, some were present in patients from different risk groups. These aberrations included the loss at 1q42.3 that included the *IRF2BP2* gene among others; the loss at 3q13.2, which includes *CD200* and *BTLA* genes; loss at 3q26.32; loss at 3p14.2, affecting *FHIT* gene; loss at 7q34, including T cell receptor cluster (*TRB@*); loss at 8q12.1; loss

at 8p; loss at 9p21.3, including *CDKN2A* and *CDKN2B*; loss of the *ETV6* gene at 12p13.2; Loss at 14q11.2, including *PIP4K2A* gene; loss at 17q21.32, including *CDC27* gene among others; and loss at 22q11.22, that includes the immunoglobulin lambda locus (*IGL@*). Some of these alterations might be associated with the leukemic process.

In fact, *CD200/BTLA, FHIT, CDKN2A/B* and *ETV6* are known genomic hallmarks detected in previous single nucleotide polymorphism-array studies of pediatric acute lymphoblastic leukemia [39,243,250-269]. *CD200* and *BTLA*, lost in 3 cases, belong to the immunoglobulin superfamily and regulate immune response [270,271]. *FHIT*, lost in 3 cases, is a tumor suppressor gene involved in apoptosis [272]. *CDKN2A* and *B*, lost in 4 cases, are other known tumor suppressor genes that stabilize p53 [273]. *ETV6*, deleted in 9 cases, is a transcription factor needed for hematopoiesis [274]. The role of all these genes in cell proliferation and differentiation and immune response regulation can explain why alterations involving them are recurrent in ALL.

On the other hand, deletions at the *IGL* (7 cases) and *TRB* (4 cases) loci are more likely to be related to the clonal origin of the leukemic cells than to the leukemic process in itself. Deletions at the *IGL* locus can be the result of clonal DJ rearrangements of the immunoglobulin genes, seen in nearly all cases of B-ALL [1], while deletions in the *TRB* cluster can be the result of T-cell receptor gene rearrangements, which are seen in a significant proportion of cases (up to 70%) [1]. In the normal lymphocytes, these rearrangements and deletions occur in different places in each of the cells. Consequently, these alterations will not be present in the bulk of normal cells and they will only be detected in the array in

the tumoral tissue, despite not being an alteration that occurs during the leukemic process.

Finally, other genes included in the genetic aberrations we have detected, *IRF2BP2, PIP4K2A and CDC27*, could be new ALL markers. Some of these genes have interesting functions. *IRF2BP2*, which is included in a deletion we have observed in 3 patients, is a transcriptional repressor of NFAT1, a DNA-binding protein that induces gene transcription in a wide range of cell types and tissues, including during the immune response [275]. It is also a repressor of IRF2, a member of the interferon regulatory transcription factor family [276], which is known to positively influence cell growth [277]. As a transcriptional repressor of these or even other unknown targets, the deletion of this gene could induce cell growth and proliferation, which could be important in the development of ALL. PIP4K2A, the precursor to second messengers of the phosphoinositide signal transduction pathways, which was deleted in 3 cases, is thought to be involved in the regulation of secretion, cell proliferation, differentiation and motility, and has been associated with breast cancer outcome [278]. If this gene has a role in cell proliferation and differentiation, its deletion could be an important step in order to deregulate these processes and promote ALL. *CDC27*, which was lost in 2 patients, is a component of anaphase-promoting complex (APC). This protein was shown to interact with mitotic checkpoint proteins including Mad2, p55CDC and BUBR1, and thus may be involved in controlling the timing of mitosis [279]. The deletion of this gene could lead to a deregulation in the mitotic process, which could lead to increased proliferation, which could explain its putative role in ALL.

In our study, some aberrations were only found in patients with the TEL-AML1 translocation, independent of their risk group. Those included the losses at 4q31.23 (2 cases), 4q31.3 (2 cases) and 12q21.33 (3 cases) in regions that included no genes. These could represent changes recurrent in the TEL-AML1 leukemia process. In previous reports, other authors have proposed that the nature and frequency of individual lesions varies significantly between B-ALL subtypes [7]. This suggests that the different first hallmarks need different secondary changes in order to lead to ALL development.

An important result of our study is the fact that we have detected recurrent aberrations that could contribute to distinguish the standard risk (loss at 7p14.1, that includes the *TRG* locus, and the loss at 14q24.2, that affects the *DPF3* gene) and high risk patients (12q23.1 loss affecting *ANKS1B* gene). Among these, *DPF3*, deleted in 2 standard risk patients, functions in association with the BAF chromatin remodeling complex to initiate gene transcription [280]. Consequently, alterations in this gene could affect the expression of a wide range of genes, including those involved in proliferation, cell death or drug resistance and could have a role in ALL prognosis. On the other hand, *ANKS1B*, lost in 2 high risk patients, encodes a multi-domain and multi-functional protein that that has been also associated with microtubules function [281]. The expression of this gene has been shown to be elevated in patients with pre-B cell acute lymphocytic leukemia associated with t(1;19) translocation [282], which, along with our result, suggests that the deregulation of this protein, possibly through its role in microtubule stability which could affect mitosis and promote proliferation, may have role in ALL and in the aggressiveness and resistance of the disease. These alterations could be new genetic markers with a potential to implement or

substitute other markers, such as karyotyping that is quite often failed, for risk group classification.

Another remarkable result is the detection of alterations that distinguish standard-risk patients who remain in this group (1-1) from those who were changed to high-risk (1-2). These aberrations included the gain at 1q21.3, including *S100A11* gene among others; the gain at 1q25.1 that includes only the *TNR* gene; the loss or gain at 5q33.3 affecting *EBF1* gene; loss at 10q25.1-q25.2, including *ADD3* gene; and loss at 12q12, which contains *ARID2* gene.

The most known of these genes is *EBF1*, which was altered in a 1-2 patient and two high risk patients. It is critical for commitment to the B cell lineage and early development [283] and is a common target of alteration in ALL [7,258,263,284]. It has been described that ALL patients with BCR-ABL-like expression, which have a bad prognosis, high risk patients and relapses often had deletions in B-cell differentiation genes including *EBF1* [284-286], suggesting a possible contribution for the development of relapse and a potential prognostic value. Accordingly, our results suggest an association of the alterations in this gene and bad prognosis. We have additionally seen that they could also help distinguishing those patients that have been incorrectly assigned to the standard risk group and should have been included in high risk. *S100A11*, which was gained in a 1-2 risk patient and 2 high risk patients, may function in motility, invasion, and tubulin polymerization. Chromosomal rearrangements and altered expression of this gene have been implicated in tumor metastasis [287]. In this case, its role in tubuline polymerization could affect the mitotic and proliferation process, which could explain a putative role in ALL prognosis. Tenascin-R (*TNR*) , which was also gained in a 1-2 patient and 2 high risk patients, is an extracellular

matrix protein expressed primarily in the central nervous system [288]. In this case, it is difficult to explain the association of this gene and ALL risk stratification, but it could have another unknown function in lymphoblasts or the region in which it is located might have a regulatory function in ALL. The same can be said about *ADD3*, which was lost in a 1-2 patient and a high risk patient and belongs to a family of membrane skeletal proteins involved in the assembly of spectrin-actin network in erythrocytes and at sites of cell-cell contact in epithelial tissues [289]. *ARID2*, which was lost in a 1-2 patient and 2 high risk patients, is a subunit of the BAF chromatin-remodeling complex, which facilitates ligand-dependent transcriptional activation by nuclear receptors and is a new tumor suppressor gene in hepatocellular carcinoma [290]. Due to its role in transcriptional activation, it could also act as a tumor suppressor in ALL and its deletion could be associated with a worse prognosis. Our results suggest that some of the alterations found in these regions could be new markers that could be used in order to improve risk group stratification.

On the other hand, we did not find any recurrent alteration to differentiate the high-risk patients who remain in this risk group from patients switching to very-high-risk. This could be due to the low number of patients in the 2-3 category (n=2) and, especially, in the very high risk group (n=1), which make difficult to find recurrent patterns of alteration.

In conclusion, the results of this pilot study suggest that risk groups classification could be improved in patients with pediatric B-ALL through the analysis of new genetic markers. Loss at 7p14.1 and 12q23.1 could implement risk group stratification and others such as 1q21.3 and 1q25.1 gain, 5q33.3 gain or loss, 10q25.1-q25.2 and 12q12 loss could improve this classification. It would be

really interesting to analyze the utility of these alterations for a better risk group stratification in larger populations.

GENETIC VARIANTS IN THE GERMINAL LINE AND THEIR IMPLICATION IN TREATMENT TOXICITY

In order to select markers to predict the toxic effect of LAL/SHOP therapy, in this study, we evaluated the influence of polymorphisms in key genes on toxicity in a group of children diagnosed with B-ALL and treated according to the standardized LAL/SHOP protocol.

In the last years, several authors have carried out studies in order to search pharmacogenetic markers of toxicity in pediatric ALL. As methotrexate and 6-mercaptopurine are the backbone of pediatric ALL therapy, there has been a great interest in analyzing polymorphisms in the genes involved in their metabolic and transport pathways. In addition, as ALL patients are treated with complex multidrug regimens, polymorphisms in genes encoding enzymes affecting the clearance of several drugs have also been studied.

However, the results are usually controversial. This lack of replication could be due to small or non-homogeneous populations, differences in treatment protocols among studies or the toxicity criteria used.

Among the strengths of our retrospective study was avoiding the problems observed in previous studies. In our study we have worked with a large population, a homogeneous diagnostic of B-ALL, a standardized treatment

protocol, LAL/SHOP, followed by all patients, and objective and well recorded data.

Drug detoxifying enzymes

In the group of drug detoxifying enzymes, we did not find any statistically significant association between the 5 polymorphisms analyzed in *GSTM1*, *GSTT1*, *GSTP1*, *CYP1A1* and *NQO1* and toxicity in induction or consolidation.

Previous works had reported some controversial associations between polymorphisms in drug detoxifying enzymes and toxicity. Homozygous deletion of *GSTM1* has been previously related to decreased hepatotoxicity [68,82], but in other studies they did not find any association with this parameter [73,100]. By contrast, the same genotype has been associated with increased hyperbilirubinemia[98] and severe infections [95], associations which were not confirmed in another study [82]. *GSTT1* deletion has been associated with increased hyperbilirubinemia [82], although this association was not found in another study which reported an association with increased gastrointestinal toxicity [73]. Finally, GSTP1 GG genotype was associated with central nervous system toxicity [73] but was not associated with this parameter in another study [82] in pediatric ALL patients.

In summary, our results and the controversy observed in previous reports do not point to a clear role of these polymorphisms in toxicity in pediatric ALL.

6-mercaptopurine pathway

In the 6-mercaptopurine pathway, we did not find any patient with the *TPMT*-deficient homozygous genotype and we did not find any significant association between *TPMT* heterozygous genotype and any of the toxicity parameters studied in the consolidation phase.

It has been described that *TPMT* deficient homozygous patients are in great risk of toxicity and need 6-mercaptopurine dose reduction. However, in our group we did not find any patient with this genotype, which was expectable due to the low frequency of this genotype in the population (1/300). Consequently, we could not assess this effect.

Whether patients with one functional allele would benefit from dose reduction is less clear. Some authors find association between *TPMT* heterozygosity and increased toxicity [114,120,127,128,130,291] while others don't [72,85,119,122,123,126,129]. It has been proposed that heterozygous *TPMT* patients treated at conventional doses (75 mg/m2 per day) are at higher risk of hematopoietic toxicity [114], while those treated with lower doses (60mg/m2 per day)[85] do not exhibit a higher rate of hematopoietic toxicity and would therefore not be expected to benefit from dose reduction [46]. In our study, patients are treated with the LAL/SHOP protocol, in which patients are treated with 30 mg/m2 per day in consolidation and 60mg/m2 per day in maintenance. This would be in agreement with that statement as these would fit among the lower doses and we did not find any association with toxicity.

MTX pathway

MTX is very important in pediatric ALL therapy. It is given in all the phases of treatment, including high doses in the consolidation phase. However, it is also a drug known for its toxicity. In some patients, the toxic effects are so severe that the dose must be reduced or the treatment stopped, which can reduce the survival. For drugs such as MTX, with a very narrow therapeutic index, every effort should be made to minimize interpatient variability in drug exposure in order to maximize the benefit while keeping the risk of serious adverse effects at an acceptable level.

Using MTX plasma levels as an objective and quantifiable toxicity marker, we found a statistically significant association with the *SLCO1B1* rs11045879 CC homozygous risk genotype and we did not find any association with polymorphisms in *MTHFR*, *SHMT1, TS, ABCB1, ABCG2 and RFC1* genes. When we analyzed in depth the MTX transporters pathway, in which *SLCO1B1* is included, we found stronger associations between MTX toxicity and *ABCC2* and *ABCC4* SNPs.

Several studies have investigated the relationship between genetic variation and MTX-related toxicity with controversial results. One of the problems we face when we want to compare studies is the use of non-objective and non-easily quantifiable toxicity criteria, whose determination may vary among clinicians.

We have used the MTX plasma levels as a good and objective MTX-related toxicity marker. We assessed that MTX plasma levels were strongly linked to the development of global toxicity. In fact, 70.6% of the patients with high MTX

plasma levels 72h after drug infusion developed toxicity, while only 39.7% of the patients with low MTX plasma levels showed toxicity events (OR= 3.64; p= 0.004). Other authors also used this parameter in their studies [66,68,73,101]. One of the advantages of using MTX plasma concentration as toxicity marker is that it can be directly associated with methotrexate, and, if needed, treatment adjustments could be performed in the future in a specific way. Another advantage of using this parameter is its availability in the patients' files and the fact that we can avoid the lack of homogeneity in the toxicity data collection, as it is an objectively quantifiable data.

In recent years, the relationship between genetic polymorphisms and MTX toxicity has been a controversial topic in childhood ALL. In our study, we selected polymorphisms of several genes from the MTX metabolism with functional effect and whose implication in ALL toxicity had been previously suggested by other authors and still are under discussion: *MTHFR*, *SHMT1*, *TS*, *ABCB1*, *ABCG2*, *RFC1*, and *SLCO1B1*.

In our study, we found a significant association between the *SLCO1B1* rs11045879 CC genotype and increased MTX plasma levels. We did not find any association between *MTHFR*, *SHMT1*, *TS*, *ABCB1*, *ABCG2* and *RFC1* polymorphisms and MTX toxicity.

With our results, we conclude that the polymorphisms analyzed in *MTHFR*, *TS*, *SHMT1*, *ABCB1*, *ABCG2* and *RFC1* are not useful markers for toxicity in pediatric ALL. Previous associations found in the literature could be due to the analysis of small or non-homogeneous samples, the use of non objective toxicity markers or differences in the protocol of treatment.

For instance, the *MTHFR* 677T and A1298C alleles encode MTHFR proteins with decreased enzymatic activity. People with the *MTHFR* 677CT and 677TT genotype exhibit 60% and 30%, respectively, of the normal MTHFR activity [45,292]. The *MTHFR* A1298C polymorphism is responsible for a milder decrease in MTHFR activity, with 1298CC homozygous individuals having 60% of the normal activity [293]. These polymorphisms have been proposed as putative markers of increased toxicity for MTX dose individualization. In our study, we did not find any significant association between *MTHFR* polymorphisms and MTX plasma levels. In fact, we observed a tendency towards lower MTX plasma levels in the group of patients with the CC genotype that did not reach statistical significance (p= 0.06). In addition, the results published by other authors are controversial. In this context, we decided to perform a review and meta-analysis to assess its role in MTX toxicity.

We identified 24 studies which investigated *MTHFR* C677T SNP and MTX related toxicity in pediatric ALL patients and 16 of these also studied the A1298C polymorphism. We categorized the 24 studies in 3 groups according to the level of association between *MTHFR* SNPs and MTX toxicity: those that found no association, those that found an association between the *MTHFR* polymorphism analyzed and a significant increase in toxicity, and those that found an association between the polymorphism and a significant decrease in toxicity.

We could not see that any ethnicity were overrepresented in any of the 3 groups of studies. Studies with both European and Asiatic populations could be found in all the groups. Additionally, the relationship with toxicity was not dependent of MTX dosage, as both high and low doses of MTX were found in all three study groupings.

We could also see that different studies analyzed toxicity according to different criteria. That is why we performed an in-depth analysis for each toxicity criterion. We could observe that the associations found were usually with different toxicity criteria and that only a few associations (increased mucositis and *MTHFR* 677T and increased hepatic toxicity and thrombocytopenia and *MTHFR* C677T and A1298C), were found in at least 2 studies. Even in these cases, the studies that found an association were a minority in comparison to the studies that did not find any association or found the opposite.

For example, we found 16 studies that analyzed *MTHFR* C677T polymorphism in association with hepatic toxicity. Three of them found an association between the 677TT genotype and increased hepatic toxicity [77,78,82]. However, two of these studies do not have a very high statistical power, and the other 13 studies that analyzed this parameter found no association between 677TT genotype and hepatic toxicity [68,72,76,79,81,83,88,96,97,100-103]. Therefore we conclude that the 677TT genotype does not appear to be a good predictor of hepatic toxicity in response to MTX treatment for ALL.

When we performed the meta-analyses with the available data, we could not find any significant association except for a slight protective effect of the *MTHFR* 1298CC genotype for leucopenia

According to the published data analyzed above and the meta-analysis we have performed, the 677T and 1298C alleles do not seem to be good MTX toxicity markers in pediatric ALL patients. If anything, the 1298C allele seems to be more likely a protective factor rather than a toxicity marker. These results combined with the fact that works that could not be included in the meta-analyses due to

lack of data, are, in general, those which found no association with toxicity, we conclude that there is no evidence to support the use of either the *MTHFR* C677T or the A1298C SNP as MTX toxicity markers.

Taking into account these results and the results from a recent study reporting that patients receiving higher doses of MTX have better survival [79], patients might benefit from higher MTX doses in spite of their *MTHFR* genotype.

On the other hand, another interesting result in our study was that all the patients with the *SLCO1B1* rs11045879 CC genotype had high MTX plasma concentrations (p=0.008 at 72 h following MTX infusion). When we corrected for multiple comparisons, the statistical significance was lost, which was expected due to the frequency of the risk genotype, although we must emphasize that the p-value (p= 0.08) is near the significance level. Also, the rs4149081 AA genotype was always associated with high MTX plasma concentrations, although this association did not reach statistical significance (p=0.057). In addition, the 3 individuals with the rs11045879 CC genotype and the 2 patients with the rs4149081 AA genotype developed toxicity during the consolidation therapy. Both SNPs, rs4149081 and rs1104579, are in linkage disequilibrium. Consequently, the fact that both SNPs are associated with toxicity suggests the implication of these SNPs or other SNPs in the same linkage block in MTX clearance and an important role for SLCO1B1 in MTX toxicity.

In a recent work, using a genome-wide approach, the polymorphisms rs4149081 and rs11045879 of *SLCO1B1* gene have been strongly associated with MTX clearance [66], being the first time that this transporter was proposed as a candidate gene in clinical pharmacogenetic studies of MTX. Now, our findings

confirm the association found by Treviño and colleagues between *SLCO1B1* and MTX plasma levels, suggesting that *SLCO1B1* polymorphisms may influence methotrexate-related toxicity in pediatric ALL.

The relationship between SLCO1B1 and MTX clearance can be understood if we consider its function. SLCO1B1 is localized at the sinusoidal membrane of hepatocytes, and its transcript has been detected in enterocytes. SLCO1B1 mediates uptake of substrates from sinusoidal blood, resulting in their excretion, likely via biliary excretion [66]. Moreover, SLCO1B1 has been shown to transport methotrexate *in vitro* [294] and, by using a transgenic mouse model, SLCO1B1 has also shown to be an important transporter for MTX in vivo, with a rate-limiting role for plasma elimination [295]. This supports a putative role of genetic polymorphisms in SLCO1B1 on plasma pharmacokinetics of MTX in ALL patients.

In summary, identifying the rs4149081 and rs11045879 *SLCO1B1* polymorphisms in children with ALL could be a useful tool for monitoring patients at risk of low methotrexate clearance in order to avoid MTX-related toxicity. However, further studies with larger populations would be necessary.

In this context, we thought that it would be also of great interest to study the implication of other polymorphisms in *SLCO1B1* and other related genes in MTX toxicity.

However, there are not studies that analyze in depth polymorphisms in the genes involved in MTX transport and toxicity. That is the reason why we decided to evaluate the correlation of 384 polymorphisms in 12 key genes involved in

the MTX transport pathway with toxicity in a larger group of 151 children diagnosed with B-ALL and treated according to the standardized LAL/SHOP protocol. This way, we have assessed the most important genes involved in the MTX transport in order to detect novel markers that could play a role in the interindividual differences observed in MTX toxicity in pediatric ALL patients.

We have found significant association with MTX toxicity for 21 polymorphisms ($p<0.05$) from 7 genes. The association between rs11045879 in *SLCO1B1* and MTX clearance still remains in both the univariate and the multivariate analysis but we have found other stronger matches. It should be noted that, from those 21 significant polymorphisms, 6 were located in *ABCC4* and 4 in *ABCC2*, which represents half of the significant SNPs. When we applied the FDR correction, 2 polymorphisms (rs9516519 and rs3740065) in those 2 genes (*ABCC4* and *ABCC2*) remained statistically significant. When haplotypes were analyzed, we found 15 significant and, after p correction, haplotype GCGGG in *ABCC2* remained statistically significant ($p= 0.0360$). All these results point to a relevant role of *ABCC4* and *ABCC2* polymorphisms in MTX toxicity in pediatric ALL.

Although we chose MTX clearance due to its direct linkage to MTX and because it is an objective and quantifiable toxicity parameter, we also analyzed other toxicity parameters, such as renal toxicity, hepatic toxicity or mucositis. However, the associations were not so clear. This may be due to the reduced number of patients in some of the categories. In fact, 5 SNPs of our subset of significant polymorphisms (rs3740065, rs2619312, rs1678392, rs2622621, rs4149035) were slightly associated with renal toxicity. As renal toxicity is not very frequent (9.4%), with a larger population this association might have been more evident. On the other hand, there could also be a masking effect due to

the other drugs that are given and that could also be the cause of these toxicities. That is why they are not as suitable MTX toxicity parameters as MTX clearance.

The SNP rs9516519 in *ABCC4*, that showed the stronger association with MTX toxicity in our study ($p=3x10^{-4}$), is located in a putative microRNA mir-367 binding site. The G allele, which is associated with a decrease in toxicity, disrupts the putative binding site. Consequently, the loss of a miRNA binding site could explain an increased ABCC4 function and the consequent decrease in MTX toxicity. From the other 5 SNPs in *ABCC4* with a milder association with MTX toxicity, rs2619312, rs1678392, rs10219913 and rs7317112 are located in putative intronic enhancers and CpG sites, that could carry changes in the methylation pattern and could affect *ABCC4* expression [296], and rs9302061 is an upstream tag-SNP.

As far as we know, none of the associated polymorphisms had been included before in pharmacogenetic studies. Even in the GWAS study that has been carried out [66] using theAffymetrix 500K platform, these polymorphisms are not well represented. This could explain the fact that there have been no matches until now. Anyway, different studies have reported associations between *ABCC4* polymorphisms and toxicity of different drugs such as cyclophosphamide [193], bisphosphonate [297] or thiopurines [298]. This gives strength to the idea that polymorphisms in this gene can affect its ability to eliminate its substrates. In the only study that analyzed *ABCC4* polymorphisms in pediatric ALL up to now, Ansari et al. studied only 4 *ABCC4* polymorphisms and found an association between the TC genotype in the upstream polymorphism rs868853 and decreased MTX plasma levels [192]. Although, we did not replicate this association,

the cumulative evidence contributes to the idea that polymorphisms in *ABCC4* can be new predictors of MTX toxicity.

ABCC4 encodes multidrug resistance protein 4 (MRP4), a member of the ATP-binding cassette family of membrane transporters involved in the efflux of endogenous and xenobiotic molecules [299]. Among others, MRP4 is able to transport folates and MTX. Due to its ability to pump MTX out, MRP4 has been described as a resistance factor for this drug in *in vitro* experiments with *ABCC4*-transfected cells [300,301]. Consequently, we could expect that if *ABCC4* is more expressed or active, we would have a higher resistance to MTX and less toxicity.

The SNP rs3740065 in *ABCC2*, associated with MTX toxicity in our study ($p=2x10^{-3}$), is located in a putative intronic enhancer. The C allele, which increases MTX toxicity, creates a putative cap signal for transcription initiation in intron 29. This polymorphism has been previously associated with gastrointestinal MTX toxicity in rheumatoid arthritis patients [189]. The rs717620 polymorphism is located in the 5'UTR region and is also associated with MTX toxicity in our study. Rau et al, the only authors that have studied the association between 4 *ABCC2* polymorphisms and toxicity in a small population of ALL patients, also found an association between rs717620 polymorphism and MTX clearance [176]. Other studies have also reported association between this polymorphism and toxicity produced by other drugs [165,167,168,171,175]. Regarding the other SNPs in *ABCC2* associated with MTX toxicity in our study, rs3740066 is a synonimous SNP and has already been associated with toxicity by other drugs [165,178,186] and rs12826 is a downstream regulatory polymorphism that, as far as we know, had not been studied before.

In our study we also found an association between the GCGGG haplotype (rs3740066; rs3740065; rs12826; rs12762549; rs11190298) in *ABCC2* and MTX toxicity. In this risk haplotype, the risk alleles of rs3740066, rs3740065 and rs12826, which were previously associated in the individual analysis, were included. Consequently, the involvement of these SNPs on the risk of developing MTX toxicity was strengthened by the haplotype association analysis.

ABCC2 encodes multidrug resistance protein 2 (MRP2), another member of the ATP-binding cassette family. MRP2 is primarily expressed in the body at critical sites of uptake and elimination, including liver canalicular membranes and kidney proximal tubules. The apical subcellular localization of MRP2 at these sites implicates the pump in hepatobiliary and urinary elimination. The substrate selectivity of MRP2 includes a range of anticancer agents, including MTX [302,303]. A dose-dependent role for MRP2 in the disposition of MTX was suggested by experiments showing increased drug levels in plasma and in the contents of the small intestine when MTX was administered to Mrp2−/− mice at high, but not low, concentrations. This indicates that MRP2 is involved in the elimination of MTX as a result of its function in liver canaliculi and/or intestine [300].

In conclusion, this study identified mainly two significant polymorphisms and one haplotype in two MTX transporter genes, *ABCC4* and *ABCC2*, associated with MTX clearance in pediatric ALL patients. We have provided additional insight into the possible genetic modulation of treatment responses in childhood ALL. Identifying these polymorphisms in children with ALL could be a useful tool for monitoring patients at risk of low-MTX clearance in order to

avoid MTX-related toxicity. Further functional analysis and replication in independent cohort are needed to support the validity of this pilot study.

Polymorphisms in microRNAs and microRNAs biogenesis machinery in drug toxicity

It has been proposed that miRNA-related SNPs interfering with miRNA function may lead to drug resistance or to drug sensitivity [151]. For instance, a SNP 829C>T near the miR-24 binding site in the 3'UTR of *DHFR* has been shown to alter miR-24 function and increase DHFR expression and MTX resistance [152]. In our study, in the MTX transport pathway we observed that the polymorphism with the strongest association with MTX toxicity in *ABCC4* created a new miRNA binding site. However, there are very few studies analyzing the role of polymorphisms in miRNAs and miRNA biogenesis genes and, until now, none of them have been carried out in pediatric ALL. That is the reason why we decided to broaden our study and analyze polymorphisms in pre-miRNAs and in miRNAs biogenesis pathway.

We found 30 statistically significant associations with toxicity during the induction phase of treatment (16 were located in processing genes and 14 in pre-miRNAs). We also found 31 statistically significant associations and toxicity during the consolidation phase (23 in processing genes and 8 in pre-miRNAs). Of these, the association between rs639174 in *DROSHA* and vomits remained statistically significant after FDR correction. This is an intronic SNP in LD with rs7731057, an intronic SNP with a putative role in transcriptional regulation (TR).

Surprisingly, other polymorphisms in *DROSHA* were associated with toxicity in induction and consolidation: rs10035440 (in LD with rs7720494, intronic TR), rs6877842 (in LD with rs17494568, situated in an upstream regulatory region), rs2287584 (synonymous with a putative role in splicing regulation), rs639174 (in LD with rs7731057, intronic TR), rs4867329 (in LD with rs9292427, intronic TR), rs3805500 (in LD with rs573156, rs10068052, rs496493, rs4867069, rs492176, rs6885959, rs516001, rs4867343, rs3828635, rs7737174, rs7726209, rs3763075, rs3792828 and rs1564381, all of them intronic SNPs with a putative role in transcriptional regulation), rs10719 (synonymous with a putative role in splicing regulation) and rs7735863 (in LD with rs10052174, intronic TR). *DROSHA* (*RNASEN*) encodes an RNAse III enzyme, involved in pri-miRNAs maturation into pre-miRNAs [304]. Several SNPs in this gene, including some of our matches, have been associated with reduced *DROSHA* mRNA expression and with miRNA expression changes (rs640831, in LD with rs3805500) [242] and with survival in lung cancer (haplotype that includes rs642321 and rs3805516, in LD with rs10719, rs493760, rs640831, in LD with rs3805500, rs7735863 and rs10520985) [242] and renal cell carcinoma (rs10719 and rs6877842) [305]. All these results together suggest that inherited variation in this gene can affect miRNA expression levels and function and this could affect the expression of genes involved in response to treatment.

Interestingly, we also found an association between the SNP rs56103835 in mir-453 (also known as mir-323b-5p) and MTX clearance. This miRNA has as putative target genes *ABCC1*, *ABCC2* and *ABCC4*. The SNP rs56103835, in which G allele is associated with higher risk of toxicity, is in the pre-miRNA, and thus could influence miRNA biogenesis and levels of mature miRNA. If mir-453 is up-

regulated, it would decrease the activity of these genes, the higher toxicity observed could be explained.

Other significant associations were between *XPO5* rs34324334 and hyperbilirubinemia, *TNRC6A* rs6497759 and mir-300 rs12894467 and hepatic toxicity, *CNOT1* rs11866002 and mucositis, *GEMIN3* rs197388 and renal toxicity, *GEMIN4* rs3744741 and hepatic toxicity and *EIF2C1* rs595961 and vomits in induction and *TNRC6B* rs9611280 and hepatic toxicity in consolidation. Among these genes and polymorphisms, only a few have been studied in relation to treatment response in other settings. *GEMIN4* rs3744741 has been associated with prostate cancer severity [306] and survival in renal cell carcinoma [305] and a polymorphism in *XPO5* has been associated with survival in multiple myeloma patients [307].

Knowing that literature about the function of these genes and their implication in pharmacogenetics is scarce, our results and the previous reports that relate some of these genes with treatment outcome in cancer indicate that these genes and polymorphisms may be of relevance in the study of drug response.

In conclusion, we have found several associations between polymorphism in pre-miRNAs and genes involved in miRNAs biogenesis and toxicity during induction and consolidation, especially with polymorphisms in *DROSHA*. These results open a new promising field of investigation, involving the study of miRNA-related polymorphisms in pediatric ALL treatment.

CONCLUSIONS

CONCLUSIONS

In pediatric Acute Lymphoblastic Leukemia:

1. The 14q24.2 deletion could be a new marker for the standard risk group and 12q23.1 deletion for the high risk group.

2. A total of 5 new markers, the 1q21.3 and 1q25.1 gain, gain or loss, 10q25.1-q25.2 and 12q12 deletion and 5q33.3 alteration, could improve standard risk characterization.

3. There is no evidence to support the use of *MTHFR* C677T and A1298C SNPs as MTX toxicity markers.

4. SNPs in the MTX transporters, as rs11045879 in *SLCO1B1*, rs9516519 in *ABCC4* and rs3740065 in *ABCC2* could be useful tools to avoid MTX-related toxicity.

5. The SNP rs56103835 in mir-453, that could regulate *ABCC1*, *ABCC2* and *ABCC4*, could also be a new new marker of MTX toxicity. rs639174 in *DROSHA*, a gene of miRNA processing, is strongly associated with vomits. These two results suggest that miRNA-related SNPs could be a useful tool for toxicity studies.

Final conclusions:

B-ALL treatment could be improved using new genetic markers to predict the effectiveness in the tumor and the risk of toxicity in the individual.

We open a new promising field of investigation, involving the study of miRNA-related polymorphisms in pediatric ALL treatment.

REFERENCES

REFERENCES

1.Swerdlov SH, Campo E, Harris NL, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: IARC; 2008.

2.Ferlay J, Bray F, Pisani P, Parkin D. GLOBOCAN 2002: cancer incidence, mortality and prevalence worldwide. Lyon: IARC; 2002.

3.Redaelli A, Laskin BL, Stephens JM, Botteman MF, Pashos CL. A systematic literature review of the clinical and epidemiological burden of acute lymphoblastic leukaemia (ALL). *Eur J Cancer Care (Engl)*. 2005;14(1):53-62.

4.Koppen IJ, Hermans FJ, Kaspers GJ. Folate related gene polymorphisms and susceptibility to develop childhood acute lymphoblastic leukaemia. *Br J Haematol*. 2010;148(1):3-14.

5.Johnston WT, Lightfoot TJ, Simpson J, Roman E. Childhood cancer survival: a report from the United Kingdom Childhood Cancer Study. *Cancer Epidemiol*. 2010;34(6):659-666.

6.Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. *Lancet*. 2008;371(9617):1030-1043.

7.Mullighan CG. Genomic profiling of B-progenitor acute lymphoblastic leukemia. *Best Pract Res Clin Haematol*. 2011;24(4):489-503.

8.Advani AS, Hunger SP, Burnett AK. Acute leukemia in adolescents and young adults. *Semin Oncol*. 2009;36(3):213-226.

9.Graux C. Biology of acute lymphoblastic leukemia (ALL): clinical and therapeutic relevance. *Transfus Apher Sci*. 2011;44(2):183-189.

10.Mori H, Colman SM, Xiao Z, et al. Chromosome translocations and covert leukemic clones are generated during normal fetal development. *Proc Natl Acad Sci U S A*. 2002;99(12):8242-8247.

11.Greaves MF. Biological models for leukaemia and lymphoma. *IARC Sci Publ*. 2004(157):351-372.

12.Greaves M. Infection, immune responses and the aetiology of childhood leukaemia. *Nat Rev Cancer*. 2006;6(3):193-203.

13.Eden T. Aetiology of childhood leukaemia. *Cancer Treat Rev*. 2010;36(4):286-297.

14.Hashimoto M, Yamashita Y, Mori N. Immunohistochemical detection of CD79a expression in precursor T cell lymphoblastic lymphoma/leukaemias. *J Pathol*. 2002;197(3):341-347.

15.Cobaleda C, Gutiérrez-Cianca N, Pérez-Losada J, et al. A primitive hematopoietic cell is the target for the leukemic transformation in human philadelphia-positive acute lymphoblastic leukemia. *Blood*. 2000;95(3):1007-1013.

16.Heerema NA, Raimondi SC, Anderson JR, et al. Specific extra chromosomes occur in a modal number dependent pattern in pediatric acute lymphoblastic leukemia. *Genes Chromosomes Cancer*. 2007;46(7):684-693.

17.Sutcliffe MJ, Shuster JJ, Sather HN, et al. High concordance from independent studies by the Children's Cancer Group (CCG) and Pediatric Oncology Group (POG) associating favorable prognosis with combined trisomies 4, 10, and 17 in children with NCI Standard-Risk B-precursor Acute Lymphoblastic Leukemia: a Children's Oncology Group (COG) initiative. *Leukemia*. 2005;19(5):734-740.

18.Harrison CJ, Moorman AV, Broadfield ZJ, et al. Three distinct subgroups of hypodiploidy in acute lymphoblastic leukaemia. *Br J Haematol*. 2004;125(5):552-559.

19.Nachman JB, Heerema NA, Sather H, et al. Outcome of treatment in children with hypodiploid acute lymphoblastic leukemia. *Blood*. 2007;110(4):1112-1115.

20.Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *J Clin Oncol*. 2011;29(5):551-565.

21.Conter V, Aricò M, Basso G, et al. Long-term results of the Italian Association of Pediatric Hematology and Oncology (AIEOP) Studies 82, 87, 88, 91 and 95 for childhood acute lymphoblastic leukemia. *Leukemia*. 2010;24(2):255-264.

22.Möricke A, Zimmermann M, Reiter A, et al. Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. *Leukemia*. 2010;24(2):265-284.

23.Kamps WA, van der Pal-de Bruin KM, Veerman AJ, Fiocco M, Bierings M, Pieters R. Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004. *Leukemia*. 2010;24(2):309-319.

24.Mitchell C, Richards S, Harrison CJ, Eden T. Long-term follow-up of the United Kingdom medical research council protocols for childhood acute lymphoblastic leukaemia, 1980-2001. *Leukemia*. 2010;24(2):406-418.

25.Liang DC, Yang CP, Lin DT, et al. Long-term results of Taiwan Pediatric Oncology Group studies 1997 and 2002 for childhood acute lymphoblastic leukemia. *Leukemia*. 2010;24(2):397-405.

26.Tsuchida M, Ohara A, Manabe A, et al. Long-term results of Tokyo Children's Cancer Study Group trials for childhood acute lymphoblastic leukemia, 1984-1999. *Leukemia*. 2010;24(2):383-396.

27.Pui CH, Campana D, Pei D, et al. Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N Engl J Med*. 2009;360(26):2730-2741.

28.Pui CH, Pei D, Sandlund JT, et al. Long-term results of St Jude Total Therapy Studies 11, 12, 13A, 13B, and 14 for childhood acute lymphoblastic leukemia. *Leukemia*. 2010;24(2):371-382.

29.Schmiegelow K, Forestier E, Hellebostad M, et al. Long-term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. *Leukemia*. 2010;24(2):345-354.

30.Stark B, Nirel R, Avrahami G, et al. Long-term results of the Israeli National Studies in childhood acute lymphoblastic leukemia: INS 84, 89 and 98. *Leukemia*. 2010;24(2):419-424.

31.Silverman LB, Stevenson KE, O'Brien JE, et al. Long-term results of Dana-Farber Cancer Institute ALL Consortium protocols for children with newly diagnosed acute lymphoblastic leukemia (1985-2000). *Leukemia*. 2010;24(2):320-334.

32.Stary J, Jabali Y, Trka J, et al. Long-term results of treatment of childhood acute lymphoblastic leukemia in the Czech Republic. *Leukemia*. 2010;24(2):425-428.

33.Escherich G, Horstmann MA, Zimmermann M, Janka-Schaub GE, group Cs. Cooperative study group for childhood acute lymphoblastic leukaemia (COALL): long-term results of trials 82,85,89,92 and 97. *Leukemia*. 2010;24(2):298-308.

34.Gaynon PS, Angiolillo AL, Carroll WL, et al. Long-term results of the children's cancer group studies for childhood acute lymphoblastic leukemia 1983-2002: a Children's Oncology Group Report. *Leukemia*. 2010;24(2):285-297.

35.Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med*. 2004;350(15):1535-1548.

36.Harrison CJ. Cytogenetics of paediatric and adolescent acute lymphoblastic leukaemia. *Br J Haematol*. 2009;144(2):147-156.

37.Okuda T, Shurtleff SA, Valentine MB, et al. Frequent deletion of p16INK4a/MTS1 and p15INK4b/MTS2 in pediatric acute lymphoblastic leukemia. *Blood*. 1995;85(9):2321-2330.

38.Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004;306(5694):269-271.

39.Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*. 2007;446(7137):758-764.

40.Mullighan CG. Genomic analysis of acute leukemia. *Int J Lab Hematol*. 2009;31(4):384-397.

41.Mullighan CG, Su X, Zhang J, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med*. 2009;360(5):470-480.

42.Bardini M, Spinelli R, Bungaro S, et al. DNA copy-number abnormalities do not occur in infant ALL with t(4;11)/MLL-AF4. *Leukemia*. 2010;24(1):169-176.

43.Bardini M, Galbiati M, Lettieri A, et al. Implementation of array based whole-genome high-resolution technologies confirms the absence of secondary copy-number alterations in MLL-AF4-positive infant ALL patients. *Leukemia*. 2011;25(1):175-178.

44.Ley TJ, Mardis ER, Ding L, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature*. 2008;456(7218):66-72.

45.Cheok MH, Evans WE. Acute lymphoblastic leukaemia: a model for the pharmacogenomics of cancer therapy. *Nat Rev Cancer*. 2006;6(2):117-129.

46.Stanulla M, Schaeffeler E, Flohr T, et al. Thiopurine methyltransferase (TPMT) genotype and early treatment response to mercaptopurine in childhood acute lymphoblastic leukemia. *JAMA*. 2005;293(12):1485-1489.

47.Ansari M, Krajinovic M. Pharmacogenomics in cancer treatment defining genetic bases for inter-individual differences in responses to chemotherapy. *Curr Opin Pediatr*. 2007;19(1):15-22.

48.Paugh SW, Stocco G, Evans WE. Pharmacogenomics in pediatric leukemia. *Curr Opin Pediatr*. 2010;22(6):703-710.

49.Cheok MH, Pottier N, Kager L, Evans WE. Pharmacogenetics in acute lymphoblastic leukemia. *Semin Hematol*. 2009;46(1):39-51.

50.Pui CH, Relling MV, Evans WE. Role of pharmacogenomics and pharmacodynamics in the treatment of acute lymphoblastic leukaemia. *Best Pract Res Clin Haematol*. 2002;15(4):741-756.

51.Consortium GP. A map of human genome variation from population-scale sequencing. *Nature*. 2010;467(7319):1061-1073.

52.Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. *Science*. 2002;296(5576):2225-2229.

53.Goldstein DB, Weale ME. Population genomics: linkage disequilibrium holds the key. *Curr Biol*. 2001;11(14):R576-579.

54.Reich DE, Cargill M, Bolk S, et al. Linkage disequilibrium in the human genome. *Nature*. 2001;411(6834):199-204.

55.Risch N, Merikangas K. The future of genetic studies of complex human diseases. *Science*. 1996;273(5281):1516-1517.

56.Sachidanandam R, Weissman D, Schmidt SC, et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature*. 2001;409(6822):928-933.

57.Goldstein DB, Cavalleri GL. Genomics: understanding human diversity. *Nature*. 2005;437(7063):1241-1242.

58.Howie BN, Carlson CS, Rieder MJ, Nickerson DA. Efficient selection of tagging single-nucleotide polymorphisms in multiple populations. *Hum Genet*. 2006;120(1):58-68.

59.Relling MV, Altman RB, Goetz MP, Evans WE. Clinical implementation of pharmacogenomics: overcoming genetic exceptionalism. *Lancet Oncol*. 2010;11(6):507-509.

60.Paugh SW, Stocco G, McCorkle JR, Diouf B, Crews KR, Evans WE. Cancer pharmacogenomics. *Clin Pharmacol Ther*. 2011;90(3):461-466.

61.Wall AM, Rubnitz JE. Pharmacogenomic effects on therapy for acute lymphoblastic leukemia in children. *Pharmacogenomics J*. 2003;3(3):128-135.

62.Gorlick R, Goker E, Trippett T, Waltham M, Banerjee D, Bertino JR. Intrinsic and acquired resistance to methotrexate in acute leukemia. *N Engl J Med*. 1996;335(14):1041-1048.

63.Krajinovic M, Lemieux-Blanchard E, Chiasson S, Primeau M, Costea I, Moghrabi A. Role of polymorphisms in MTHFR and MTHFD1 genes in the outcome of childhood acute lymphoblastic leukemia. *Pharmacogenomics J*. 2004;4(1):66-72.

64.Krajinovic M, Moghrabi A. Pharmacogenetics of methotrexate. *Pharmacogenomics*. 2004;5(7):819-834.

65.Swerts K, De Moerloose B, Dhooge C, Laureys G, Benoit Y, Philippé J. Prognostic significance of multidrug resistance-related proteins in childhood acute lymphoblastic leukaemia. *Eur J Cancer*. 2006;42(3):295-309.

66.Treviño LR, Shimasaki N, Yang W, et al. Germline genetic variation in an organic anion transporter polypeptide associated with methotrexate pharmacokinetics and clinical effects. *J Clin Oncol*. 2009;27(35):5972-5978.

67.Abe T, Unno M, Onogawa T, et al. LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers. *Gastroenterology*. 2001;120(7):1689-1699.

68.Imanishi H, Okamura N, Yagi M, et al. Genetic polymorphisms associated with adverse events and elimination of methotrexate in childhood acute lymphoblastic leukemia and malignant lymphoma. *J Hum Genet*. 2007;52(2):166-171.

69.Laverdière C, Chiasson S, Costea I, Moghrabi A, Krajinovic M. Polymorphism G80A in the reduced folate carrier gene and its relationship to methotrexate plasma levels and outcome of childhood acute lymphoblastic leukemia. *Blood*. 2002;100(10):3832-3834.

70.Ashton LJ, Gifford AJ, Kwan E, et al. Reduced folate carrier and methylenetetrahydrofolate reductase gene polymorphisms: associations with clinical outcome in childhood acute lymphoblastic leukemia. *Leukemia*. 2009;23(7):1348-1351.

71.Faganel Kotnik B, Grabnar I, Bohanec Grabar P, Dolžan V, Jazbec J. Association of genetic polymorphism in the folate metabolic pathway with methotrexate pharmacokinetics and toxicity in childhood acute lymphoblastic leukaemia and malignant lymphoma. *Eur J Clin Pharmacol*. 2011;67(10):993-1006.

72.Shimasaki N, Mori T, Torii C, et al. Influence of MTHFR and RFC1 polymorphisms on toxicities during maintenance chemotherapy for childhood acute lymphoblastic leukemia or lymphoma. *J Pediatr Hematol Oncol*. 2008;30(5):347-352.

73.Kishi S, Cheng C, French D, et al. Ancestry and pharmacogenetics of antileukemic drug toxicity. *Blood*. 2007;109(10):4151-4157.

74.Shimasaki N, Mori T, Samejima H, et al. Effects of methylenetetrahydrofolate reductase and reduced folate carrier 1 polymorphisms on high-dose methotrexate-induced toxicities in children with acute lymphoblastic leukemia or lymphoma. *J Pediatr Hematol Oncol*. 2006;28(2):64-68.

75.Pietrzyk JJ, Bik-Multanowski M, Balwierz W, et al. Additional genetic risk factor for death in children with acute lymphoblastic leukemia: a common polymorphism of the MTHFR gene. *Pediatr Blood Cancer*. 2009;52(3):364-368.

76.Aplenc R, Thompson J, Han P, et al. Methylenetetrahydrofolate reductase polymorphisms and therapy response in pediatric acute lymphoblastic leukemia. *Cancer Res*. 2005;65(6):2482-2487.

77.El-Khodary NM, El-Haggar SM, Eid MA, Ebeid EN. Study of the pharmacokinetic and pharmacogenetic contribution to the toxicity of high-dose methotrexate in children with acute lymphoblastic leukemia. *Med Oncol*. 2011.

78.Tantawy AA, El-Bostany EA, Adly AA, Abou El Asrar M, El-Ghouroury EA, Abdulghaffar EE. Methylene tetrahydrofolate reductase gene polymorphism in Egyptian children with acute lymphoblastic leukemia. *Blood Coagul Fibrinolysis*. 2010;21(1):28-34.

79.Salazar J, Altés A, Del Río E, et al. Methotrexate consolidation treatment according to pharmacogenetics of MTHFR ameliorates event-free survival in childhood acute lymphoblastic leukaemia. *Pharmacogenomics J*. 2011.

80.D'Angelo V, Ramaglia M, Iannotta A, et al. Methotrexate toxicity and efficacy during the consolidation phase in paediatric acute lymphoblastic leukaemia and MTHFR polymorphisms as pharmacogenetic determinants. *Cancer Chemother Pharmacol*. 2011;68(5):1339-1346.

81.Liu SG, Li ZG, Cui L, Gao C, Li WJ, Zhao XX. Effects of methylenetetrahydrofolate reductase gene polymorphisms on toxicities during consolidation therapy in pediatric acute lymphoblastic leukemia in a Chinese population. *Leuk Lymphoma*. 2011;52(6):1030-1040.

82.Sepe DM, McWilliams T, Chen J, et al. Germline genetic variation and treatment response on CCG-1891. *Pediatr Blood Cancer*. 2012;58(5):695-700.

83.Huang L, Tissing WJ, de Jonge R, van Zelst BD, Pieters R. Polymorphisms in folate-related genes: association with side effects of high-dose methotrexate in childhood acute lymphoblastic leukemia. *Leukemia*. 2008;22(9):1798-1800.

84.Krajinovic M, Costea I, Primeau M, Dulucq S, Moghrabi A. Combining several polymorphisms of thymidylate synthase gene for pharmacogenetic analysis. *Pharmacogenomics J*. 2005;5(6):374-380.

85.Rocha JC, Cheng C, Liu W, et al. Pharmacogenetics of outcome in children with acute lymphoblastic leukemia. *Blood*. 2005;105(12):4752-4758.

86.Krajinovic M, Costea I, Chiasson S. Polymorphism of the thymidylate synthase gene and outcome of acute lymphoblastic leukaemia. *Lancet*. 2002;359(9311):1033-1034.

87.da Silva Silveira V, Canalle R, Scrideli CA, et al. Polymorphisms of xenobiotic metabolizing enzymes and DNA repair genes and outcome in childhood acute lymphoblastic leukemia. *Leuk Res*. 2009;33(7):898-901.

88.Erčulj N, Kotnik BF, Debeljak M, Jazbec J, Dolžan V. Influence of folate pathway polymorphisms on high-dose methotrexate-related toxicity and survival in childhood acute lymphoblastic leukemia. *Leuk Lymphoma*. 2012.

89.Stanulla M, Schäffeler E, Arens S, et al. GSTP1 and MDR1 genotypes and central nervous system relapse in childhood acute lymphoblastic leukemia. *Int J Hematol*. 2005;81(1):39-44.

90.Rao DN, Anuradha C, Vishnupriya S, et al. Association of an MDR1 gene (C3435T) polymorphism with acute leukemia in India. *Asian Pac J Cancer Prev*. 2010;11(4):1063-1066.

91.Jamroziak K, Młynarski W, Balcerczak E, et al. Functional C3435T polymorphism of MDR1 gene: an impact on genetic susceptibility and clinical outcome of childhood acute lymphoblastic leukemia. *Eur J Haematol*. 2004;72(5):314-321.

92.Yang YL, Lin DT, Chang SK, et al. Pharmacogenomic variations in treatment protocols for childhood acute lymphoblastic leukemia. *Pediatr Blood Cancer*. 2010;54(2):206-211.

93.Erdélyi DJ, Kámory E, Zalka A, et al. The role of ABC-transporter gene polymorphisms in chemotherapy induced immunosuppression, a retrospective study in childhood acute lymphoblastic leukaemia. *Cell Immunol*. 2006;244(2):121-124.

94.Erdilyi DJ, Kámory E, Csókay B, et al. Synergistic interaction of ABCB1 and ABCG2 polymorphisms predicts the prevalence of toxic encephalopathy during anticancer chemotherapy. *Pharmacogenomics J*. 2008;8(5):321-327.

95.Marino S, Verzegnassi F, Tamaro P, et al. Response to glucocorticoids and toxicity in childhood acute lymphoblastic leukemia: role of polymorphisms of genes involved in glucocorticoid response. *Pediatr Blood Cancer*. 2009;53(6):984-991.

96.Karathanasis NV, Stiakaki E, Goulielmos GN, Kalmanti M. The role of the methylenetetrahydrofolate reductase 677 and 1298 polymorphisms in Cretan children with acute lymphoblastic leukemia. *Genet Test Mol Biomarkers*. 2011;15(1-2):5-10.

97.Chatzidakis K, Goulas A, Athanassiadou-Piperopoulou F, Fidani L, Koliouskas D, Mirtsou V. Methylenetetrahydrofolate reductase C677T polymorphism: association with risk for childhood acute lymphoblastic leukemia and response during the initial phase of chemotherapy in greek patients. *Pediatr Blood Cancer*. 2006;47(2):147-151.

98.Kishi S, Griener J, Cheng C, et al. Homocysteine, pharmacogenetics, and neurotoxicity in children with leukemia. *J Clin Oncol*. 2003;21(16):3084-3091.

99.Pakakasama S, Kanchanakamhaeng K, Kajanachumpol S, et al. Genetic polymorphisms of folate metabolic enzymes and toxicities of high dose methotrexate in children with acute lymphoblastic leukemia. *Ann Hematol*. 2007;86(8):609-611.

100.Horinouchi M, Yagi M, Imanishi H, et al. Association of genetic polymorphisms with hepatotoxicity in patients with childhood acute lymphoblastic leukemia or lymphoma. *Pediatr Hematol Oncol*. 2010;27(5):344-354.

101.Kantar M, Kosova B, Cetingul N, et al. Methylenetetrahydrofolate reductase C677T and A1298C gene polymorphisms and therapy-related toxicity in children treated for acute lymphoblastic leukemia and non-Hodgkin lymphoma. *Leuk Lymphoma*. 2009;50(6):912-917.

102.van Kooten Niekerk PB, Schmiegelow K, Schroeder H. Influence of methylene tetrahydrofolate reductase polymorphisms and coadministration of antimetabolites on toxicity after high dose methotrexate. *Eur J Haematol*. 2008;81(5):391-398.

103.Costea I, Moghrabi A, Laverdiere C, Graziani A, Krajinovic M. Folate cycle gene variants and chemotherapy toxicity in pediatric patients with acute lymphoblastic leukemia. *Haematologica*. 2006;91(8):1113-1116.

104.Krynetski EY, Schuetz JD, Galpin AJ, Pui CH, Relling MV, Evans WE. A single point mutation leading to loss of catalytic activity in human thiopurine S-methyltransferase. *Proc Natl Acad Sci U S A*. 1995;92(4):949-953.

105.Krynetski EY, Tai HL, Yates CR, et al. Genetic polymorphism of thiopurine S-methyltransferase: clinical importance and molecular mechanisms. *Pharmacogenetics*. 1996;6(4):279-290.

106.Deininger M, Szumlanski CL, Otterness DM, Van Loon J, Ferber W, Weinshilboum RM. Purine substrates for human thiopurine methyltransferase. *Biochem Pharmacol*. 1994;48(11):2135-2138.

107.Eichelbaum M, Ingelman-Sundberg M, Evans WE. Pharmacogenomics and individualized drug therapy. *Annu Rev Med*. 2006;57:119-137.

108.McLeod HL, Krynetski EY, Relling MV, Evans WE. Genetic polymorphism of thiopurine methyltransferase and its clinical relevance for childhood acute lymphoblastic leukemia. *Leukemia*. 2000;14(4):567-572.

109.Carroll WL, Bhojwani D, Min DJ, et al. Pediatric acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program*. 2003:102-131.

110.Yates CR, Krynetski EY, Loennechen T, et al. Molecular diagnosis of thiopurine S-methyltransferase deficiency: genetic basis for azathioprine and mercaptopurine intolerance. *Ann Intern Med*. 1997;126(8):608-614.

111.Lennard L, Lilleyman JS, Van Loon J, Weinshilboum RM. Genetic variation in response to 6-mercaptopurine for childhood acute lymphoblastic leukaemia. *Lancet*. 1990;336(8709):225-229.

112.Evans WE, Horner M, Chu YQ, Kalwinsky D, Roberts WM. Altered mercaptopurine metabolism, toxic effects, and dosage requirement in a thiopurine methyltransferase-deficient child with acute lymphocytic leukemia. *J Pediatr*. 1991;119(6):985-989.

113.Evans WE, Hon YY, Bomgaars L, et al. Preponderance of thiopurine S-methyltransferase deficiency and heterozygosity among patients intolerant to mercaptopurine or azathioprine. *J Clin Oncol*. 2001;19(8):2293-2301.

114.Relling MV, Hancock ML, Rivera GK, et al. Mercaptopurine therapy intolerance and heterozygosity at the thiopurine S-methyltransferase gene locus. *J Natl Cancer Inst*. 1999;91(23):2001-2008.

115.Lehne G, Bjørheim J, Saeter G. [Anticancer drug dosing--pharmacogenomic biomarkers or body surface area?]. *Tidsskr Nor Laegeforen*. 2007;127(8):1040-1044.

116.Hawwa AF, Collier PS, Millership JS, et al. Population pharmacokinetic and pharmacogenetic analysis of 6-mercaptopurine in paediatric patients with acute lymphoblastic leukaemia. *Br J Clin Pharmacol*. 2008;66(6):826-837.

117.Schmiegelow K, Forestier E, Kristinsson J, et al. Thiopurine methyltransferase activity is related to the risk of relapse of childhood acute lymphoblastic leukemia: results from the NOPHO ALL-92 study. *Leukemia*. 2009;23(3):557-564.

118.Samochatova EV, Chupova NV, Rudneva A, et al. TPMT genetic variations in populations of the Russian Federation. *Pediatr Blood Cancer*. 2009;52(2):203-208.

119.Desire S, Balasubramanian P, Bajel A, et al. Frequency of TPMT alleles in Indian patients with acute lymphatic leukemia and effect on the dose of 6-mercaptopurine. *Med Oncol*. 2010;27(4):1046-1049.

120.Dokmanovic L, Urosevic J, Janic D, et al. Analysis of thiopurine S-methyltransferase polymorphism in the population of Serbia and Montenegro and mercaptopurine therapy tolerance in childhood acute lymphoblastic leukemia. *Ther Drug Monit*. 2006;28(6):800-806.

121.Tumer TB, Ulusoy G, Adali O, Sahin G, Gozdasoglu S, Arinç E. The low frequency of defective TPMT alleles in Turkish population: a study on pediatric patients with acute lymphoblastic leukemia. *Am J Hematol*. 2007;82(10):906-910.

122.Fakhoury M, Andreu-Gallien J, Mahr A, et al. Should TPMT genotype and activity be used to monitor 6-mercaptopurine treatment in children with acute lymphoblastic leukaemia? *J Clin Pharm Ther*. 2007;32(6):633-639.

123.Silva MR, de Oliveira BM, Viana MB, Murao M, Romanha AJ. Thiopurine S-methyltransferase (TPMT) gene polymorphism in Brazilian children with acute lymphoblastic leukemia: association with clinical and laboratory data. *Ther Drug Monit*. 2008;30(6):700-704.

124.Niedzielska E, Niedzielska M, Chybicka A. [Allelic variants of TPMT and the risk of leucopenia and neutropenia in patients treated for acute leukaemia]. *Med Wieku Rozwoj*. 2009;13(3):180-186.

125.Kapoor G, Sinha R, Naithani R, Chandgothia M. Thiopurine S-methyltransferase gene polymorphism and 6-mercaptopurine dose intensity in Indian children with acute lymphoblastic leukemia. *Leuk Res*. 2010;34(8):1023-1026.

126.Dokmanović L, Janić D, Krstovski N, Zukić B, Tosić N, Pavlović S. [Importance of genotyping of thiopurine S-methyltransferase in children with acute lymphoblastic leukaemia during maintenance therapy]. *Srp Arh Celok Lek*. 2008;136(11-12):609-616.

127.Peregud-Pogorzelski J, Tetera-Rudnicka E, Kurzawski M, et al. Thiopurine S-methyltransferase (TPMT) polymorphisms in children with acute lymphoblastic leukemia, and the need for reduction or cessation of 6-mercaptopurine doses during maintenance therapy: the Polish multicenter analysis. *Pediatr Blood Cancer*. 2011;57(4):578-582.

128.Albayrak M, Konyssova U, Kaya Z, et al. Thiopurine methyltransferase polymorphisms and mercaptopurine tolerance in Turkish children with acute lymphoblastic leukemia. *Cancer Chemother Pharmacol*. 2011;68(5):1155-1159.

129.Wan Rosalina WR, Teh LK, Mohamad N, et al. Polymorphism of ITPA 94C>A and risk of adverse effects among patients with acute lymphoblastic leukaemia treated with 6-mercaptopurine. *J Clin Pharm Ther*. 2012;37(2):237-241.

130.Dervieux T, Médard Y, Verpillat P, et al. Possible implication of thiopurine S-methyltransferase in occurrence of infectious episodes during maintenance therapy for childhood lymphoblastic leukemia with mercaptopurine. *Leukemia*. 2001;15(11):1706-1712.

131.Borst L, Buchard A, Rosthøj S, et al. Gene dose effects of GSTM1, GSTT1 and GSTP1 polymorphisms on outcome in childhood acute lymphoblastic leukemia. *J Pediatr Hematol Oncol*. 2012;34(1):38-42.

132.Krajinovic M, Labuda D, Mathonnet G, et al. Polymorphisms in genes encoding drugs and xenobiotic metabolizing enzymes, DNA repair enzymes, and response to treatment of childhood acute lymphoblastic leukemia. *Clin Cancer Res*. 2002;8(3):802-810.

133.Bolufer P, Collado M, Barragán E, et al. The potential effect of gender in combination with common genetic polymorphisms of drug-metabolizing enzymes on the risk of developing acute leukemia. *Haematologica*. 2007;92(3):308-314.

134.Eyada TK, El Ghonemy EG, El Ghoroury EA, El Bassyouni SO, El Masry MK. Study of genetic polymorphism of xenobiotic enzymes in acute leukemia. *Blood Coagul Fibrinolysis*. 2007;18(5):489-495.

135.Takanashi M, Morimoto A, Yagi T, et al. Impact of glutathione S-transferase gene deletion on early relapse in childhood B-precursor acute lymphoblastic leukemia. *Haematologica*. 2003;88(11):1238-1244.

136.Anderer G, Schrappe M, Brechlin AM, et al. Polymorphisms within glutathione S-transferase genes and initial response to glucocorticoids in childhood acute lymphoblastic leukaemia. *Pharmacogenetics*. 2000;10(8):715-726.

137.Stanulla M, Schrappe M, Brechlin AM, Zimmermann M, Welte K. Polymorphisms within glutathione S-transferase genes (GSTM1, GSTT1, GSTP1) and risk of relapse in childhood B-cell precursor acute lymphoblastic leukemia: a case-control study. *Blood*. 2000;95(4):1222-1228.

138.Gatedee J, Pakakassama S, Muangman S, Pongstaporn W. Glutathione S-transferase P1 genotypes, genetic susceptibility and outcome of therapy in thai

childhood acute lymphoblastic leukemia. *Asian Pac J Cancer Prev*. 2007;8(2):294-296.

139.Suneetha KJ, Nancy KN, Rajalekshmy KR, Rama R, Sagar TG, Rajkumar T. Role of glutathione-s-transferase and CYP1A1*2A polymorphisms in the therapy outcome of south Indian acute lymphoblastic leukemia patients. *Indian J Med Paediatr Oncol*. 2011;32(1):25-29.

140.Stanulla M, Dynybil C, Bartels DB, et al. The NQO1 C609T polymorphism is associated with risk of secondary malignant neoplasms after treatment for childhood acute lymphoblastic leukemia: a matched-pair analysis from the ALL-BFM study group. *Haematologica*. 2007;92(11):1581-1582.

141.Bolufer P, Collado M, Barragan E, et al. Profile of polymorphisms of drug-metabolising enzymes and the risk of therapy-related leukaemia. *Br J Haematol*. 2007;136(4):590-596.

142.Gong J, Tong Y, Zhang HM, et al. Genome-wide identification of SNPs in microRNA genes and the SNP effects on microRNA target binding and biogenesis. *Hum Mutat*. 2012;33(1):254-263.

143.Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: the implications for cancer research. *Nat Rev Cancer*. 2010;10(6):389-402.

144.Munker R, Calin GA. MicroRNA profiling in cancer. *Clin Sci (Lond)*. 2011;121(4):141-158.

145.Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell*. 2003;115(7):787-798.

146.Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-297.

147.Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res*. 2011;39(Database issue):D152-157.

148.Slaby O, Bienertova-Vasku J, Svoboda M, Vyzula R. Genetic polymorphisms and microRNAs: new direction in molecular epidemiology of solid cancer. *J Cell Mol Med*. 2012;16(1):8-21.

149.Sun G, Yan J, Noltner K, et al. SNPs in human miRNA genes affect biogenesis and function. *RNA*. 2009;15(9):1640-1651.

150.Schotte D, De Menezes RX, Moqadam FA, et al. MicroRNA characterize genetic diversity and drug resistance in pediatric acute lymphoblastic leukemia. *Haematologica*. 2011;96(5):703-711.

151.Mishra PJ, Bertino JR. MicroRNA polymorphisms: the future of pharmacogenomics, molecular epidemiology and individualized medicine. *Pharmacogenomics*. 2009;10(3):399-416.

152.Mishra PJ, Humeniuk R, Longo-Sorbello GS, Banerjee D, Bertino JR. A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. *Proc Natl Acad Sci U S A*. 2007;104(33):13513-13518.

153.Bao L, Zhou M, Wu L, et al. PolymiRTS Database: linking polymorphisms in microRNA target sites with complex traits. *Nucleic Acids Res*. 2007;35(Database issue):D51-54.

154.Ziebarth JD, Bhattacharya A, Chen A, Cui Y. PolymiRTS Database 2.0: linking polymorphisms in microRNA target sites with human diseases and complex traits. *Nucleic Acids Res*. 2012;40(Database issue):D216-221.

155.Hiard S, Charlier C, Coppieters W, Georges M, Baurain D. Patrocles: a database of polymorphic miRNA-mediated gene regulation in vertebrates. *Nucleic Acids Res*. 2010;38(Database issue):D640-651.

156.Larson RA, Wang Y, Banerjee M, et al. Prevalence of the inactivating 609C-->T polymorphism in the NAD(P)H:quinone oxidoreductase (NQO1) gene in patients with primary and therapy-related myeloid leukemia. *Blood*. 1999;94(2):803-807.

157.Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl*. 1995;4(6):357-362.

158.Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. Vol. 57: J Royal Stat

Soc Series B; 1995.

159.van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17(12):2257-2317.

160.Warren RB, Smith RL, Campalani E, et al. Genetic variation in efflux transporters influences outcome to methotrexate therapy in patients with psoriasis. *J Invest Dermatol*. 2008;128(8):1925-1929.

161.Innocenti F, Kroetz DL, Schuetz E, et al. Comprehensive pharmacogenetic analysis of irinotecan neutropenia and pharmacokinetics. *J Clin Oncol*. 2009;27(16):2604-2614.

162.Siedlinski M, Boezen HM, Boer JM, Smit HA, Postma DS. ABCC1 polymorphisms contribute to level and decline of lung function in two population-based cohorts. *Pharmacogenet Genomics*. 2009;19(9):675-684.

163.Lee SH, Lee MS, Lee JH, et al. MRP1 polymorphisms associated with citalopram response in patients with major depression. *J Clin Psychopharmacol*. 2010;30(2):116-125.

164.Wang H, Jin G, Liu G, et al. Genetic susceptibility of lung cancer associated with common variants in the 3' untranslated regions of the adenosine

triphosphate-binding cassette B1 (ABCB1) and ABCC1 candidate transporter genes for carcinogen export. *Cancer*. 2009;115(3):595-607.

165.Choi JH, Ahn BM, Yi J, et al. MRP2 haplotypes confer differential susceptibility to toxic liver injury. *Pharmacogenet Genomics*. 2007;17(6):403-415.

166.Akamine Y, Miura M, Sunagawa S, Kagaya H, Yasui-Furukori N, Uno T. Influence of drug-transporter polymorphisms on the pharmacokinetics of fexofenadine enantiomers. *Xenobiotica*. 2010;40(11):782-789.

167.Lubomirov R, di Iulio J, Fayet A, et al. ADME pharmacogenetics: investigation of the pharmacokinetics of the antiretroviral agent lopinavir coformulated with ritonavir. *Pharmacogenet Genomics*. 2010;20(4):217-230.

168.Ohmann EL, Burckart GJ, Brooks MM, et al. Genetic polymorphisms influence mycophenolate mofetil-related adverse events in pediatric heart transplant patients. *J Heart Lung Transplant*. 2010;29(5):509-516.

169.Sun N, Sun X, Chen B, et al. MRP2 and GSTP1 polymorphisms and chemotherapy response in advanced non-small cell lung cancer. *Cancer Chemother Pharmacol*. 2010;65(3):437-446.

170.Ufer M, Mosyagin I, Muhle H, et al. Non-response to antiepileptic pharmacotherapy is associated with the ABCC2 -24C>T polymorphism in young and adult patients with epilepsy. *Pharmacogenet Genomics*. 2009;19(5):353-362.

171.Rodríguez-Nóvoa S, Labarga P, Soriano V, et al. Predictors of kidney tubular dysfunction in HIV-infected patients treated with tenofovir: a pharmacogenetic study. *Clin Infect Dis*. 2009;48(11):e108-116.

172.Miura M, Satoh S, Inoue K, Saito M, Habuchi T, Suzuki T. Telmisartan pharmacokinetics in Japanese renal transplant recipients. *Clin Chim Acta*. 2009;399(1-2):83-87.

173.Miura M, Satoh S, Inoue K, et al. Influence of SLCO1B1, 1B3, 2B1 and ABCC2 genetic polymorphisms on mycophenolic acid pharmacokinetics in Japanese renal transplant recipients. *Eur J Clin Pharmacol*. 2007;63(12):1161-1169.

174.Han JY, Lim HS, Yoo YK, et al. Associations of ABCB1, ABCC2, and ABCG2 polymorphisms with irinotecan-pharmacokinetics and clinical outcome in patients with advanced non-small cell lung cancer. *Cancer*. 2007;110(1):138-147.

175.Daly AK, Aithal GP, Leathart JB, Swainsbury RA, Dang TS, Day CP. Genetic susceptibility to diclofenac-induced hepatotoxicity: contribution of UGT2B7, CYP2C8, and ABCC2 genotypes. *Gastroenterology*. 2007;132(1):272-281.

176.Rau T, Erney B, Göres R, Eschenhagen T, Beck J, Langer T. High-dose methotrexate in pediatric acute lymphoblastic leukemia: impact of ABCC2 polymorphisms on plasma concentrations. *Clin Pharmacol Ther*. 2006;80(5):468-476.

177.Naesens M, Kuypers DR, Verbeke K, Vanrenterghem Y. Multidrug resistance protein 2 genetic polymorphisms influence mycophenolic acid exposure in renal allograft recipients. *Transplantation*. 2006;82(8):1074-1084.

178.Han B, Gao G, Wu W, et al. Association of ABCC2 polymorphisms with platinum-based chemotherapy response and severe toxicity in non-small cell lung cancer patients. *Lung Cancer*. 2011;72(2):238-243.

179.Lévesque E, Benoit-Biancamano MO, Delage R, Couture F, Guillemette C. Pharmacokinetics of mycophenolate mofetil and its glucuronide metabolites in healthy volunteers. *Pharmacogenomics*. 2008;9(7):869-879.

180.Kim WJ, Lee JH, Yi J, et al. A nonsynonymous variation in MRP2/ABCC2 is associated with neurological adverse drug reactions of carbamazepine in patients with epilepsy. *Pharmacogenet Genomics*. 2010;20(4):249-256.

181.Fujita K, Nagashima F, Yamamoto W, et al. Association of ATP-binding cassette, sub-family C, number 2 (ABCC2) genotype with pharmacokinetics of irinotecan in Japanese patients with metastatic colorectal cancer treated with irinotecan plus infusional 5-fluorouracil/leucovorin (FOLFIRI). *Biol Pharm Bull*. 2008;31(11):2137-2142.

182.Zhang WX, Chen B, Jin Z, et al. Influence of uridine diphosphate (UDP)-glucuronosyltransferases and ABCC2 genetic polymorphisms on the pharmacokinetics of mycophenolic acid and its metabolites in Chinese renal transplant recipients. *Xenobiotica*. 2008;38(11):1422-1436.

183.Ranganathan P, Culverhouse R, Marsh S, et al. Methotrexate (MTX) pathway gene polymorphisms and their effects on MTX toxicity in Caucasian and African American patients with rheumatoid arthritis. *J Rheumatol*. 2008;35(4):572-579.

184.Haenisch S, May K, Wegner D, Caliebe A, Cascorbi I, Siegmund W. Influence of genetic polymorphisms on intestinal expression and rifampicin-type induction of ABCC2 and on bioavailability of talinolol. *Pharmacogenet Genomics*. 2008;18(4):357-365.

185.Izzedine H, Hulot JS, Villard E, et al. Association between ABCC2 gene haplotypes and tenofovir-induced proximal tubulopathy. *J Infect Dis*. 2006;194(11):1481-1491.

186.Han JY, Lim HS, Park YH, Lee SY, Lee JS. Integrated pharmacogenetic prediction of irinotecan pharmacokinetics and toxicity in patients with advanced non-small cell lung cancer. *Lung Cancer*. 2009;63(1):115-120.

187.Sookoian S, Castaño G, Gianotti TF, Gemma C, Pirola CJ. Polymorphisms of MRP2 (ABCC2) are associated with susceptibility to nonalcoholic fatty liver disease. *J Nutr Biochem*. 2009;20(10):765-770.

188.Lang T, Schroth W, Brauch H, Schwab M. ABCC2 and clinical outcome of tamoxifen therapy. *J Clin Oncol*. 2010;28(25):e448; author reply e449.

189.Stamp LK, Chapman PT, O'Donnell JL, et al. Polymorphisms within the folate pathway predict folate concentrations but are not associated with disease activity in rheumatoid arthritis patients on methotrexate. *Pharmacogenet Genomics*. 2010;20(6):367-376.

190.Kiyotani K, Mushiroda T, Imamura CK, et al. Significant effect of polymorphisms in CYP2D6 and ABCC2 on clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients. *J Clin Oncol*. 2010;28(8):1287-1293.

191.Kiyotani K, Mushiroda T, Kubo M, Zembutsu H, Sugiyama Y, Nakamura Y. Association of genetic polymorphisms in SLCO1B3 and ABCC2 with docetaxel-induced leukopenia. *Cancer Sci*. 2008;99(5):967-972.

192.Ansari M, Sauty G, Labuda M, et al. Polymorphisms in multidrug resistance-associated protein gene 4 is associated with outcome in childhood acute lymphoblastic leukemia. *Blood*. 2009;114(7):1383-1386.

193.Low SK, Kiyotani K, Mushiroda T, Daigo Y, Nakamura Y, Zembutsu H. Association study of genetic polymorphism in ABCC4 with cyclophosphamide-induced adverse drug reactions in breast cancer patients. *J Hum Genet*. 2009;54(10):564-571.

194.Cha PC, Mushiroda T, Zembutsu H, et al. Single nucleotide polymorphism in ABCG2 is associated with irinotecan-induced severe myelosuppression. *J Hum Genet*. 2009;54(10):572-580.

195.Kim KA, Joo HJ, Park JY. ABCG2 polymorphisms, 34G>A and 421C>A in a Korean population: analysis and a comprehensive comparison with other populations. *J Clin Pharm Ther*. 2010;35(6):705-712.

196.Bailey KM, Romaine SP, Jackson BM, et al. Hepatic metabolism and transporter gene variants enhance response to rosuvastatin in patients with acute myocardial infarction: the GEOSTAT-1 Study. *Circ Cardiovasc Genet*. 2010;3(3):276-285.

197.Gunjaca G, Boban M, Pehlić M, et al. Predictive value of 8 genetic loci for serum uric acid concentration. *Croat Med J*. 2010;51(1):23-31.

198.Tomlinson B, Hu M, Lee VW, et al. ABCG2 polymorphism is associated with the low-density lipoprotein cholesterol response to rosuvastatin. *Clin Pharmacol Ther*. 2010;87(5):558-562.

199.Stark K, Reinhard W, Grassl M, et al. Common polymorphisms influencing serum uric acid levels contribute to susceptibility to gout, but not to coronary artery disease. *PLoS One*. 2009;4(11):e7729.

200.Keskitalo JE, Pasanen MK, Neuvonen PJ, Niemi M. Different effects of the ABCG2 c.421C>A SNP on the pharmacokinetics of fluvastatin, pravastatin and simvastatin. *Pharmacogenomics*. 2009;10(10):1617-1624.

201.Woodward OM, Köttgen A, Coresh J, Boerwinkle E, Guggino WB, Köttgen M. Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. *Proc Natl Acad Sci U S A*. 2009;106(25):10338-10342.

202.Keskitalo JE, Zolk O, Fromm MF, Kurkinen KJ, Neuvonen PJ, Niemi M. ABCG2 polymorphism markedly affects the pharmacokinetics of atorvastatin and rosuvastatin. *Clin Pharmacol Ther*. 2009;86(2):197-203.

203.Müller PJ, Dally H, Klappenecker CN, et al. Polymorphisms in ABCG2, ABCC3 and CNT1 genes and their possible impact on chemotherapy outcome of lung cancer patients. *Int J Cancer*. 2009;124(7):1669-1674.

204.Kim IS, Kim HG, Kim DC, et al. ABCG2 Q141K polymorphism is associated with chemotherapy-induced diarrhea in patients with diffuse large B-cell

lymphoma who received frontline rituximab plus cyclophosphamide/doxorubicin/vincristine/prednisone chemotherapy. *Cancer Sci*. 2008;99(12):2496-2501.

205.Petain A, Kattygnarath D, Azard J, et al. Population pharmacokinetics and pharmacogenetics of imatinib in children and adults. *Clin Cancer Res*. 2008;14(21):7102-7109.

206.Dehghan A, Köttgen A, Yang Q, et al. Association of three genetic loci with uric acid concentration and risk of gout: a genome-wide association study. *Lancet*. 2008;372(9654):1953-1961.

207.Urquhart BL, Ware JA, Tirona RG, et al. Breast cancer resistance protein (ABCG2) and drug disposition: intestinal expression, polymorphisms and sulfasalazine as an in vivo probe. *Pharmacogenet Genomics*. 2008;18(5):439-448.

208.Yamasaki Y, Ieiri I, Kusuhara H, et al. Pharmacogenetic characterization of sulfasalazine disposition based on NAT2 and ABCG2 (BCRP) gene polymorphisms in humans. *Clin Pharmacol Ther*. 2008;84(1):95-103.

209.Hu LL, Wang XX, Chen X, et al. BCRP gene polymorphisms are associated with susceptibility and survival of diffuse large B-cell lymphoma. *Carcinogenesis*. 2007;28(8):1740-1744.

210.Cusatis G, Gregorc V, Li J, et al. Pharmacogenetics of ABCG2 and adverse reactions to gefitinib. *J Natl Cancer Inst*. 2006;98(23):1739-1742.

211.Hahn NM, Marsh S, Fisher W, et al. Hoosier Oncology Group randomized phase II study of docetaxel, vinorelbine, and estramustine in combination in hormone-refractory prostate cancer with pharmacogenetic survival analysis. *Clin Cancer Res*. 2006;12(20 Pt 1):6094-6099.

212.Zhang W, Yu BN, He YJ, et al. Role of BCRP 421C>A polymorphism on rosuvastatin pharmacokinetics in healthy Chinese males. *Clin Chim Acta*. 2006;373(1-2):99-103.

213.Zamboni WC, Ramanathan RK, McLeod HL, et al. Disposition of 9-nitrocamptothecin and its 9-aminocamptothecin metabolite in relation to ABC transporter genotypes. *Invest New Drugs*. 2006;24(5):393-401.

214.Takahashi N, Miura M, Scott SA, et al. Influence of CYP3A5 and drug transporter polymorphisms on imatinib trough concentration and clinical response among patients with chronic phase chronic myeloid leukemia. *J Hum Genet*. 2010;55(11):731-737.

215.Thomas F, Rochaix P, White-Koning M, et al. Population pharmacokinetics of erlotinib and its pharmacokinetic/pharmacodynamic relationships in head and neck squamous cell carcinoma. *Eur J Cancer*. 2009;45(13):2316-2323.

216.Miura M, Kagaya H, Satoh S, et al. Influence of drug transporters and UGT polymorphisms on pharmacokinetics of phenolic glucuronide metabolite of mycophenolic acid in Japanese renal transplant recipients. *Ther Drug Monit*. 2008;30(5):559-564.

217.Tamura A, Wakabayashi K, Onishi Y, et al. Re-evaluation and functional classification of non-synonymous single nucleotide polymorphisms of the human ATP-binding cassette transporter ABCG2. *Cancer Sci*. 2007;98(2):231-239.

218.Korenaga Y, Naito K, Okayama N, et al. Association of the BCRP C421A polymorphism with nonpapillary renal cell carcinoma. *Int J Cancer*. 2005;117(3):431-434.

219.Sai K, Saito Y, Maekawa K, et al. Additive effects of drug transporter genetic polymorphisms on irinotecan pharmacokinetics/pharmacodynamics in Japanese cancer patients. *Cancer Chemother Pharmacol*. 2010;66(1):95-105.

220.Campa D, Pardini B, Naccarati A, et al. A gene-wide investigation on polymorphisms in the ABCG2/BRCP transporter and susceptibility to colorectal cancer. *Mutat Res*. 2008;645(1-2):56-60.

221.Badagnani I, Castro RA, Taylor TR, et al. Interaction of methotrexate with organic-anion transporting polypeptide 1A2 and its genetic variants. *J Pharmacol Exp Ther*. 2006;318(2):521-529.

222.Link E, Parish S, Armitage J, et al. SLCO1B1 variants and statin-induced myopathy--a genomewide study. *N Engl J Med*. 2008;359(8):789-799.

223.Lopez-Lopez E, Martin-Guerrero I, Ballesteros J, et al. Polymorphisms of the SLCO1B1 gene predict methotrexate-related toxicity in childhood acute lymphoblastic leukemia. *Pediatr Blood Cancer*. 2011;57(4):612-619.

224.Kang TW, Kim HJ, Ju H, et al. Genome-wide association of serum bilirubin levels in Korean population. *Hum Mol Genet*. 2010;19(18):3672-3678.

225.Faganel Kotnik B, Dolzan V, Grabnar I, Jazbec J. Relationship of the reduced folate carrier gene polymorphism G80A to methotrexate plasma concentration, toxicity, and disease outcome in childhood acute lymphoblastic leukemia. *Leuk Lymphoma*. 2010;51(4):724-726.

226.Hayashi H, Fujimaki C, Daimon T, Tsuboi S, Matsuyama T, Itoh K. Genetic polymorphisms in folate pathway enzymes as a possible marker for predicting the outcome of methotrexate therapy in Japanese patients with rheumatoid arthritis. *J Clin Pharm Ther*. 2009;34(3):355-361.

227.Bi XH, Zhao HL, Zhang ZX, Zhang JW. Association of RFC1 A80G and MTHFR C677T polymorphisms with Alzheimer's disease. *Neurobiol Aging*. 2009;30(10):1601-1607.

228.Drozdzik M, Rudas T, Pawlik A, Gornik W, Kurzawski M, Herczynska M. Reduced folate carrier-1 80G>A polymorphism affects methotrexate treatment outcome in rheumatoid arthritis. *Pharmacogenomics J*. 2007;7(6):404-407.

229.Wang L, Chen W, Wang J, et al. Reduced folate carrier gene G80A polymorphism is associated with an increased risk of gastroesophageal cancers in a Chinese population. *Eur J Cancer*. 2006;42(18):3206-3211.

230.Dufficy L, Naumovski N, Ng X, et al. G80A reduced folate carrier SNP influences the absorption and cellular translocation of dietary folate and its association with blood pressure in an elderly population. *Life Sci*. 2006;79(10):957-966.

231.Yates Z, Lucock M. G80A reduced folate carrier SNP modulates cellular uptake of folate and affords protection against thrombosis via a non homocysteine related mechanism. *Life Sci*. 2005;77(22):2735-2742.

232.O'Byrne MR, Au KS, Morrison AC, et al. Association of folate receptor (FOLR1, FOLR2, FOLR3) and reduced folate carrier (SLC19A1) genes with meningomyelocele. *Birth Defects Res A Clin Mol Teratol*. 2010;88(8):689-694.

233.Adjei AA, Salavaggione OE, Mandrekar SJ, et al. Correlation between polymorphisms of the reduced folate carrier gene (SLC19A1) and survival after pemetrexed-based therapy in non-small cell lung cancer: a North Central Cancer Treatment Group-based exploratory study. *J Thorac Oncol*. 2010;5(9):1346-1353.

234.Clague J, Lippman SM, Yang H, et al. Genetic variation in MicroRNA genes and risk of oral premalignant lesions. *Mol Carcinog*. 2010;49(2):183-189.

235.Liang D, Meyer L, Chang DW, et al. Genetic variants in MicroRNA biosynthesis pathways and binding sites modify ovarian cancer risk, survival, and treatment response. *Cancer Res*. 2010;70(23):9765-9776.

236.Yang H, Dinney CP, Ye Y, Zhu Y, Grossman HB, Wu X. Evaluation of genetic variants in microRNA-related genes and risk of bladder cancer. *Cancer Res*. 2008;68(7):2530-2537.

237.Lee HC, Kim JG, Chae YS, et al. Prognostic impact of microRNA-related gene polymorphisms on survival of patients with colorectal cancer. *J Cancer Res Clin Oncol*. 2010;136(7):1073-1078.

238.Wilker EH, Baccarelli A, Suh H, Vokonas P, Wright RO, Schwartz J. Black carbon exposures, blood pressure, and interactions with single nucleotide polymorphisms in MicroRNA processing genes. *Environ Health Perspect*. 2010;118(7):943-948.

239.Horikawa Y, Wood CG, Yang H, et al. Single nucleotide polymorphisms of microRNA machinery genes modify the risk of renal cell carcinoma. *Clin Cancer Res*. 2008;14(23):7956-7962.

240.Ye Y, Wang KK, Gu J, et al. Genetic variations in microRNA-related genes are novel susceptibility loci for esophageal cancer risk. *Cancer Prev Res (Phila)*. 2008;1(6):460-469.

241.Zhang X, Yang H, Lee JJ, et al. MicroRNA-related genetic variations as predictors for risk of second primary tumor and/or recurrence in patients with early-stage head and neck cancer. *Carcinogenesis*. 2010;31(12):2118-2123.

242.Rotunno M, Zhao Y, Bergen AW, et al. Inherited polymorphisms in the RNA-mediated interference machinery affect microRNA expression and lung cancer survival. *Br J Cancer*. 2010;103(12):1870-1874.

243.Kuiper RP, Schoenmakers EF, van Reijmersdal SV, et al. High-resolution genomic profiling of childhood ALL reveals novel recurrent genetic lesions affecting pathways involved in lymphocyte differentiation and cell cycle progression. *Leukemia*. 2007;21(6):1258-1266.

244.Lilljebjörn H, Heidenblad M, Nilsson B, et al. Combined high-resolution array-based comparative genomic hybridization and expression profiling of ETV6/RUNX1-positive acute lymphoblastic leukemias reveal a high incidence of

cryptic Xq duplications and identify several putative target genes within the commonly gained region. *Leukemia*. 2007;21(10):2137-2144.

245.Strefford JC, Worley H, Barber K, et al. Genome complexity in acute lymphoblastic leukemia is revealed by array-based comparative genomic hybridization. *Oncogene*. 2007;26(29):4306-4318.

246.Kuchinskaya E, Heyman M, Nordgren A, et al. Array-CGH reveals hidden gene dose changes in children with acute lymphoblastic leukaemia and a normal or failed karyotype by G-banding. *Br J Haematol*. 2008;140(5):572-577.

247.Rabin KR, Man TK, Yu A, et al. Clinical utility of array comparative genomic hybridization for detection of chromosomal abnormalities in pediatric acute lymphoblastic leukemia. *Pediatr Blood Cancer*. 2008;51(2):171-177.

248.Usvasalo A, Elonen E, Saarinen-Pihkala UM, et al. Prognostic classification of patients with acute lymphoblastic leukemia by using gene copy number profiles identified from array-based comparative genomic hybridization data. *Leuk Res*. 2010;34(11):1476-1482.

249.Sulong S, Moorman AV, Irving JA, et al. A comprehensive analysis of the CDKN2A gene in childhood acute lymphoblastic leukemia reveals genomic deletion, copy number neutral loss of heterozygosity, and association with specific cytogenetic subgroups. *Blood*. 2009;113(1):100-107.

250.Lilljebjörn H, Soneson C, Andersson A, et al. The correlation pattern of acquired copy number changes in 164 ETV6/RUNX1-positive childhood acute lymphoblastic leukemias. *Hum Mol Genet*. 2010;19(16):3150-3158.

251.Okamoto R, Ogawa S, Nowak D, et al. Genomic profiling of adult acute lymphoblastic leukemia by single nucleotide polymorphism oligonucleotide microarray and comparison to pediatric acute lymphoblastic leukemia. *Haematologica*. 2010;95(9):1481-1488.

252.Emerenciano M, Bungaro S, Cazzaniga G, et al. ETV6-RUNX1 fusion gene and additional genetic changes in infant leukemia: a genome-wide analysis. *Cancer Genet Cytogenet*. 2009;193(2):86-92.

253.Kearney L, Gonzalez De Castro D, Yeung J, et al. Specific JAK2 mutation (JAK2R683) and multiple gene deletions in Down syndrome acute lymphoblastic leukemia. *Blood*. 2009;113(3):646-648.

254.Bungaro S, Dell'Orto MC, Zangrando A, et al. Integration of genomic and gene expression data of childhood ALL without known aberrations identifies subgroups with specific genetic hallmarks. *Genes Chromosomes Cancer*. 2009;48(1):22-38.

255.Kawamata N, Ogawa S, Zimmermann M, et al. Cloning of genes involved in chromosomal translocations by high-resolution single nucleotide polymorphism genomic microarray. *Proc Natl Acad Sci U S A*. 2008;105(33):11921-11926.

256.Kawamata N, Ogawa S, Seeger K, et al. Molecular allelokaryotyping of relapsed pediatric acute lymphoblastic leukemia. *Int J Oncol*. 2009;34(6):1603-1612.

257.Paulsson K, Forestier E, Lilljebjörn H, et al. Genetic landscape of high hyperdiploid childhood acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*. 2010;107(50):21719-21724.

258.Paulsson K, Cazier JB, Macdougall F, et al. Microdeletions are a general feature of adult and adolescent acute lymphoblastic leukemia: Unexpected similarities with pediatric disease. *Proc Natl Acad Sci U S A*. 2008;105(18):6708-6713.

259.Lundin C, Hjorth L, Behrendtz M, et al. High frequency of BTG1 deletions in acute lymphoblastic leukemia in children with down syndrome. *Genes Chromosomes Cancer*. 2012;51(2):196-206.

260.Paulsson K, Heidenblad M, Mörse H, Borg A, Fioretos T, Johansson B. Identification of cryptic aberrations and characterization of translocation breakpoints using array CGH in high hyperdiploid childhood acute lymphoblastic leukemia. *Leukemia*. 2006;20(11):2002-2007.

261.Davidsson J, Paulsson K, Lindgren D, et al. Relapsed childhood high hyperdiploid acute lymphoblastic leukemia: presence of preleukemic ancestral clones and the secondary nature of microdeletions and RTK-RAS mutations. *Leukemia*. 2010;24(5):924-931.

262.Xu W, Lu X, Kim Y, et al. Deletion of 14q24.1 approximately q24.3 in a patient with acute lymphoblastic leukemia: a hidden chromosomal anomaly detected by array-based comparative genomic hybridization. *Cancer Genet Cytogenet*. 2008;185(1):43-46.

263.Kuiper RP, Waanders E, van der Velden VH, et al. IKZF1 deletions predict relapse in uniformly treated pediatric precursor B-ALL. *Leukemia*. 2010;24(7):1258-1264.

264.Irving JA, Bloodworth L, Bown NP, Case MC, Hogarth LA, Hall AG. Loss of heterozygosity in childhood acute lymphoblastic leukemia detected by genome-wide microarray single nucleotide polymorphism analysis. *Cancer Res*. 2005;65(8):3053-3058.

265.Usvasalo A, Savola S, Räty R, et al. CDKN2A deletions in acute lymphoblastic leukemia of adolescents and young adults: an array CGH study. *Leuk Res*. 2008;32(8):1228-1235.

266.Yasar D, Karadogan I, Alanoglu G, et al. Array comparative genomic hybridization analysis of adult acute leukemia patients. *Cancer Genet Cytogenet*. 2010;197(2):122-129.

267.Kim M, Choi JE, She CJ, et al. PAX5 deletion is common and concurrently occurs with CDKN2A deletion in B-lineage acute lymphoblastic leukemia. *Blood Cells Mol Dis*. 2011;47(1):62-66.

268.Ko DH, Jeon Y, Kang HJ, et al. Native ETV6 deletions accompanied by ETV6-RUNX1 rearrangements are associated with a favourable prognosis in childhood acute lymphoblastic leukaemia: a candidate for prognostic marker. *Br J Haematol*. 2011;155(4):530-533.

269.Waanders E, Scheijen B, van der Meer LT, et al. The origin and nature of tightly clustered BTG1 deletions in precursor B-cell acute lymphoblastic leukemia support a model of multiclonal evolution. *PLoS Genet*. 2012;8(2):e1002533.

270.Najar M, Raicevic G, Jebbawi F, et al. Characterization and functionality of the CD200-CD200R system during mesenchymal stromal cell interactions with T-lymphocytes. *Immunol Lett*. 2012.

271.McGrath MM, Najafian N. The role of coinhibitory signaling pathways in transplantation and tolerance. *Front Immunol*. 2012;3:47.

272.Romero I, Martinez M, Garrido C, et al. The tumour suppressor Fhit positively regulates MHC class I expression on cancer cells. *J Pathol*. 2012.

273.Zhang J, Pickering CR, Holst CR, Gauthier ML, Tlsty TD. p16INK4a modulates p53 in primary human mammary epithelial cells. *Cancer Res*. 2006;66(21):10325-10331.

274.Eguchi-Ishimae M, Eguchi M, Maki K, et al. Leukemia-related transcription factor TEL/ETV6 expands erythroid precursors and stimulates hemoglobin synthesis. *Cancer Sci*. 2009;100(4):689-697.

275.Carneiro FR, Ramalho-Oliveira R, Mognol GP, Viola JP. Interferon regulatory factor 2 binding protein 2 is a new NFAT1 partner and represses its transcriptional activity. *Mol Cell Biol*. 2011;31(14):2889-2901.

276.Childs KS, Goodbourn S. Identification of novel co-repressor molecules for Interferon Regulatory Factor-2. *Nucleic Acids Res*. 2003;31(12):3016-3026.

277.Koeppel M, van Heeringen SJ, Smeenk L, Navis AC, Janssen-Megens EM, Lohrum M. The novel p53 target gene IRF2BP2 participates in cell survival during the p53 stress response. *Nucleic Acids Res*. 2009;37(2):322-335.

278.Myhre S, Mohammed H, Tramm T, et al. In silico ascription of gene expression differences to tumor and stromal cells in a model to study impact on breast cancer outcome. *PLoS One*. 2010;5(11):e14002.

279.Singh N, Wiltshire TD, Thompson JR, Mer G, Couch FJ. Molecular basis for the association of microcephalin (MCPH1) protein with the cell division cycle protein 27 (Cdc27) subunit of the anaphase-promoting complex. *J Biol Chem*. 2012;287(4):2854-2862.

280.Zeng L, Zhang Q, Li S, Plotnikov AN, Walsh MJ, Zhou MM. Mechanism and regulation of acetylated histone binding by the tandem PHD finger of DPF3b. *Nature*. 2010;466(7303):258-262.

281.Zanic M, Stear JH, Hyman AA, Howard J. EB1 recognizes the nucleotide state of tubulin in the microtubule lattice. *PLoS One*. 2009;4(10):e7585.

282.Casagrande G, te Kronnie G, Basso G. The effects of siRNA-mediated inhibition of E2A-PBX1 on EB-1 and Wnt16b expression in the 697 pre-B leukemia cell line. *Haematologica*. 2006;91(6):765-771.

283.Vilagos B, Hoffmann M, Souabni A, et al. Essential role of EBF1 in the generation and function of distinct mature B cell types. *J Exp Med*. 2012;209(4):775-792.

284.Yang JJ, Bhojwani D, Yang W, et al. Genome-wide copy number profiling reveals molecular evolution from diagnosis to relapse in childhood acute lymphoblastic leukemia. *Blood*. 2008;112(10):4178-4183.

285.Den Boer ML, van Slegtenhorst M, De Menezes RX, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. *Lancet Oncol*. 2009;10(2):125-134.

286.Harvey RC, Mullighan CG, Wang X, et al. Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. *Blood*. 2010;116(23):4874-4884.

287.Meding S, Balluff B, Elsner M, et al. Tissue Based Proteomics Reveals FXYD3, S100A11 and GSTM3 as Novel Markers for Regional Lymph Node Metastasis in Colon Cancer. *J Pathol*. 2012.

288.El Ayachi I, Fernandez C, Baeza N, De Paula AM, Pesheva P, Figarella-Branger D. Spatiotemporal distribution of tenascin-R in the developing human cerebral cortex parallels neuronal migration. *J Comp Neurol*. 2011;519(12):2379-2389.

289.Dimke H, San-Cristobal P, de Graaf M, et al. γ-Adducin stimulates the thiazide-sensitive NaCl cotransporter. *J Am Soc Nephrol*. 2011;22(3):508-517.

290.Li M, Zhao H, Zhang X, et al. Inactivating mutations of the chromatin remodeling gene ARID2 in hepatocellular carcinoma. *Nat Genet*. 2011;43(9):828-829.

291.Adam de Beaumais T, Fakhoury M, Medard Y, et al. Determinants of mercaptopurine toxicity in paediatric acute lymphoblastic leukemia maintenance therapy. *Br J Clin Pharmacol*. 2011;71(4):575-584.

292.Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*. 1995;10(1):111-113.

293.Weisberg IS, Jacques PF, Selhub J, et al. The 1298A-->C polymorphism in methylenetetrahydrofolate reductase (MTHFR): in vitro expression and association with homocysteine. *Atherosclerosis*. 2001;156(2):409-415.

294.Tirona RG, Leake BF, Merino G, Kim RB. Polymorphisms in OATP-C: identification of multiple allelic variants associated with altered transport activity among European- and African-Americans. *J Biol Chem*. 2001;276(38):35669-35675.

295.van de Steeg E, van der Kruijssen CM, Wagenaar E, et al. Methotrexate pharmacokinetics in transgenic mice with liver-specific expression of human organic anion-transporting polypeptide 1B1 (SLCO1B1). *Drug Metab Dispos*. 2009;37(2):277-281.

296.Samuelsson J, Alonso S, Ruiz-Larroya T, Cheung TH, Wong YF, Perucho M. Frequent somatic demethylation of RAPGEF1/C3G intronic sequences in gastrointestinal and gynecological cancer. *Int J Oncol*. 2011;38(6):1575-1577.

297.Nicoletti P, Cartsos VM, Palaska PK, Shen Y, Floratos A, Zavras AI. Genomewide pharmacogenetics of bisphosphonate-induced osteonecrosis of the jaw: the role of RBMS3. *Oncologist*. 2012;17(2):279-287.

298.Krishnamurthy P, Schwab M, Takenaka K, et al. Transporter-mediated protection against thiopurine-induced hematopoietic toxicity. *Cancer Res*. 2008;68(13):4983-4989.

299.Abla N, Chinn LW, Nakamura T, et al. The human multidrug resistance protein 4 (MRP4, ABCC4): functional analysis of a highly polymorphic gene. *J Pharmacol Exp Ther*. 2008;325(3):859-868.

300.Kruh GD, Belinsky MG, Gallo JM, Lee K. Physiological and pharmacological functions of Mrp2, Mrp3 and Mrp4 as determined from recent studies on gene-disrupted mice. *Cancer Metastasis Rev*. 2007;26(1):5-14.

301.Chen ZS, Lee K, Walther S, et al. Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. *Cancer Res*. 2002;62(11):3144-3150.

302.Nies AT, Keppler D. The apical conjugate efflux pump ABCC2 (MRP2). *Pflugers Arch*. 2007;453(5):643-659.

303.Gerk PM, Vore M. Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. *J Pharmacol Exp Ther*. 2002;302(2):407-415.

304.Phua SL, Sivakamasundari V, Shao Y, et al. Nuclear accumulation of an uncapped RNA produced by Drosha cleavage of a transcript encoding miR-10b and HOXD4. *PLoS One*. 2011;6(10):e25689.

305.Lin J, Horikawa Y, Tamboli P, Clague J, Wood CG, Wu X. Genetic variations in microRNA-related genes are associated with survival and recurrence in patients with renal cell carcinoma. *Carcinogenesis*. 2010;31(10):1805-1812.

306.Liu J, Wei M, He Y, et al. Genetic Variants in the MicroRNA Machinery Gene GEMIN4 Are Associated with Risk of Prostate Cancer: A Case-control Study of the Chinese Han Population. *DNA Cell Biol*. 2012.

307.Fernandez de Larrea C, Navarro A, Tejero R, et al. Impact of MIRSNPS on survival and progression in patients with multiple myeloma undergoing autologous stem cell transplantation. *Clin Cancer Res*. 2012.

ANNEX III