



UNIVERSITÀ  
DEGLI STUDI DI TRIESTE

**XVI CICLO DEL DOTTORATO DI RICERCA IN  
SCIENZE DELLA RIPRODUZIONE**

Indirizzo: MEDICINA MATERNO INFANTILE  
PERINATOLOGIA

**Sviluppo di strategie farmacologiche per la  
personalizzazione della terapia della leucemia  
linfoblastica acuta nel bambino**

Settore scientifico disciplinare: BIO/14 - FARMACOLOGIA

DOTTORANDA

Margherita Londero

COORDINATORE

Prof. Giuliana Decorti

SUPERVISORE DI TESI

Prof. Giuliana Decorti

ANNO ACCADEMICO 2012 / 2013

## **Abstract**

L'attività dell'enzima tiopurina-S-metil transferasi (TPMT) è un determinante importante di eventi avversi severi durante il trattamento della leucemia linfoblastica acuta (LLA) con l'antimetabolita mercaptopurina. Recentemente è stato dimostrato che la proteina PACSIN2 modula l'attività di TPMT e la tossicità indotta da mercaptopurina, mediante un meccanismo molecolare che si ipotizza riguardi la regolazione dell'autofagia.

Nell'ambito del protocollo italiano per il trattamento della LLA AIEOP 2009, si vogliono sviluppare strategie farmacologiche (farmacogenetiche, farmacocinetiche e farmacodinamiche) *in vitro* da integrare agli attuali parametri di risposta del paziente per personalizzare la terapia. Queste strategie comprendono la valutazione dell'attività e di polimorfismi genetici di enzimi importanti per la biotrasformazione della mercaptopurina, ovvero TPMT ed inosina trifosfato-pirofosfatasi (ITPA), della concentrazione dei metaboliti attivi della mercaptopurina e della sensibilità *in vitro* dei blasti dei pazienti raccolti alla diagnosi e trattati con diversi farmaci antitumorali. Si vuole poi validare l'effetto dei polimorfismi di PACSIN2 sull'attività dell'enzima TPMT. I dati preliminari ottenuti sostengono il ruolo dei polimorfismi d'interesse sulla farmacocinetica della mercaptopurina. In particolare, la casistica considerata finora valida un contributo dello SNP di PACSIN2 rs2413739 sull'attività enzimatica di TPMT. Lo studio è in continuo aggiornamento. Il suo ampliamento e l'integrazione dei dati farmacologici con i dati clinici dei pazienti contribuiranno a

comprendere l'impatto di queste variabili farmacocinetiche/farmacogenomiche sull'efficacia e la tossicità del trattamento con tiopurine.

Per determinare se PACSIN2 e l'autofagia contribuiscono alla variabilità interindividuale nell'attività di TPMT e nella suscettibilità alla tossicità da mercaptopurina abbiamo eseguito degli esperimenti in cellule con meccanismo di autofagia alterato (ovvero fibroblasti murini embrionali, MEF, da topi con ATG7 disattivato) e alterazione di PACSIN2 (cellule NALM6 con silenziamento di PACSIN2). Le cellule con meccanismo di autofagia alterato esprimono costitutivamente livelli più alti di PACSIN2 endogeno; questo avviene anche per altre proteine correlate all'autofagia come p62. Il trattamento con rapamicina induce la degradazione di PACSIN2 nelle cellule con autofagia funzionante, ma non in quelle con meccanismo di autofagia alterato. Il silenziamento dell'espressione di PACSIN2 ha indotto un aumento nel livello basale di autofagia, come documentato dall'accumulo di LC3-II e autofagosomi. La sequenza proteica di PACSIN2 contiene due siti di legame per LC3 e la co-immunoprecipitazione di PACSIN2 e LC3 dimostra l'interazione delle due proteine nelle linee cellulari NALM6. La stabilità di TPMT è diminuita quando l'espressione di PACSIN2 è alterata, in confronto a cellule con livelli normali di PACSIN2. Qui dimostriamo che PACSIN2 è bona fide una proteina dell'autofagia e che il suo ruolo come modulatore dell'autofagia influenza la variabilità interindividuale nell'attività di TPMT.

## INTRODUZIONE

### **La leucemia linfoblastica acuta**

Il termine “leucemia” indica una neoplasia maligna dovuta ad una proliferazione incontrollata dei precursori degli elementi corpuscolati del sangue con arresto della differenziazione, che determina un’invasione nel sangue e nei tessuti periferici (De Vita et al., 1997). In base alla linea cellulare da cui evolve il clone leucemico e alla velocità di progressione della malattia si definiscono quattro tipologie principali: la *leucemia linfoblastica acuta* (LLA), la *leucemia linfatica cronica*, la *leucemia mieloide acuta* e la *leucemia mieloide cronica*.

#### *Quali sono le dimensioni del problema?*

Secondo il Rapporto dell’Associazione Italiana Registri Tumori (AIRTUM) del 2012 la LLA è il tipo di leucemia più frequente in età pediatrica (80%) e rappresenta il 24% dei tumori registrati in Italia nel periodo 2003-2008 per la fascia di età 0-19 anni.

In particolare sempre secondo la stessa fonte il tasso di incidenza della LLA in Italia è di 36,7 casi per milione (IC95%:34,2-39,3) nella fascia d’età 0-19 anni con un picco tra i bambini di età compresa tra 1-4 anni (81,6 casi per milione; 87,9 nei maschi e 74,9 nelle femmine) (Figura 1).

- L'AIEOP (Associazione Italiana Ematologia e Oncologia Pediatrica)

In Italia il trattamento della LLA nella popolazione pediatrica è standardizzato secondo le linee guida dell'Associazione Italiana di Ematologia e Oncologia Pediatrica (AIEOP). Questa associazione dal 1975 promuove l'elaborazione di protocolli per la diagnosi e la cura dei pazienti pediatrici affetti da patologia oncoematologica. La rete nazionale AIEOP è costituita oggi da 54 centri clinici, nei quali nel solo triennio 2008-2010 sono stati trattati 4488 bambini (0-14 anni), pari al 92% della totalità dei casi attesi in Italia (stime di incidenza AIRTUM).

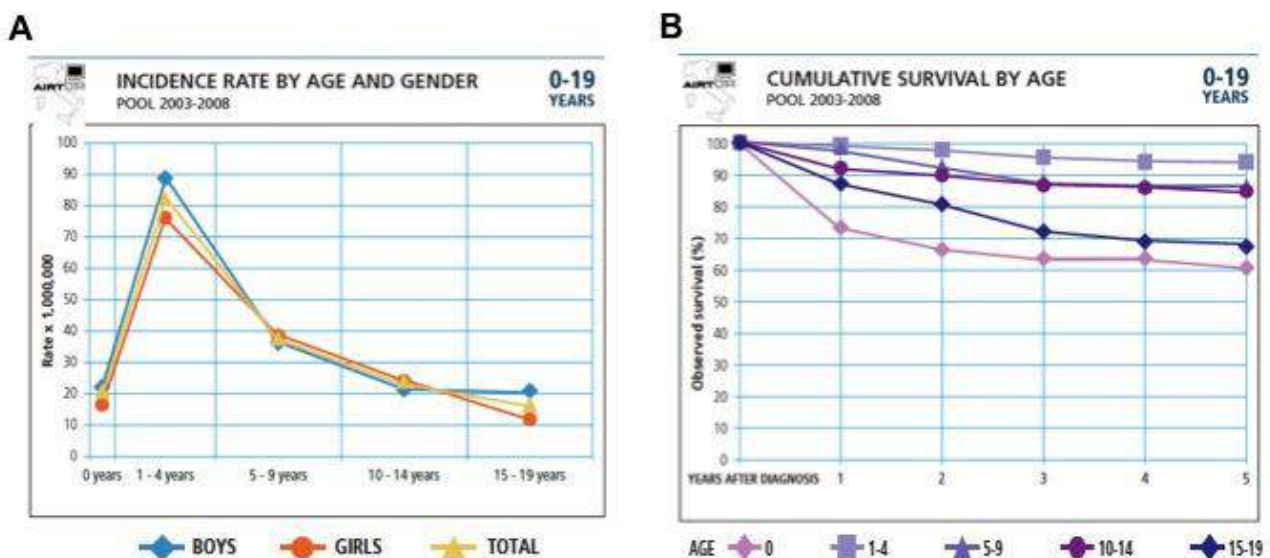


Figura 1 : LLA pediatrica in Italia (periodo 2003-2008, Rapporto ARTIUM 2012). A) incidenza della malattia suddivisa per fasce d'età e genere; B) sopravvivenza cumulativa a 5 anni dalla diagnosi per fasce d'età.

## **Protocollo AIEOP LLA 2009**

Il protocollo AIEOP LLA 2009 (identificativo NCT01117441, <http://clinicaltrials.gov>) è il protocollo collaborativo internazionale attualmente in vigore in Italia per il trattamento di bambini e adolescenti affetti da LLA; i criteri di eleggibilità sono rappresentati da:

- diagnosi di leucemia linfoblastica acuta di nuova diagnosi di tipo non-B matura
- non LLA Ph+ (BCR/ABL o t(9;22)-positiva)
- età compresa fra 1 anno (> 365 giorni) e 18 anni (sino a 17 anni e 364 giorni)
- non evidenza di gravidanza o periodo di allattamento
- non partecipazione in altri studi clinici
- paziente arruolato in un centro AIEOP partecipante (con approvazione CE) allo studio
- consenso informato firmato.

In base al protocollo i pazienti vengono stratificati in tre gruppi di rischio (standard, medio e alto) e trattati con regimi terapeutici di intensità differente in base alla categoria di appartenenza.

L'attribuzione ad una fascia di rischio viene effettuata in base a criteri biologici e clinici. In particolare rientrano in una classificazione di alto rischio i soggetti con:

- Prednisone poor response (PPR), intesa come una inadeguata clearance dei blasti dal sangue periferico al giorno +8, ovvero una conta superiore a 1000 cellule/mm<sup>3</sup>.
- Positività della Malattia Residua Minima (MRM) valutata al giorno +15 sul sangue periferico mediante tecniche citofluorimetriche e al giorno +33 sul sangue midollare mediante PCR-quantitativa, utilizzando oligonucleotidi sequenza-specifici e sonde fluorescenti con sensibilità da 10<sup>-3</sup> a 10<sup>-4</sup> (capaci cioè di individuare 1 blasto leucemico ogni 1000-10.000 cellule normali nel midollo osseo). La MRM ha dimostrato una netta superiorità rispetto ai fattori prognostici convenzionali nella stratificazione dei pazienti in gruppi di rischio, ossia si è rivelata maggiormente predittiva di ricaduta (Schrappe et al., 2011).
- Positività per MLL/AF4 o t(4;11)
- Ipodiploidia (< 45 cromosomi)

Il trattamento della LLA si fonda sull'approccio polichemioterapico, ovvero sull'utilizzo di più farmaci antitumorali combinati tra loro in regimi terapeutici di differente intensità in base alla classe di rischio del paziente. Questo approccio, oltre a consentire una maggiore efficacia terapeutica, permette anche la riduzione dei dosaggi dei singoli farmaci impiegati e quindi della loro potenziale tossicità. Il trattamento della LLA è organizzato per fasi terapeutiche, di seguito descritte. Nella figura viene proposta una loro rappresentazione schematica per il protocollo AIEOP LLA 2009.

**Pre-fase:** inizia il giorno della diagnosi della malattia e dura 7 giorni. Consiste in una finestra monoterapica con prednisone (PDN) ad alte dosi ed un'unica somministrazione di metotressato intratecale al giorno +1. E' stato dimostrato che un'adeguata risposta del paziente (PGR) a questa prima settimana di trattamento a base di glucocorticoidi rappresenta uno dei fattori prognostici più importanti di successo della cura (Gajjar et al., 1995).

**Induzione della remissione:** Questa fase, che ha lo scopo di ottenere la *remissione completa* della malattia, dura 8 settimane, è strutturata in due protocolli successivi (IA: dal giorno 8 al giorno 36, IB: dal giorno 36 al giorno 64) e consiste nella somministrazione quotidiana di PDN ad alte dosi per via orale dal giorno 8 al 29 (seguito da una settimana di scalo dello steroide) e di

mercaptopurina ad alte dosi *per os* dal giorno 36 al giorno 64. In associazione con questi, viene somministrato il metotressato per via intratecale (giorno 12, 33, 45 e 59) e per via endovenosa, vengono somministrate la vincristina (VCR) e la daunorubicina (DNR, entrambe al giorno 8, 15, 22, 29), la PEG-asparaginasi (PEG-L-Asp, giorno 12, 26, 36 e 64), la ciclofosfamida (CPM, giorno 36 e 64) e la citarabina (Ara-c, 4 cicli di 3 giorni, dal giorno 38 fino al giorno 62) (Figura 2). I pazienti che presentano tutti i fattori prognostici positivi (sono PGR, non presentano t(4;11), presentano remissione midollare completa al giorno +33 e MRM negativa ai giorni +15, +33 e +78), vengono sottoposti ad una riduzione del dosaggio della DNR e della VCR (ricevono due dosi rispetto alle 4 previste). L'ottimizzazione di questa fase ha permesso di ottenere un tasso di remissione completa superiore al 95% (Pediatric reports, XXXVII Congresso Nazionale AIEOP).

**Fase d'intensificazione o consolidamento:** E' stato dimostrato che questa fase è necessaria per la completa deplezione di cellule tumorali dall'organismo e diminuisce il rischio di ricaduta (Pui et al., 2006). Il trattamento inizia quando viene ripristinata la normale ematopoiesi del paziente dopo l'induzione e consiste nella somministrazione giornaliera di mercaptopurina a basse dosi *per os* per circa 8 settimane, associata a un'infusione bi-settimanale di metotressato ad alte dosi. La co-somministrazione di questi due farmaci ha mostrato un effetto sinergico *in vitro* dovuto al fatto che il

metotressato inibisce le prime tappe della sintesi delle purine (Liliemark et al.,1992). Per i pazienti ad alto rischio (HR) questa fase consiste in tre blocchi polichemioterapici ad alte dosi della durata di sei giorni ciascuno, in cui viene somministrato quotidianamente desametasone (DXM) *per os* in associazione con altri farmaci come descritto in figura 4.

**Fase di reinduzione:** Questa fase, molto simile alla fase di induzione, si differenzia da quest'ultima per la durata inferiore (6 settimane) (Protocollo II). Rispetto ai protocolli IA e IB, vengono utilizzati il DXM al posto del PDN, la doxorubicina (DOX) piuttosto che la DNR e la tioguanina invece della mercaptopurina (Figura 3). I pazienti HR seguono un protocollo di reinduzione modificato (Protocollo III) (Figura 3) della durata di circa un mese che prevede la somministrazione quotidiana di DXM *per os* dal giorno 1 al 14 (seguito da una settimana di scalo dello steroide) in associazione con singole dosi endovenose di VCR, DOX (entrambe al giorno 1 e 8) e PEG-L-Asp (al giorno 1). È previsto inoltre l'uso continuato di tioguanina *per os* dal giorno 15 al giorno 28 insieme a singole dosi di metotressato intratecale (giorni 17 e 24) e a due infusioni di Ara-c endovena della durata di 3 giorni (giorni 17 e 24). Il protocollo III risulta più intenso rispetto al protocollo II in quanto viene ripetuto tre volte, intervallando il "mantenimento ad interim" costituito da 29 giorni di terapia con mercaptopurina (somministrata giornalmente) e metotressato (somministrato settimanalmente).

**Fase di mantenimento:** Il trattamento si conclude con questa fase che prevede la somministrazione giornaliera di mercaptopurina e metotressato (*per os*) per un periodo che varia dai 18 ai 20 mesi (in modo da ottenere una durata complessiva del trattamento di 2 anni dall'esordio della malattia). Questa fase è anche necessaria in quanto preventiva nei confronti di un'eventuale ricaduta.

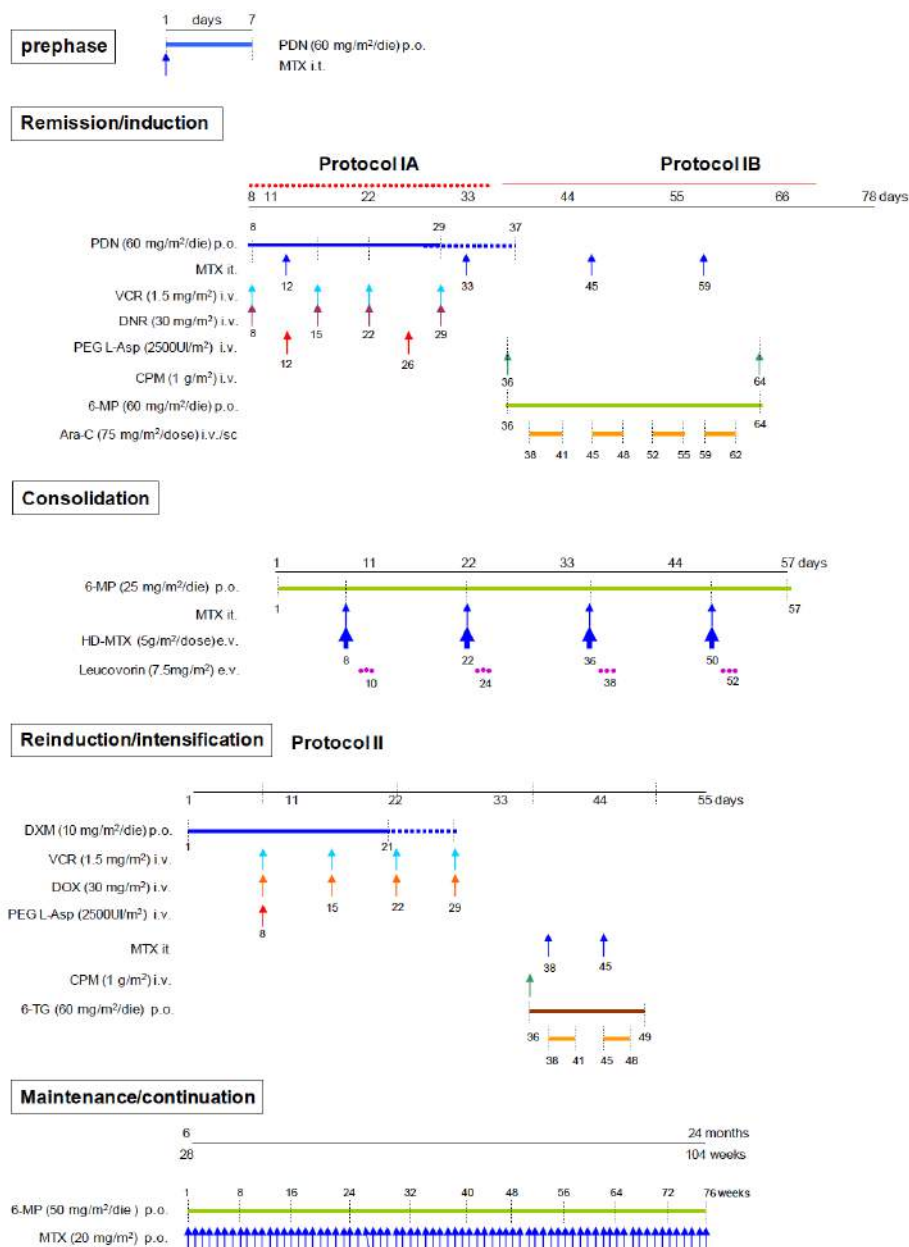


Figura 2: Rappresentazione schematica delle diverse fasi secondo protocollo AIEOP-BFM ALL 2009. 6-MP = mercaptopurina, 6-TG = -tioguanina, Ara-C = citarabina, CPM = ciclofosfamida, DNR = daunorubicina, DOX = doxorubicina, DXM = desametasone, HD-metotressato = metotrexato ad alte dosi, metotressato = metotrexato, PDN = prednisone, PEG-L-ASP = PEG-asparaginasi, VCR = vincristina.

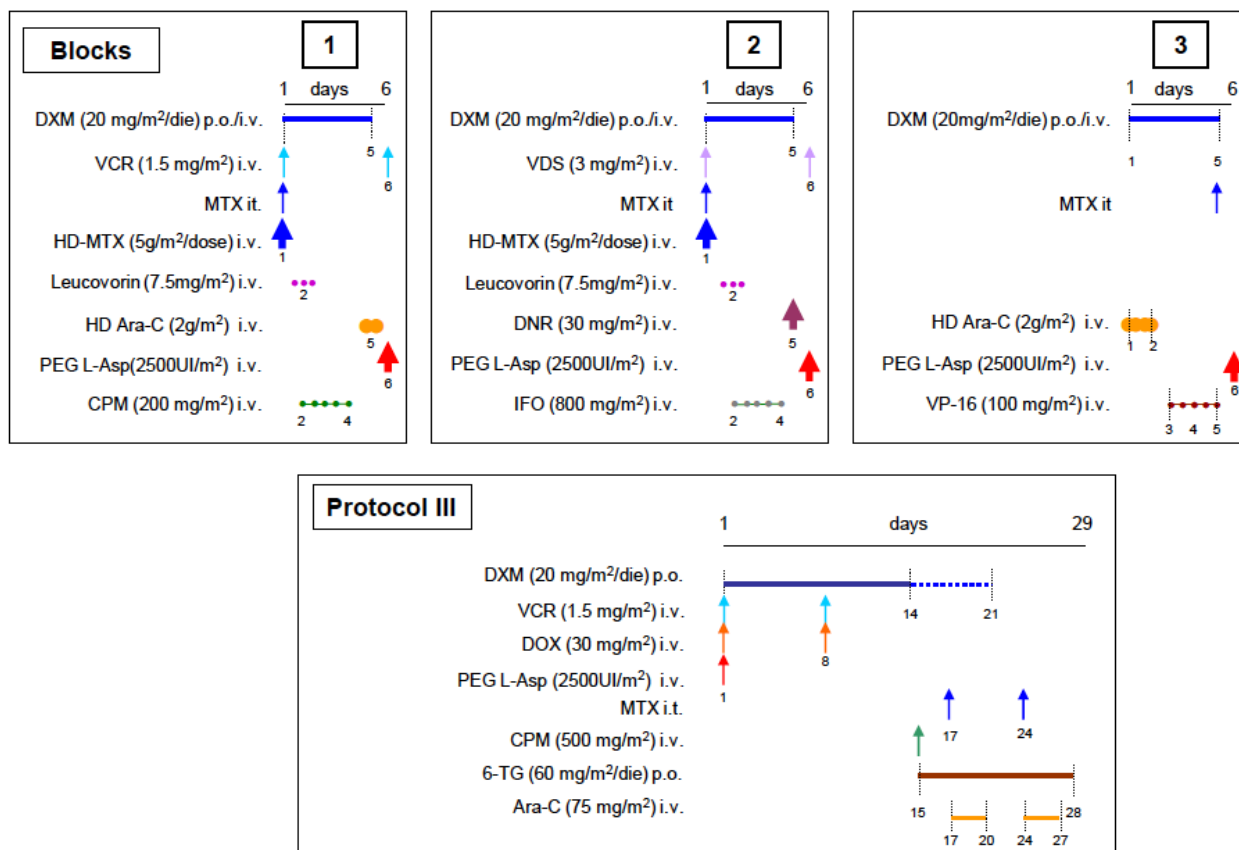


Figura 3:: Schema dei blocchi e della fase di reinduzione per i pazienti high-risk AIEOP-BFM ALL 2009, 6-TG = tioguanina, Ara-C = citarabina, CPM = ciclofosfamide, DNR = daunorubicina, DOX = doxorubicina, DXM = desametasone, HD-Arac = citarabina ad alte dosi, HD-metotressato = metotrexato ad alte dosi, IFO = ifosfamide, MTX = metotrexato, PDN = prednisone, PEG-L-ASP = PEG-asparaginasi, VCR = vincristina, VDS = vindesina, VP16 = etoposide.

## Tattamento diretto contro le ricadute del sistema nervoso centrale (SNC)

L'introduzione della radioterapia craniale (RTC) combinata con chemioterapia intratecale ha consentito di ottenere una riduzione della ricaduta a livello del SNC dal 50% dei casi al 10% circa. L'uso di questa metodica si basa sul presupposto che il SNC è difficilmente raggiungibile dai chemioterapici somministrati per via sistemica. Tuttavia in anni recenti si è gradualmente modificata

questa strategia per ridurre gli effetti tossici legati al trattamento preventivo (Pui et al., 2009), ed attualmente la RTC viene somministrata solo ai pazienti a più alto rischio di recidiva mentre in tutti i pazienti viene somministrato il metotressato per via intratecale.

Un punto rilevante nel trattamento delle LLA pediatriche è rappresentato dalle risposte terapeutiche inattese, che si verificano in circa il 20% dei casi (Fullmer et al., 2009): pazienti a basso rischio possono non reagire in modo adeguato o sviluppare tossicità acute, mentre pazienti ad alto rischio possono guarire senza complicazioni, nonostante il loro quadro clinico sia sfavorevole. Tra le possibili cause di questa variabilità interindividuale potrebbe esserci la presenza di polimorfismi dei geni coinvolti nel metabolismo o nella distribuzione dei farmaci utilizzati.

Il problema della recidiva durante la terapia o precocemente dopo la sospensione della stessa rappresenta ancora una delle complicazioni più rilevanti (Kerst et al., 2005; Fullmer et al., 2009).

Le recidive sono probabilmente dovute alla persistenza di un clone

leucemico "resistente" alla terapia convenzionale. I pazienti ricaduti vengono inseriti nel protocollo a più alto rischio e l'approccio terapeutico più efficace per la maggior parte di essi è rappresentato dal trapianto di midollo osseo allogenico (Pediatric Reports, XXXVII Congresso Nazionale AIEOP, 2012).

### **Personalizzazione della terapia**

Da cosa nasce l'esigenza di personalizzare la terapia dei pazienti pediatrici affetti da LLA?

Per rispondere a questa domanda è necessario considerare che benchè la percentuale di cura dei pazienti pediatrici affetti da tale patologia sia alta (secondo il rapporto AIRTUM 2012 la sopravvivenza in bambini diagnosticati fra 1 e 4 anni è del 93%), a parità di diagnosi e di trattamento vi è ancora un 20% di risposta terapeutica inattesa (Fullmer et al, 2009). In particolare, i problemi principali restano quelli della ricaduta (intesa come ripresa di malattia successiva all'ottenimento della remissione completa) e della tossicità.

Analizzando i dati relativi ai pazienti pediatrici trattati secondo il protocollo AIEOP BFM 2000 emerge che il 15.3% (306) dei pazienti arruolati ha presentato una ricaduta, e che il 63% dei 785 pazienti arruolati nel periodo compreso fra il 2002 e il 2006 ha presentato una tossicità di grado severo (III-IV). Tra i pazienti trattati dall'Oncoematologia dell'IRCCS Burlo Garofolo in particolare nello stesso periodo la percentuale di tossicità grave è stata del 36%.

### **Ruolo della farmacogenetica**

Al fine di prevenire la tossicità indotta dalle terapie quale contributo si può ottenere dalla farmacogenetica?

A questo proposito uno degli esempi più interessanti di applicazione della farmacogenetica nella pratica clinica in campo oncologico è la personalizzazione della terapia con mercaptopurina sulla base del genotipo di TPMT. I farmaci mercaptopurina e tioguanina vengono metilati direttamente dall'enzima tiopurinametiltransferasi (TPMT). Si stima che circa un paziente ogni 300 abbia attività TPMT completamente assente (difetto in omozigosi) e che circa il 10% della popolazione generale abbia attività ridotta, dovuta a un

polimorfismo in eterozigosi (Weinshilboum, 2001). E' stato dimostrato che somministrare la stessa dose di farmaco ai pazienti senza tenere in considerazione l'assetto genetico espone i soggetti a livelli di esposizione sistemica al farmaco e di tossicità differenti (Cheek and Evans, 2006). Infatti i soggetti con ridotta o nulla attività TPMT sono esposti a concentrazioni più alte di farmaci analoghi purinici e dei metaboliti attivi; questo li espone a un maggiore rischio di sviluppare tossicità acuta.

In particolare gli effetti avversi sono la mielodepressione e più raramente la tossicità epatica, proporzionalmente al deficit di attività TPMT. Un gruppo internazionale ha quindi messo a punto un protocollo di modulazione del dosaggio della terapia con mercaptopurina sulla base del genotipo di TPMT. Attualmente negli Stati Uniti i soggetti omozigoti mutati per TPMT ricevono una drastica riduzione del dosaggio della 6 MP mentre i soggetti eterozigoti per la mutazione ricevono una dose ridotta pari a 60 mg/m<sup>2</sup>. E' stato dimostrato che questo approccio permette di ottimizzare la terapia riducendo gli effetti collaterale senza inficiare l'efficacia del trattamento (Relling et al, 2013).

Anche nei protocolli pediatrici di trattamento della LLA in Italia viene effettuato un aggiustamento della dose di mercaptopurina sulla base del genotipo di TPMT, con la differenza che la riduzione della terapia è prevista unicamente per i pazienti che presentano attività TPMT assente per difetti genetici in omozigosi. In particolare in questi soggetti viene somministrata una dose iniziale di mercaptopurina pari al 25% della dose standard; se tale dose risulta ben tollerata il dosaggio non viene modificato per 4 settimane. Successivamente la dose viene modulata gradualmente (es. ogni 2-3 settimane) sulla base delle conte ematologiche.

### **PACSIN2 e attività di TPMT**

Un precedente studio (Stocco et al., 2012) ha documentato che PACSIN2 modula l'attività di TPMT e che uno SNP in PACSIN2 è associato in maniera significativa con l'incidenza di tossicità gastrointestinale da mercaptopurina in bambini affetti da LLA. Benchè l'importanza dei polimorfismi genetici di TPMT nel determinare la risposta alla mercaptopurina e la tossicità ematologica nei bambini affetti da LLA siano ben documentate

( clinicamente ma anche dal punto di vista del meccanismo molecolare) (Relling et al., 2013), questo studio rappresenta la prima evidenza *in vivo* che anche la variante genetica di un secondo gene (PACSIN2) può influenzare l'attività di TPMT.

PACSIN2 è stato identificato da analisi genome-wide per SNP e di espressione genica delle cellule umane HapMap (Peters et al., 2009, Wheeler et al., 2012), per le quali è stata determinata l'attività di TPMT (Jones et al., 2007) e l'associazione è stata validata nei pazienti. Infatti, il gene più significativo per l'associazione con l'attività di TPMT nelle linee cellulari HapMap basate sugli SNPs e sull'espressione genica (dopo l'aggiustamento per il genotipo di TPMT) è stato PACSIN2, che è portatore di uno SNP (rs2413739) che è poi risultato significativamente associato sia con l'attività di TPMT sia con la tossicità gastrointestinale severa in bambini che ricevono terapia con mercaptopurina per il trattamento della LLA. Studi molecolari *in vitro* hanno indicato che, il knock-down dell'mRNA di PACSIN2 in cellule umane leucemiche (NALM6) con iperespressione di TPMT porta a un'attività significativamente

inferiore di TPMT ed una minore produzione dell'mRNA di questo gene.

PACSIN2, anche chiamato syndapin II, è un membro delle proteine substrato della protein chinasi C e della casein chinasi nei neuroni; esse sono coinvolte nel legare il citoscheletro di actina con la formazione di vescicole, regolando la polimerizzazione della tubulina ed esercitano la loro funzione prevalentemente attraverso interazioni proteina-proteina con differenti substrati, come la dinamina o N-WASP (Kessels et al., 2004). Ci sono dati che indicano come PACSIN abbia un ruolo in vari processi biologici, comprendenti l'endocitosi (Modregger et al., 2000), il controllo del ciclo cellulare (Meng et al., 2011) e l'autofagia (Szyniarowski et al., 2011).

Per identificare ulteriori geni e processi biologici potenzialmente alterati da PACSIN2, lo studio ha identificato i geni la cui espressione è cambiata significativamente in cellule umane di leucemia nelle quali il gene PACSIN2 è stato silenziato, mediante array Affymetrix GeneChip, rivelando che processi/pathways significativamente alterati dal knock-down di PACSIN2 in termini di

espressione genica sono quelli che riguardano l'actina e il citoscheletro (Kessels et al., 2004), l'organizzazione delle membrane (Wang et al., 2009) e l'autofagia (Szyanirowski et al., 2011). L'identificazione dell'autofagia come processo alterato dal silenziamento di PACSIN2 è stata principalmente determinata dalla riduzione nell'espressione di SH3GLB1; questa proteina, anche conosciuta come endofilina B1/BIF-1, interagisce con UVRAG-Beclin 1, e contribuisce all'attivazione dell'autofagia (Takahashi et al., 2007); è plausibile che SH3GLB1 abbia un ruolo nella fissione delle membrane dal Golgi durante la formazione dell'autofagosoma (Takahashi et al., 2011). L'effetto di PACSIN2 sull'autofagia è ulteriormente supportato dai cambiamenti significativi che sono stati osservati in un secondo gene C13orf18 (anche conosciuto come KIAA0226-like/PPP1R2P4), che è stato recentemente dimostrato essere coinvolto nell'autofagia (Behrends et al., 2010). Il ruolo di PACSIN2 nell'autofagia potrebbe essere coinvolto nel meccanismo dei suoi effetti sull'attività di TPMT, dal momento che l'autofagia è stata collegata alla degradazione della proteina di TPMT codificata dalla variante TPMT\*3A e in misura minore dall'allele wild-type

TPMT\*1 (Li et al., 2008). PACSIN2 potrebbe dunque influenzare l'attività di TPMT attraverso un effetto sui livelli di mRNA e/o sulla degradazione proteica di TPMT, mediata dall'autofagia. Ulteriori studi sono necessari per delucidare i meccanismi molecolari attraverso i quali PACSIN2 altera l'attività di TPMT ed in particolare il suo contributo nell'autofagia.

## SCOPO DELLA TESI

Questa tesi si inserisce all'interno di un progetto più articolato dell'ospedale pediatrico IRCSS Burlo Garofolo che ha lo scopo di sviluppare strategie farmacologiche per la personalizzazione delle diverse fasi del protocollo AIEOP LLA 2009 tramite un approccio multifattoriale che consiste nella valutazione, per ogni paziente arruolato prospetticamente, di:

- Variabili farmacogenomiche, analizzando i polimorfismi genetici a carico di enzimi coinvolti nella biotrasformazione dei farmaci, in particolare quelli coinvolti nel metabolismo della mercaptopurina;
- Parametri farmacodinamici, tramite il saggio di citotossicità *in vitro* sul pannello di chemioterapici in uso nella fase d'induzione nel protocollo AIEOP LLA 2009, messo a punto sulla base dei protocolli sperimentali proposti al St.Jude Children's Research Hospital di Memphis (USA);
- Parametri farmacocinetici della mercaptopurina e del metotressato nelle fasi di consolidamento e mantenimento: per la mercaptopurina attraverso la misurazione delle concentrazioni

plasmatiche dei metaboliti attivi e misurazione dell'attività degli enzimi ITPA e TPMT coinvolti nel suo metabolismo, mentre per il metotressato, attraverso la misurazione della clearance plasmatica in seguito alle infusioni ad alte dosi.

- Risposta clinica del paziente alle varie fasi della terapia. In particolare, i dati raccolti dalle cartelle cliniche da parte dei medici oncologi pediatri, comprendono: le caratteristiche demografiche dei pazienti, la risposta al PDN dopo la prima settimana di terapia, la MRM al giorno +15, +33, +78, eventuali tossicità (classificate secondo i criteri del National Cancer Institute statunitense) sviluppate nelle varie fasi terapeutiche, i dosaggi delle tiopurine e i dati delle metotressatemie e l'outcome finale.

Verranno valutate le associazioni tra i vari parametri farmacologici (farmacogenomici, farmacodinamici e farmacocinetici) e la risposta clinica del paziente. I parametri farmacologici di ogni paziente, saranno inoltre correlati tra di loro. Il progetto prevede il coinvolgimento di altri centri italiani affiliati all'AIEOP per aumentarne la casistica. Nello specifico, questa tesi si è occupata

delle genotipizzazioni dei pazienti finora arruolati, di ottimizzare il saggio per la fase d'induzione (sensibilità *in vitro* dei blasti) e per il consolidamento e il mantenimento (misura dei metaboliti tiopurinici e dell'attività enzimatica di ITPA e TPMT).

Inoltre, fra le caratteristiche genetiche candidate, questa tesi ha l'obiettivo di validare l'associazione fra l'attività di TPMT ed il polimorfismo genetico rs2413739 del gene PACSIN2, recentemente descritta in una popolazione di pazienti statunitense. Questo stesso studio ha indicato che il meccanismo molecolare con cui PACSIN2 influenza l'attività di TPMT e l'incidenza di tossicità gastrointestinale severa durante la fase di consolidamento, potrebbe essere la modulazione dell'autofagia. Sono stati quindi eseguiti una serie di esperimenti al fine di chiarire il potenziale ruolo di PACSIN2 nella modulazione dell'autofagia.

## **POPOLAZIONE DI STUDIO E MATERIALI E METODI**

### **Pazienti**

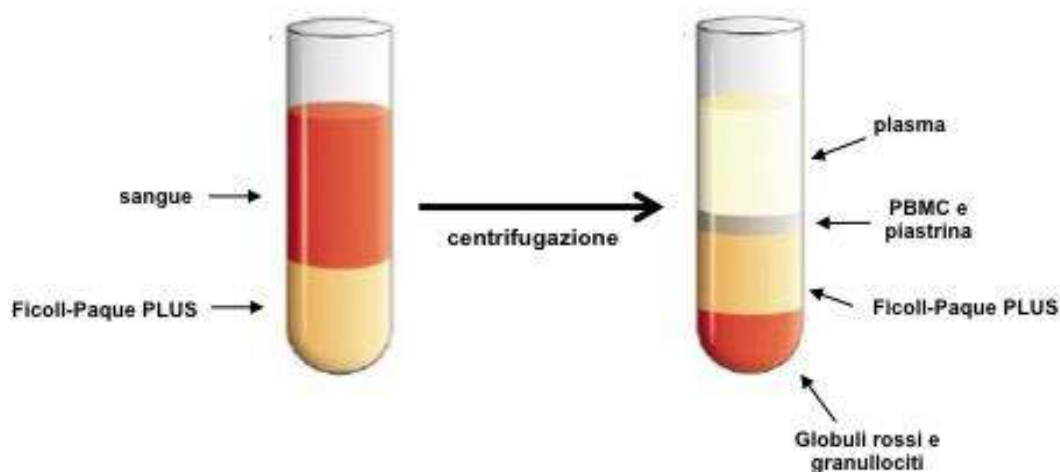
Sono rientrati nello studio pazienti all'esordio in età pediatrica (da 1 a 18 anni) affetti da LLA, arruolati presso il reparto di ematologia degli Ospedali pediatrici Burlo Garofolo di Trieste, Regina Margherita di Torino, San Gerardo di Monza e Bambino Gesù di Roma e trattati secondo il protocollo AIEOP LLA 2009.

Per ogni paziente, sono stati isolati i linfoblasti dall'aspirato midollare (prelevato alla diagnosi) per l'estrazione del DNA tumorale e la valutazione della loro sensibilità *in vitro* ai chemioterapici. Sono stati eseguiti inoltre dei prelievi di sangue periferico durante la fase di consolidamento della terapia (prima di ogni infusione bisettimanale di metotressato ad alte dosi) e durante la fase di mantenimento (in corrispondenza delle visite programmate di controllo al 3°, 9° e 15° mese), per l'estrazione del DNA somatico e per la quantificazione dei metaboliti tiopurinici e la valutazione dell'attività di ITPA. Lo studio è stato sottoposto ad approvazione da parte dei Comitati Etici dei centri partecipanti ed è

stato ottenuto il consenso informato dei tutori legali di tutti i pazienti arruolati.

### **Colture cellulari primarie: isolamento di linfoblasti da sangue midollare dei pazienti**

I linfoblasti dei pazienti sono stati isolati dall'aspirato midollare alla diagnosi, mediante centrifugazione in gradiente di densità su Ficoll Paque™ Plus (GE Healthcare, densità: 1,077 g/ml). Il sangue midollare è stato diluito 1:3 o 1:4 con il buffer HHH e 10 ml di campione diluito sono stati stratificati su 5 ml di Ficoll; il tutto è stato poi centrifugato a 800xg per 40 minuti a 4°C, ottenendo così la separazione dei vari elementi del sangue per migrazione differenziale (Figura 4).



*Figura 4: Separazione degli elementi del sangue per centrifugazione in gradiente di densità su Ficoll Paque™ Plus.*

I blasti e le cellule mononucleate concentrate nell'anello localizzato tra il plasma e il Ficoll sono stati recuperati e lavati 2 volte con 5 ml di buffer HHH (centrifughe intermedie a 300 xg per 5 minuti). Al termine dei lavaggi è stata effettuata la lisi dei globuli rossi rimasti sul pellet cellulare aggiungendo 2 ml di un buffer apposito (RBC Lysis Buffer) con un'incubazione massima di 4 minuti a 4°C. Per bloccare la reazione di lisi sono stati aggiunti 13 ml di terreno completo per blasti e si è centrifugato il tutto a 300xg per 5 minuti a 4°C. Le cellule sono state poi risospese in 2 ml dello stesso terreno ed è stata eseguita la conta vitale tramite Trypan Blue. I linfoblasti sono stati poi diluiti per ottenere la concentrazione finale di  $2 \times 10^6$  cellule/ml necessaria per il saggio MTT. Tutti i passaggi sono stati eseguiti in condizioni di sterilità.

## REAGENTI UTILIZZATI

- Buffer HHH: 0,1% Eparina Sodica (fiale da 5.000U.I./ml, Biologi Italia Laboratories), 1% HEPES Buffer 1M (H0887, Sigma-Aldrich), 10% di soluzione Hanks (H6648, Sigma-Aldrich).

Conservazione a 4°C;

- RBC Lysis Buffer: 1ml Buffer A, 1ml Buffer B, 8ml di H<sub>2</sub>O sterile e 5% (0.5ml) di FBS (Fetal Bovine Serum; F7524, Sigma-Aldrich). Da preparare fresco;
- Buffer A: 0.083 g/ml di NH<sub>4</sub>Cl e 0.37 mg/ml di Na<sub>2</sub>EDTA in 100 ml di H<sub>2</sub>O sterile. Conservazione a 4°C;
- Buffer B: 0.1M KHCO<sub>3</sub> in H<sub>2</sub>O sterile. Conservazione a 4°C;
- Terreno completo per blasti: RPMI 1640 Dutch modified (R7638, Sigma-Aldrich) addizionato con 10% di FBS heat inactivated (F4135, Sigma-Aldrich), 1% di soluzione di Penicillina/Streptomicina (P0781, Sigma-Aldrich), 1% di antimicotico (Amphotericin B; A2942, Sigma-Aldrich), 1% L-glutammina (Euroclone), 1% di ITS (I3146, Sigma-Aldrich) e 0.4% di gentamicina (G1397, Sigma-Aldrich).

## **Test di vitalità**

### *Saggio di esclusione del Trypan Blue*

Questo test permette di stimare la quantità di cellule vitali in una popolazione tramite l'utilizzo del Trypan Blue, un colorante che penetra nelle cellule danneggiate facendole assumere un colore blu

molto intenso e viene escluso invece dalle cellule con membrana intatta. La conta cellulare viene eseguita mediante emocitometro (camera contaglobuli di Burker) e microscopio ottico.

### *Saggio MTT*

Il saggio del bromuro di 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) è un saggio colorimetrico sensibile e quantitativo che misura la vitalità delle cellule (Mosmann, 1983). Esso si basa sulla capacità dell'enzima mitocondriale succinato deidrogenasi di convertire l'MTT (sostanza di colore giallo solubile in acqua) in un sale insolubile di colore blu scuro, il formazano (Figura 5). Dal momento che questa reazione può avvenire solo in cellule metabolicamente attive, la quantità di formazano prodotta risulta proporzionale al numero di cellule vive.



*Figura 5: Conversione del bromuro di 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio in formazano.*

La procedura del saggio MTT viene di seguito descritta in riferimento alla popolazione di studio considerata o alla linea cellulare.

### *Saggio MTT su blasti di pazienti*

I linfoblasti isolati dal sangue midollare di pazienti affetti da LLA sono stati posti in coltura in piastre sterili da 96 pozzetti seminando  $1.6 \times 10^5$  cellule per pozzetto in presenza o meno di farmaco in un volume finale di 100  $\mu$ l. Ciascuna piastra da 96 pozzetti presenta:

- i controlli (6 replicati) in cui a 80 $\mu$ l della sospensione di blasti ( $2 \times 10^6$ /ml in terreno completo) sono stati aggiunti 20 $\mu$ l di terreno RPMI–PSF;
- i trattati (in triplicato) in cui a 80 $\mu$ l della sospensione di blasti ( $2 \times 10^6$ /ml in terreno completo) sono stati aggiunti 20 $\mu$ l delle soluzioni a concentrazioni nota di ogni farmaco in RPMI-PSF (Tabella 1);
- i bianchi (6 replicati) in cui a 80 $\mu$ l del terreno completo per i blasti sono stati aggiunti 20 $\mu$ l di terreno RPMI–PSF. Nel caso delle tiopurine tioguanina e mercaptopurina, sono stati preparati anche dei bianchi specifici in cui a 20  $\mu$ l di ogni concentrazione di farmaco impiegata sono stati aggiunti 80 $\mu$ l di terreno completo per blasti.

La Tabella 1 riporta i farmaci testati in ordine di priorità e i range di concentrazioni finali raggiunte nei pozzetti con rispettivo fattore di diluizione seriale.

<b>Farmaci</b>	<b>Range di concentrazioni finali nel pozzetto (µg/ml)</b>	<b>Fattore di diluizione seriale</b>
PDN	0,008 – 250	8
VCR	0,05 - 50	4
ASP	0,03 – 10*	4
DNR	0,002 – 2	5
mercaptipurina	15,6 – 500	4
tioguanina	1,56 - 50	2
ARA-C	0,01 - 10	2
DXM	0,0002 - 6	8

*Tabella 1: Tabella riassuntiva dei farmaci utilizzati per il saggio MTT sui linfoblasti presentati in ordine prioritario, con il corrispettivo range di concentrazioni testati e il fattore di diluizione seriale utilizzato. 6-TG = tioguanina, Ara-C = citarabina, DNR = daunorubicina, DXM = desametasone, PDN = prednisone, Asp = asparaginasi, VCR = vincristina. \*concentrazione espressa in IU/ml.*

Le piastre così preparate sono state poste in termostato a 37°C per 96 ore in atmosfera contenente il 5% di CO<sub>2</sub>. Quattro ore prima

dello scadere del trattamento, ad ogni pozzetto sono stati aggiunti 10µl di MTT (5mg/ml) e le piastre sono state incubate nuovamente in termostato per permettere la formazione del sale di formazano. Il precipitato è stato quindi disciolto aggiungendo 100µl di isopropanolo acidificato e mantenuto a temperatura ambiente per 5 minuti in modo da permettere la sua completa solubilizzazione. Per valutare la quantità di sale prodotta dalle cellule vitali è stata misurata la densità ottica del contenuto di ciascun pozzetto mediante uno spettrofotometro per piastre (MicroPlate Reader, Bio-Tek Instruments) effettuando una doppia lettura alla lunghezza d'onda di 570 nm e di 630 nm. La percentuale di cellule vitali è stata calcolata come rapporto tra la densità ottica delle cellule trattate rispetto alla media delle cellule di controllo secondo la formula  $(Abs_{trattato} - Abs_{media\ controlli}) / Abs_{media\ controlli} \times 100$ , dopo opportuna sottrazione dei bianchi. Tali percentuali sono state riportate in grafico in funzione delle concentrazioni di farmaco usate, espresse in molarità e riportate in ascissa in scala logaritmica. I dati sono stati analizzati utilizzando il programma GraphPad Prism tramite una regressione non lineare ottenendo una curva (sigmoide) dose-

risposta da cui si è potuto estrapolare il valore di IC<sub>50</sub>, ossia la concentrazione di farmaco che riduce la vitalità cellulare del 50%. Qualora la curva dose-risposta non scenda sotto il 50% si ritiene che ci sia una resistenza e quindi viene assegnato un valore di IC<sub>50</sub> pari al doppio della concentrazione più alta usata nel pozzetto. Secondo letteratura può essere assegnato un punteggio numerico che va da 1 (sensibile) a 3 (resistente) per definire la sensibilità *in vitro* al PDN, VCR e ASP (PVA score) o insieme alla DNR (PVDA score). In particolare, il PVDA score viene valutato durante la fase di induzione della terapia IA (giorno +8 al giorno +33) (Den Boer et al., 2003).

- REAGENTI UTILIZZATI

- MTT (M2128, Sigma-Aldrich) 5mg/ml in NaCl 0.9%;
- Isopropanolo acidificato: 2-propanolo (I9516, Sigma-Aldrich) acidificato con HCl 0.04N, preparato almeno 14 giorni prima dell'uso e conservato a temperatura ambiente;
- RPMI-PSF: RPMI 1640 Dutch modified (R7638, Sigma-Aldrich) addizionato con 1% di soluzione di Penicillina/Streptomicina (10000 U/ml e 10000 mg/l; EuroClone) e 1% di antimicotico (Amphotericin B; A2942, Sigma-Aldrich);

- Farmaci: Metil-Prednisolone (PDN, Urbason, 50mg/ml, Aventis); Vincristina (VCR, Vincristina, 1mg/ml, Pfizer); Daunorubicina (DAU, Daunoblastina, 2mg/ml Pharmacia); L-asparaginasi (ASP, Kidrolase, 2000 UI/ml, EusaPharma); citarabina (ARA-C, Aracytin, 20mg/ml, Pharmacia); Tioguanina  $\geq$  98% (6-TG; Sigma-Aldrich); Mercaptopurina monoidrata 98% (mercaptopurina; Sigma- Aldrich); desametasone (DEX; Soldesam<sup>®</sup> 4mg/ml, Laboratorio Farmacologico Milanese). Le diluizioni seriali di ciascun farmaco sono state eseguite in RPMI-PSF.

### **Estrazione di DNA da sangue**

Il DNA è stato estratto per mezzo di un kit specifico (Gene Elute Blood Genomic DNA Kit; Sigma-Aldrich) seguendo il protocollo in dotazione; questa procedura prevede l'utilizzo di colonne contenenti una resina di silice capace di legare in maniera specifica il DNA, permettendone così la purificazione. In una provetta da 1,5 ml vengono aggiunti 20  $\mu$ l di Proteinasi K (in soluzione 20 mg/ml) e 200  $\mu$ l di sangue intero periferico o sangue midollare. Si aggiungono quindi 200  $\mu$ l di Lysis Buffer C, si agita la provetta su vortex per 15 secondi ai quali segue un'incubazione in bagnetto termostato di 10 minuti a 55°C al fine di lisare le cellule. Si procede intanto alla preparazione delle colonne aggiungendo 500  $\mu$ l di soluzione "Column Preparation" e centrifugandole per 1 minuto a 12000xg. Il DNA viene precipitato aggiungendo ai lisati 200 l di

etanolo al 95%-100% (Sigma-Aldrich), trasferito successivamente nelle colonne pretrattate e centrifugato per 1 minuto a 6500xg. La colonna contenente il DNA viene lavata con 500  $\mu$ l di Pre-wash solution e centrifugata per 1 minuto a 6500xg. Viene effettuato un secondo lavaggio con 500  $\mu$ l di Wash solution e a seguire una centrifugazione di 3 minuti a 16000xg. Per ottenere il DNA genomico puro in soluzione si aggiungono 200  $\mu$ l di Elution Solution e si centrifuga per 1 minuto a 6500xg.

### **Genotipizzazioni**

Sono stati studiati gli SNP dei geni TPMT (rs1800462, rs1800460 e rs1142345), ITPA (rs1127354, rs7270101 e rs6051702), SLCO1B1 (rs4149056) e PACSIN2 (rs2413739) (Tabella 2) tramite la metodica Taqman® (Applied Biosystem). La valutazione delle delezioni del gene di GST-M1 e T1 invece è stata effettuata tramite PCR Multiplex, una variante della PCR che permette di amplificare diversi frammenti del DNA impiegando un'unica reazione e numerose coppie di inneschi, ognuna specifica per una data regione del DNA.

Per lo studio degli SNPs sono stati utilizzati i kit TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA) che contengono i due primers (Forward e Reverse) specifici per l'amplicone di interesse ed una coppia di sonde, ciascuna specifica

per uno degli alleli; inoltre una delle sonde e' marcata all'estremità 5' con il fluoroforo FAM™ e l'altra con il fluoroforo VIC®.

I campioni sono stati preparati in una piastra a 96 pozzetti (96 Well MULTIPLY® - PCRplate, Sarstedt) e in ogni pozzetto sono stati caricati:

- 1µl di DNA genomico
- 6 µl di H2O distillata filtrata
- 6 µl di TaqMan® Genotyping PCR Master Mix
- 0,3 µl di sonda 40x oppure 0,6 l di sonda 20x

Per un totale di circa 13.3 µl.

Le caratteristiche dei kit specifici per i polimorfismi considerati sono riportati nella seguente tabella:

GENE	SNP (Assay ID)	ALLELE	SEQUENZA[VIC/FAM]
TPMT	rs1800462 (C__12091552_30)	C238G	CCAACTACACTGTGTCCCCGGTCTG[C/G]AAACCTGCATAAAATCATACATTTA
TPMT	rs1800460 (C__30634116_20)	A460G	TCACCTGGATTGATGGCAACTAATG[T/C]TCCTCTATCCCAAATCATGTCAAAT
TPMT	rs1142345 (C____19567_20)	G719A	TCTCATTTACTTTTCTGTAAGTAGA[C/T]ATAACTTTTCAAAAAGACAGTCAAT
ITPA	rs1127354 (C__27465000_10)	94A>C	CGTTCAGATTCTAGGAGATAAGTTT[A/C]CATGCACCTTTGGTGGCACAGAAAAT
ITPA	rs7270101 (C__29168507_10)	IVS2+21A>C	TGACCGTATGTCTCTGTTTTGTTTT[A/C]TTTTTAAAAGATGTTGGATTCTC
ITPA	rs6051702 (C__11201721_10)	*94>A	ACTCACCATATAACAGGGTTATT[C/A]TTATATCCTCAAAGAGTGCACCTGCC
PACSIN2	rs2413739 (C__2503304_20)	C/T	GAGAGCACAAGCAGCCCTTGGGCC[C/T]GTCCGACCTCAAATAAACTTAGGC

Tabella 2: Elenco delle sequenze nucleotidiche e probes per i kit TaqMan® SNP Genotyping Assays (Applied Biosystems) utilizzati.

I risultati ottenuti sono stati visualizzati e interpretati mediante l'impiego del software SDS (Sequence Detection System) che acquisisce lo spettro di emissione del campione per tutta la durata della reazione di PCR e converte la variazione di fluorescenza del reporter in una rappresentazione in tempo reale della cinetica di amplificazione: se il genotipo analizzato è omozigote wild-type, si osserverà un'unica curva di amplificazione corrispondente alla sonda complementare all'allele 1 (es. FAM); se omozigote mutato, graficamente si osserverà un'unica curva di amplificazione corrispondente alla sonda complementare all'allele 2 (es. VIC); se il genotipo è eterozigote, entrambe le sonde emetteranno fluorescenza e si osserveranno graficamente due curve di amplificazione che avranno un andamento sigmoidale simile e affiancato (es. FAM/VIC).

### **Dosaggio dei metaboliti tiopurinici**

I livelli dei metaboliti 6-tioguaninici (TGN) e 6-metilmercatopurinici (MMPN), sono stati quantificati mediante un saggio HPLC (High Performance Liquid Chromatography), effettuato sulle emazie concentrate lisate di pazienti leucemici. Come bianco sono state utilizzate emazie concentrate di donatori di sangue non in terapia, tali analisi sono state eseguite presso il laboratorio di Tossicologia Forense dell'Ospedale Maggiore di Trieste.

*Preparazione del lisato da sangue intero*

Circa 3 - 4 ml di sangue intero sono stati prelevati da pazienti pediatrici affetti da LLA in fase di consolidamento e mantenimento trattati secondo il protocollo AIEOP 2009. Per evitare il danneggiamento degli eritrociti, la degradazione dei metaboliti e la diminuzione dell'attività enzimatica, il prelievo (in vacutainer contenenti anticoagulante) è stato mantenuto a 4 °C e processato entro le 72 ore. Al campione sono stati aggiunti 10 µl di ditionitrotolo 100 mg/ml (DTT; Sigma-Aldrich) ed è stato centrifugato a 800xg per 10 minuti a 4 °C ottenendo la separazione dei globuli rossi dagli altri componenti del sangue (plasma, leucociti, piastrine). Questi ultimi sono stati rimossi e le emazie concentrate sono state risospese e lavate per due volte con una soluzione di NaCl 0,9% pari a due volte il volume del pellet di emazie concentrate ottenuto. Gli eritrociti ottenuti dopo i lavaggi sono stati diluiti 1:200 in una soluzione di NaCl 0,9% e contati utilizzando una camera Bürker. Gli eritrociti sono stati contati nei quadratini di area 1/400 mm<sup>2</sup> (Fig 2 precedente) ed espressi come media. Poiché ogni riquadro sull'emocitometro con il coprioggetto in posizione ha una profondità di 0,1 mm e quindi un volume totale di 10<sup>-4</sup>cm<sup>3</sup> e poiché 1 cm<sup>3</sup> corrisponde ad 1 ml, la concentrazione di cellule/ml è stata calcolata mediante la seguente formula:

$$\text{eritrociti/ml} = \text{media della conta} \times \text{fattore di diluizione} \times 400 \times 10^4$$

nella quale 200 è il fattore di diluizione utilizzato per gli eritrociti, 400 è il fattore di conversione a  $\text{cm}^3$  e  $10^4$  è il fattore di conversione a ml.

Al termine dei lavaggi, i globuli rossi concentrati sono stati lisati con un volume di acqua distillata pari alla metà del volume di eritrociti ottenuto e conservati a  $-20^\circ\text{C}$ .

#### *Preparazione dei campioni per HPLC*

Il saggio prevede l'aggiunta di 10  $\mu\text{l}$  di una soluzione acquosa di DTT 50 mg/ml a 500  $\mu\text{l}$  di lisato. Dopo mescolamento, si aggiungono 50  $\mu\text{l}$  di acido perclorico al 70%, con agitazione immediata su vortex per 10 secondi. Dopo centrifugazione dei campioni a 16100 xg per 15 minuti, il sopranatante viene incubato per 1 ora a  $95^\circ\text{C}$ . Il riscaldamento prolungato a  $95^\circ\text{C}$  in ambiente acido dei lisati determina l'idrolisi dei nucleotidi con conseguente liberazione delle basi tioguaniniche e metilmercaptipuriniche; in particolare, i TGN liberano la base tioguanina, mentre i metaboliti MMPN vengono convertiti durante il riscaldamento a 4-amino-5-metiltiocarbonil imidazolo. I campioni vengono quindi messi per 10 minuti a  $-20^\circ\text{C}$  per bloccare la reazione di idrolisi e poi conservati a  $4^\circ\text{C}$  fino al momento dell'analisi (entro 24 ore). Si prepara inoltre un campione "bianco" processato come sopra descritto utilizzando lisati degli eritrociti di volontari sani.

#### *Curva di taratura*

Per l'analisi quantitativa si prepara una curva di taratura composta da diversi punti che corrispondono a diverse concentrazioni degli

standard diluiti nei lisati degli eritrociti di volontari sani. Per ciascuna sostanza è stata preparata una prima soluzione 100 mM in DMSO (il peso molecolare della tioguanina è 167,2 g/mol, mentre quello della 6-MMP è 166,2 g/mol). Ciascuna di queste due soluzioni è stata diluita 1:100 in acqua distillata. A questo punto è stata preparata un'unica soluzione contenente tioguanina 20 µM e 6-MMP 100 µM, da cui poi sono state ottenute, per diluizioni successive, le diverse concentrazioni degli standard (Tabella 3). Il tutto è stato vortexato per circa 10 secondi.

	<b>Composto</b>	<b>Concentrazione finale (M)</b>
P 1	tioguanina	300 nM
	6-MMP	1500 nM
P 2	tioguanina	600 nM
	6-MMP	30000 nM
P 3	tioguanina	1000 nM
	6-MMP	5000 nM
P 4	tioguanina	2000 nM
	6-MMP	10000 nM
P 5	tioguanina	3000 nM
	6-MMP	15000 nM

*Tabella 3: Tabella riassuntiva dei punti di taratura e delle rispettive concentrazioni finali dei composti per ogni punto. 6-MMP= 6-metilmercaptipurina.*

### *Strumento analitico e condizioni cromatografiche*

Per l'analisi dei campioni è stato utilizzato lo strumento HPLC (cromatografia liquida ad alta prestazione) della ditta Hewlett Packard serie 1260 Infinity con rivelatore UV dotato di autocampionatore, e software *Chemstation* di gestione dati. L'autocampionatore preleva un'aliquota di campione preparato come descritto in precedenza che viene inserito nel sistema fluidico e trasportato nella colonna cromatografica.

Per l'analisi dei metaboliti TGN e MMPN, la fase stazionaria è rappresentata dalla colonna analitica a fase inversa Res Elute (Zorbax Omnisfer C18, Agilent) mantenuta a temperatura ambiente in cui vengono iniettati 100 µl di campione preparato come descritto precedentemente. La fase mobile è costituita da tampone fosfato acido 0,02 M pH 3,5 (preparato con 1,3610 g di potassio diidrogeno fosfato (KH<sub>2</sub>PO<sub>4</sub>) in 500 ml di acqua con l'aggiunta di 70 µl di H<sub>2</sub>PO<sub>4</sub>).

### *Elaborazione dei dati*

Dalle curve di taratura calcolate per i due metaboliti sono stati ottenuti i coefficienti di pendenza specifici K, utilizzati per calcolare i valori delle concentrazioni dei metaboliti TGN e MMPN dei campioni. Le concentrazioni dei metaboliti TGN e MMPN si ottengono moltiplicando questi K, per le aree (A) sottostanti i picchi cromatografici:

$$[\text{MMPN}] = A_{\text{MMPN}} * K_{\text{MMPN}}$$

$$[\text{TGN}] = A_{\text{TGN}} * K_{\text{TGN}}$$

Le concentrazioni così ottenute sono convertite in pmol di metabolita formato e i risultati vengono espressi come pmol/ $8 \times 10^8$  eritrociti.

## **Dosaggio dell'attività enzimatica di ITPA**

Per monitorare l'attività di ITPA è stata effettuata una quantificazione della capacità di tale enzima di convertire *in vitro* il substrato specifico inosina trifosfato (ITP; Sigma-Aldrich, Milano, Italia) nel suo prodotto finale di reazione, l'inosina monofosfato (IMP).

Le analisi sono state effettuate su emazie concentrate lisate di pazienti leucemici tramite l'impiego della metodica HPLC (Shipkova et al., 2006). Tali analisi sono state eseguite presso il laboratorio di Tossicologia Forense dell'Ospedale Maggiore di Trieste.

### *Preparazione del lisato da sangue intero*

I campioni dei pazienti pediatrici sono stati raccolti in concomitanza ai prelievi per il dosaggio dei metaboliti tiopurinici con le stesse modalità (3 - 4 ml di sangue intero in vacutainer con anticoagulante) e i lisati degli eritrociti sono stati preparati seguendo gli stessi passaggi. Diversamente dalla metodica descritta, non viene aggiunto il DTT al sangue intero e i globuli rossi concentrati vengono diluiti 1:3 in acqua distillata e centrifugati a 12000xg per 10 minuti a 4 °C. Il sopranatante ottenuto viene conservato a -80 °C per salvaguardare la funzionalità dell'enzima.

### *Preparazione dei campioni per HPLC*

Dopo scongelamento, 200 µl del campione conservato a -80 °C sono stati prelevati e centrifugati a 13000 xg per 10 minuti. A 25 µl di sovrinatante sono stati aggiunti 170 µl di una miscela composta da: 150 µl di una soluzione acquosa di DTT 10 mM (EuroClone), 10 µl di MgCl<sub>2</sub> 1 M e 10 µl di Tris solution 100 mM a pH9 (Trizma base, minimum 99.9% titration Sigma-Aldrich). La miscela è stata vortexata e preincubata a 37 °C per 5 minuti. Sono stati quindi aggiunti 10 µl del substrato inosina trifosfato (ITP) (Sigma-Aldrich) 40 mM con agitazione immediata su vortex per 10 secondi e incubazione per 15 minuti a 37 °C, periodo in cui avverrà la conversione di ITP in IMP il quale viene quantificato mediante HPLC. Per interrompere la reazione enzimatica e deproteinizzare il campione, sono stati aggiunti 20 µl di HClO<sub>4</sub> 4M (Merck) agitando rapidamente tramite vortex. La soluzione è stata incubata in ghiaccio per 10 minuti e dopo l'aggiunta di 30 µl di K<sub>2</sub>HPO<sub>4</sub> 8,57 M (Merck) è stata vortexata e centrifugata a 13000 xg per 10 minuti al fine di neutralizzare l'ambiente acido. Il sovrinatante così ottenuto, viene aliquotato e conservato a 4 °C fino al momento dell'analisi cromatografica. La preparazione del campione è stabile per 10 ore a temperatura ambiente (Shipkova et al., 2006). Per ciascun paziente, si prepara inoltre un campione "bianco" processato come sopra descritto, senza l'aggiunta del substrato ITP per permettere la determinazione dell'IMP endogeno (Shipkova et al.,2006).

### *Curva di taratura*

Per l'analisi quantitativa dei campioni, si prepara una curva di taratura composta da sette punti che corrispondono a diverse concentrazioni degli standard opportunamente diluiti in emazie concentrate derivanti da donatori sani. Gli standard sono stati preparati a partire da IMP (Sigma-Aldrich) alla concentrazione di  $7,65 \cdot 10^{-2}$  M (il peso molecolare dell'IMP è 392.17 g/mol) dalla quale, tramite opportune diluizioni in acqua distillata, si sono ottenuti i sette punti e il controllo (Tabella 4). Conoscendo le concentrazioni dei singoli punti costituenti la retta e le aree corrispondenti, si può risalire alla concentrazione del campione incognito per confronto tra le aree.

	<b>Concentrazione finale di IMP (M)</b>
<b>Controllo</b>	$6,30 \cdot 10^{-5}$
<b>P 1</b>	$5,86 \cdot 10^{-6}$
<b>P 2</b>	$2,34 \cdot 10^{-5}$
<b>P 3</b>	$9,38 \cdot 10^{-5}$
<b>P 4</b>	$3,75 \cdot 10^{-4}$
<b>P 5</b>	$7,50 \cdot 10^{-4}$
<b>P 6</b>	$1,50 \cdot 10^{-3}$
<b>P 7</b>	$3,00 \cdot 10^{-3}$

*Tabella 4: Tabella riassuntiva dei punti di taratura e delle rispettive concentrazioni finali dei composti per ogni punto.*

Le soluzioni così preparate vengono processate allo stesso modo dei campioni.

#### *Strumento analitico e condizioni cromatografiche*

Per l'analisi dei campioni è stata utilizzata l'HPLC (Hewlett Packard) precedentemente descritta. In questo caso la fase stazionaria è rappresentata dalla colonna analitica di tipo C18 a fase inversa Aqua Perfect (CPS analitica Srl) mantenuta a temperatura ambiente, mentre la fase mobile è costituita da tampone fosfato acido 20 mmol/l a pH 2.5 (Sigma Aldrich) in 500 ml d'H<sub>2</sub>O milli-Q, e acetonitrile (Merck).

#### *Elaborazione dei dati*

Analogamente a quanto descritto per i metaboliti tiopurinici, si ottiene il coefficiente di pendenza K che, moltiplicato all'area del picco cromatografico, permette di ottenere le concentrazioni dell'IMP prodotto proporzionalmente all'attività di enzimatica di ITPA.

$$[\text{IMP}] = A_{\text{IMP}} * K_{\text{IMP}}$$

I risultati vengono espressi come  $\mu\text{mol}$  di IMP prodotto nell'arco di un'ora, tenendo conto del numero di eritrociti, espressi come emoglobina media eritrocitaria, secondo la seguente formula:

$$[\mu\text{mol IMP/g HB *h}]$$

### **Saggio dell'attività di TPMT**

L'attività di TPMT è stata determinata mediante un saggio HPLC-UV come descritto in Anglicheau et al. (2002). Il saggio enzimatico

consiste nella quantificazione della 6MMP prodotta durante l'incubazione di lisati di eritrociti dei pazienti (900 µl), preparati come precedentemente indicato con mercaptopurina in presenza di S-adenosilmetionina, quale donatore di gruppi metile. I cationi bivalenti, che potrebbero interferire con l'analisi, sono stati eliminati mediante incubazione per un'ora a 4 °C sotto agitazione, aggiungendo 100 µl di una sospensione (5 g / 100 ml) della resina Chelex 100 (Biorad, Richmond, USA). Trascorsa l'incubazione, la resina è stata separata dai lisati mediante centrifugazione a 4000 x g per 10 minuti a 4 °C. A 100 µl di lisati purificati dai cationi, vengono aggiunti 25 µl di tampone fosfato potassio (0,15 M, pH 7,5), 10 µl di acqua distillata, 5 µl di una soluzione acquosa di SAM (372 m M), 5 µl di soluzione acquosa di allopurinolo (1,5 mM) per inibire la xantina ossidasi, 5 µl di una soluzione acquosa di ditionitrotolo (DTT) (31 mM) e 5 m l di una soluzione di mercaptopurina (117,8 mM). Il mix di reazione così preparato viene incubato per un'ora a 37 °C con agitazione continua. La reazione viene fermata aggiungendo 200 m l di etanolo assoluto e lasciando i campioni per 10 minuti in ghiaccio. Dopo l'aggiunta di 355 µl di una soluzione metanolo-HCl 0,1 M (v/v) e centrifugazione a 5000 x g per 5 minuti a 4 °C, 100 m l del surnatante sono stati caricati nello strumento HPLC per la separazione e la quantificazione della 6MMP prodotta.

Lo strumento utilizzato è stato un sistema HPLC Hewlett Packard Agilent 1100 (HP, Palo Alto, USA), con una colonna analitica da

250 mm C18 a fase inversa (Varian, Palo Alto, USA), dotato di una pompa quaternaria (mod. G1311A), un detector con tecnologia ad array di diodi (mod. G1315), un autocampionatore (mod. G1313A) ed un degasatore a vuoto (mod. G1322A). La fase mobile utilizzata consiste nella miscela di acido acetico 0,1 % (fase A), acetonitrile (fase B) e acqua (fase C). La separazione è stata eseguita con una miscelazione programmata delle fasi con una durata totale pari a 26 min.

## **ESPERIMENTI *IN VITRO*: PACSIN2 E AUTOFAGIA**

### **Colture cellulari di linee stabilizzate**

I protocolli sperimentali hanno previsto l'impiego di differenti linee cellulari stabilizzate. Le linee cellulari sono coltivate in fiaschette sterili di polistirene con tappo filtrante da 25 cm<sup>2</sup> e da 75 cm<sup>2</sup>, contenenti rispettivamente 7 ml e 20 ml di terreno completo. Le colture cellulari sono state mantenute in incubatore umidificato a 37°C, in atmosfera al 5% di CO<sub>2</sub>.

### *Fibroblasti murini embrionali (MEF) da topi knock-out per Atg7*

Queste cellule MEF (Taherbhoy et al., 2011) sono state fornite del laboratorio del Dr. William Evans del St.Jude Children's Hospital di Memphis (USA) e sono state coltivate in terreno DMEM contenente 10% siero fetale bovino ed L-glutamina 20 mM.

### *RAW iperesprimenti LC3-GFP*

Queste cellule RAW (Sanjuan et al., 2007) sono state fornite del laboratorio del Dr. William Evans del St.Jude Children's Hospital di Memphis (USA) e sono state coltivate in terreno DMEM contenente 10% siero fetale bovino ed L-glutamina 20 mM.

### *HeLa iperesprimenti LC3-GFP*

Queste cellule HeLa sono state fornite del laboratorio del Dr. William Evans del St.Jude Children's Hospital di Memphis (USA) e sono state coltivate in terreno DMEM contenente 10% siero fetale bovino ed L-glutamina 20 mM.

### *NALM6 con knock-down di PACSIN2*

Queste cellule NALM6 (Stocco et al. 2012) sono state fornite del laboratorio del Dr. William Evans del St.Jude Children's Hospital di Memphis (USA) e sono state coltivate in terreno RPMI completato con 10% di siero fetale bovino ed L-glutamina 20 mM.

## **Analisi delle proteine**

### *Estrazione di proteine*

Le proteine sono state estratte a partire da un pellet contenente almeno  $5 \times 10^6$  cellule, preventivamente lavato con PBS, mediante il seguente protocollo:

- Vengono aggiunti al pellet cellulare 100 $\mu$ l di lysis buffer (500  $\mu$ l Tris HCl pH 7,4 10mM; 5mL EDTA 100mM; 5mL di NaCl 100mM;

20  $\mu$ l SDS 0,1%; 9,48mL H<sub>2</sub>O) ed 1 $\mu$ l di inibitore di proteasi (Halt<sup>TM</sup> Protease Inhibitor Cocktail (100X), Thermo Scientific).

- Il campione viene trattato mediante l'impiego di un sonicatore per circa 20 secondi, tenendolo in ghiaccio per prevenire la generazione di calore.

- La provetta viene centrifugata per 10 minuti a 16.000xg,

- Il surnatante, contenente le proteine, viene prelevato e trasferito in una nuova provetta.

Gli estratti proteici così ottenuti vengono analizzati mediante lo spettrofotometro NanoDrop (NanoDrop 2000, EuroClone®) per valutarne la concentrazione.

### *Western blotting*

Questa tecnica consente di separare proteine di diverso peso molecolare mediante la loro migrazione attraverso le maglie di un gel denaturante di poliacrilamide, sottoposto alle forze di un campo elettrico. Il processo è reso possibile dall'azione del sodio dodecil solfato (SDS), che, essendo un detergente ionico, lega le proteine conferendo loro una carica netta negativa costante per unità di massa. Viene così uniformata la carica delle proteine, le quali migreranno solo in base al loro peso molecolare. Il gel elettroforetico è formato da due parti distinte: una superiore, detta stacking gel, che serve a compattare le proteine fino a raggiungere la fase inferiore, detta running gel, costituita da maglie più dense, che permette di separare i campioni in base alla loro mobilità elettroforetica. Successivamente, le proteine vengono trasferite dal

gel ad una membrana di PVDF. Una volta fissate nella membrana, le proteine possono poi essere analizzate mediante l'impiego di anticorpi specifici. Le proteine (20 $\mu$ g) sono state caricate nei pozzetti di un gel precast al 10% di acrilamide (PAGEr Gold Precast, Lonza), dopo aver opportunamente diluito i campioni con il tampone di caricamento (61.5mM Tris-HCl pH 6.8, SDS 2.5%, glicerolo 10%, blu di bromofenolo 0.0025%) in cui viene aggiunto il 5% di  $\beta$ -mercaptoetanololo in modo da ridurre i ponti disolfuro. Un marcatore di peso molecolare (ColorBurst Electrophoresis marker, Sigma-Aldrich) è stato caricato nel gel, in modo da poter valutare l'andamento della corsa elettroforetica e allo stesso tempo analizzare il peso molecolare delle proteine di interesse confrontandole con i pesi standard. La migrazione è stata effettuata in tampone di corsa (192mM glicina, 25mM Tris-HCl pH 8.0, SDS 1% w/v) in una PAGEr MiniGel Chamber (Lonza) per un tempo di circa 2 ore ad un voltaggio costante di 125V (60mA). Terminata la corsa elettroforetica, si procede con lo step successivo, definito blotting, che prevede l'allestimento di un sandwich costituito, nell'ordine dall'anodo al catodo, da 3 fogli di carta filtro (Electode Paper Novablot PKG/500 Pharmacia), dalla membrana di PVDF (Millipore), dal gel di poliacrilamide e da 3 ulteriori fogli di carta da filtro. I fogli di carta da filtro sono stati precedentemente immersi per qualche minuto nel tampone di trasferimento (192mM glicina, 25mM Tris-HCl pH 8.0, 20% metanolo), mentre la membrana di PVDF è stata attivata in metanolo ed equilibrata nello stesso

tampone. Il trasferimento delle proteine dal gel alla membrana è stato effettuato mediante sistema semi-dry in apposito blottatore (Semi-dry Protein Blotter System, Pharmacia LKB Multiphor II), applicando una corrente di 50mA per 2 ore. Il trasferimento delle proteine dal gel è stato verificato colorando la membrana con Rosso Ponceau (0,1% Ponceau S (w/v) in 5% acido acetico (v/v), Sigma-Aldrich). Dopo il trasferimento, la membrana di PVDF è stata tagliata in base alla corsa delle proteine, identificabili grazie ai marcatori di peso molecolare, ed incubata in soluzione di bloccaggio TTBS (50mM Tris-base e 150mM NaCl, portato a pH 7,4 con HCl in acqua distillata; Tween 20 0.01%) addizionato del 5% di latte scremato in polvere (Euroclone) in agitazione per un'ora a temperatura ambiente, in modo da saturare i siti di legame aspecifici. La membrana viene quindi incubata overnight a 4°C con l'anticorpo primario specifico. In particolare sono stati utilizzati anticorpi di coniglio anti PACSIN2 AP8088b (Abgent) ed anti LC3 ab48394 (Abcam) diluiti in soluzione di bloccaggio. Al termine dell'incubazione, la membrana viene lavata 4 volte per 10 minuti in agitazione con TTBS in modo da rimuovere gli anticorpi non legati o legati in modo aspecifico. Successivamente viene effettuata un'incubazione di un'ora a 37°C con l'anticorpo secondario marcato con perossidasi (HRP). Nello specifico, sono stati utilizzati anticorpi anti IgG di coniglio coniugati con HRP (Cell Signalling Tech) diluiti, rispettivamente 1:50.000 e 1:25.000, in soluzione di bloccaggio. Sono stati quindi eseguiti 4 ulteriori lavaggi con TTBS ed i

complessi antigene-anticorpo vengono rilevati sfruttando la reazione di chemiluminescenza che si sviluppa in seguito all'incubazione della membrana con lo specifico substrato per la HRP (LiteAblot® TURBO, Euroclone). Tale reazione viene rilevata mediante lastra autoradiografica (Kodak® BioMax Light Film).

### *Imaging al microscopio confocale*

L'imaging di cellule vive è stato eseguito con il microscopio invertito Nikon TE2000-E equipaggiato con un sistema confocale C1Si (Nikon, Melville, NY), un laser all'argon a 488 nM e laser DPSS a 404 e 561 nM (Melles Griot, Carlsbad, USA). La temperatura è stata mantenuta a 37°C e la CO<sub>2</sub> al 5% utilizzando una camera di controllo ambientale (InVivo Scientific, St.Louis, USA). Le immagini sono state acquisite su colture cellulari al 70% di confluenza, utilizzando un obiettivo 40x ad immersione in olio.

### *Co-immunoprecipitazione*

Le cellule (10 milioni) sono state raccolte al 70% di confluenza, lavate due volte con PBS freddo e lisate con 500 µl di tampone di lisi blando (con detergente non ionico e privo degli agenti riducenti β-mercaptoetanololo e ditiotreitolo: 20 mM Tris HCl pH 7,8, sodio cloruro 137 mM, glicerolo 10%, Triton-X 100 1%, EDTA 2 mM). La lisi cellulare è stata effettuata mantenendo in costante agitazione per 30 minuti a 4 °C. I campioni sono stati quindi centrifugati per 20 minuti a 12000 rpm. Il surnatante è stato raccolto immediatamente per prevenire la contaminazione da parte di proteine insolubili e

messo in ghiaccio. Sul surnatante è stata effettuata la quantificazione delle proteine mediante metodica Bradford. Per effettuare l'immunoprecipitazione sono state utilizzate delle biglie apposite (TrueBlot® Anti-Rabbit Ig IP Beads, eBioscience, San Diego, USA), utilizzando gli anticorpi anti-PACSIN2, anti-LC3 o come controllo delle IgG di coniglio aspecifiche ("normal rabbit IgG", Santa Cruz, Dallas USA), utilizzando il protocollo specificato dal produttore. Come secondario per il western seguente l'immunoprecipitazione è stato fondamentale utilizzare l'anticorpo Rabbit TrueBlot® Anti-Rabbit IgG HRP (eBioscience, San Diego, USA), per ridurre il segnale aspecifico.

### **Analisi statistica**

Le analisi statistiche sono state svolte con il software R (versione 3.1.2). L'associazione tra i parametri farmacocinetici e quelli farmacogenetici è stata effettuata mediante modelli lineari della famiglia gaussiana e ad effetto misto. Prima di applicare i modelli lineari, è stata verificata la normalità della variabile dipendente di interesse, mediante osservazione dell'istogramma dei dati e applicazione del test di Shapiro; in caso di distribuzione non normale, ai dati è stata applicata la trasformazione logaritmica o radice quadrata, in modo da rendere la distribuzione normale.

## RISULTATI

### Pazienti

Sono arruolati nello studio al momento 120 pazienti pediatrici affetti da LLA all'esordio, trattati secondo il protocollo AIEOP LLA 2009 ed arruolati presso i reparti di emato-oncologia dell'Ospedale pediatrico IRCCS Burlo Garofolo di Trieste (n = 34), Regina Margherita di Torino (n = 41), San Gerardo di Monza (n = 30) e Bambino Gesù di Roma (n = 15). I dati demografici sono disponibili per 109 pazienti: 59 maschi e 50 femmine, di età media 6,52 anni (range interquartile 3,0-10,4). Alla diagnosi, i pazienti presentavano una percentuale media di blasti nell'aspirato midollare pari all'88,6% (range interquartile: 65,5-90%); l'80,6% era affetto da LLA di immunofenotipo B mentre i restanti presentavano immunofenotipo T (ad eccezione di un paziente affetto da linfoma leucemizzato). I dati anagrafici e le caratteristiche dei soggetti arruolati sono riassunti in Tabella 5.

PAZIENTI	n° totale= 120
<b>Sesso (dati disponibili per 109 pazienti)</b>	
Femmine n° (%)	50 (45,9)
Maschi n° (%)	59 (54,1)
<b>Età alla diagnosi</b> media (range interquartile)	6.57 anni (3,0-10,4)
<b>% blasti all'esordio*</b> media (range)	88,6 (62,5-90)
<b>Immunofenotipo</b>	
LLA-B n° (%)	29 (80,6)
LLA-T n°(%)	6 (19,4)

Tabella 5: Tabella riassuntiva dei dati demografici e clinici dei pazienti. \* Percentuale di cellule immature calcolata in citofluorimetria sull'aspirato midollare o su sangue periferico prelevati alla diagnosi.

Dal momento che la maggior parte di questi pazienti è attualmente ancora in terapia, la raccolta dei dati clinici da parte dei medici pediatri è ancora in corso

## **Genotipizzazioni**

I risultati della genotipizzazione per i 7 polimorfismi d'interesse sono mostrati in Figura 6. I polimorfismi studiati sono tutti in accordo con la distribuzione di questi SNPs nella popolazione caucasica e gli SNP rispettano l'equilibrio di Hardy-Weiberg (Relling et al. 2013, Marsh et al. 2009, Stocco et al. 2012). In particolare, per quanto riguarda gli SNP di TPMT, rs1142345 ed rs1800460, sono stati genotipizzati rispettivamente in 81 e 94 pazienti; anche rs1800462 è stato genotipizzato in 94 pazienti e, come atteso, nessun paziente con varianti di questo SNP è stato identificato. Per quanto riguarda gli SNP di ITPA, la genotipizzazione è stata eseguita al momento su 78, 88 e 74 pazienti, rispettivamente per rs1127354, rs7270101 (ITPA), rs6051702 (C20orf194). Per rs2413739 (PACSIN2) la genotipizzazione ha avuto successo in 84 pazienti. I polimorfismi sono stati caratterizzati per ciascun paziente sia sul DNA tumorale che somatico, non mostrando differenze tra il genotipo leucemico e germinale dello stesso individuo.

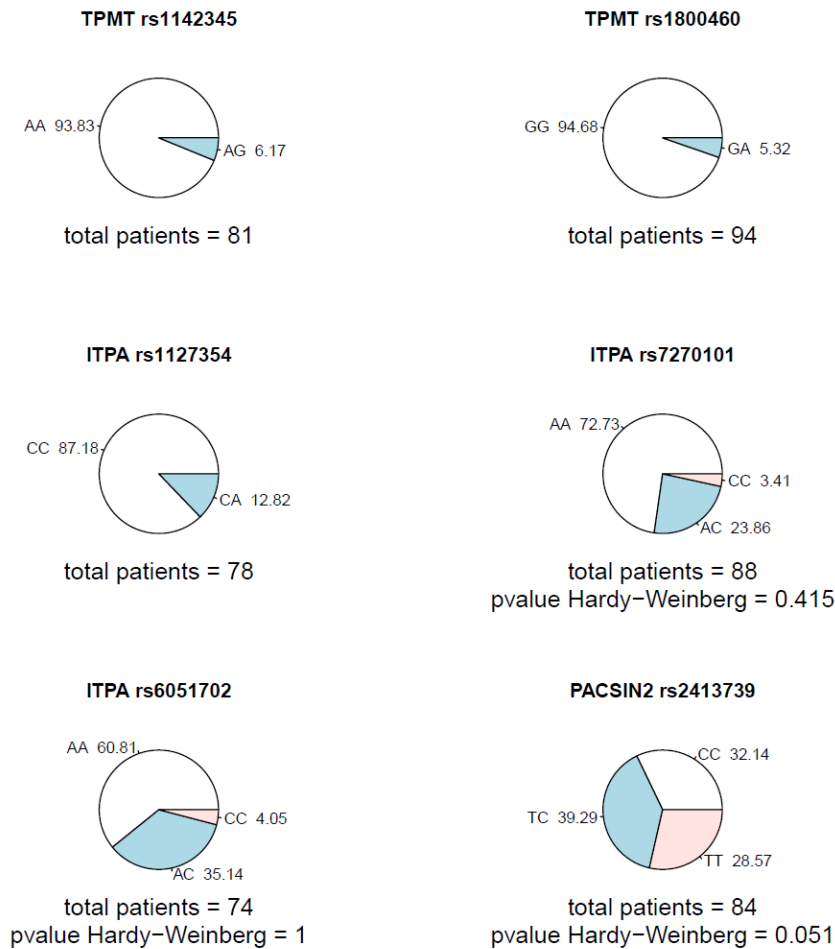


Figura 6: Distribuzione dei genotipi nella popolazione studiata.

## Sensibilità dei blasti *in vitro* e risposta clinica

Il saggio MTT è stato eseguito sui blasti di 13 pazienti. I risultati di citotossicità *in vitro* relativi a 3 pazienti sono stati scartati in quanto presentavano una percentuale di cellule immature inferiore al 70% nell'aspirato midollare (valutata tramite analisi citofluorimetrica). Dei 10 pazienti rimanenti abbiamo ricavato le IC<sub>50</sub> per tutti gli otto farmaci del pannello al di fuori di un paziente per il quale non è stato possibile ricavare le IC<sub>50</sub> di DNR, ARA-C e DXM a causa del numero insufficiente di cellule isolate.

In Tabella 6 sono riportati i valori mediani delle IC<sub>50</sub> per i farmaci testati e degli scores numerici PVA e PVDA per le relative

combinazioni; la tabella presenta inoltre i p-value di correlazione tra i dati di sensibilità *in vitro* e la risposta clinica del paziente misurata in citofluorimetria come percentuale di cellule immature residue su sangue periferico (giorno +8) o su sangue midollare (giorno +15, +33 e +78). Tale analisi viene eseguita regolarmente presso l’Ospedale pediatrico Burlo Garofolo per seguire il decorso della malattia e la risposta del paziente.

	IC <sub>50</sub> (M) mediana (range)	MRM (p-value)*			
		giorno +8	giorno +15	giorno +33	giorno +78
<b>PDN</b>	2.08x10 <sup>-4</sup> (2.11 x10 <sup>-8</sup> -1.34 x10 <sup>-3</sup> )	0.426	<b>0.086</b>	0.316	0.516
<b>VCR</b>	4.52 x10 <sup>-7</sup> (1.07 x10 <sup>-8</sup> -6.77 x10 <sup>-6</sup> )	0.143	0.294	0.701	0,741
<b>ASP</b>	1.08 x10 <sup>-2</sup> (6.35 x10 <sup>-4</sup> -20)	0.438	0.227	0.371	0.675
<b>DNR</b>	3.90 x10 <sup>-7</sup> (3.13 x10 <sup>-8</sup> -1.40 x10 <sup>-4</sup> )	0.731	0.734	0.793	0.494
<b>tioguanina</b>	2.96 x10 <sup>-5</sup> (2.47 x10 <sup>-7</sup> -5.98 x10 <sup>-4</sup> )	0.615	0.91	0.923	0.635
<b>Mercaptopurina</b>	1.15 x10 <sup>-3</sup> (1.00 x10 <sup>-5</sup> - 5.88 x10 <sup>-3</sup> )	0.781	0.462	0.569	0.911
<b>ARA-C</b>	9,50 x10 <sup>-7</sup> (4.48 x10 <sup>-8</sup> -8.22 x10 <sup>-5</sup> )	0.829	0.303	0.19	0.509
<b>DXM</b>	2.12 x10 <sup>-8</sup> (6.01 x10 <sup>-9</sup> -2.32 x10 <sup>-5</sup> )	0.885	<b>0.06</b>	0.239	0.337
<b>PVA</b>	6 (3-9)	0.514	0.127	0.462	0.457
<b>PVDA</b>	8 (4-12)	0.347	<b>0.099</b>	0.178	0.179

Tabella 6: Tabella riassuntiva delle IC<sub>50</sub> per tutti i farmaci testati; dei PVA e PVDA scores per le relative combinazioni. e dei p-value di correlazione tra i dati di citotossicità *in vitro* e la risposta clinica di 9 pazienti per cui erano disponibili i dati di citofluorimetria. \* I p-value sono stati ottenuti con il test di Pearson.

Come si può vedere dalla Tabella 6 e come illustrato in Figura 7, ad un'aumentata resistenza *in vitro* agli steroidi (PDN e DXM, ordine di grandezza  $\times 10^{-3}$ ) si tende ad avere un aumento della percentuale residua di blasti nel midollo al giorno +15: esiste infatti una tendenza di correlazione tra IC<sub>50</sub> del PDN, del DXM e del PVAD score con la MRM (p-value: 0.086, 0.06 e 0.099 rispettivamente).

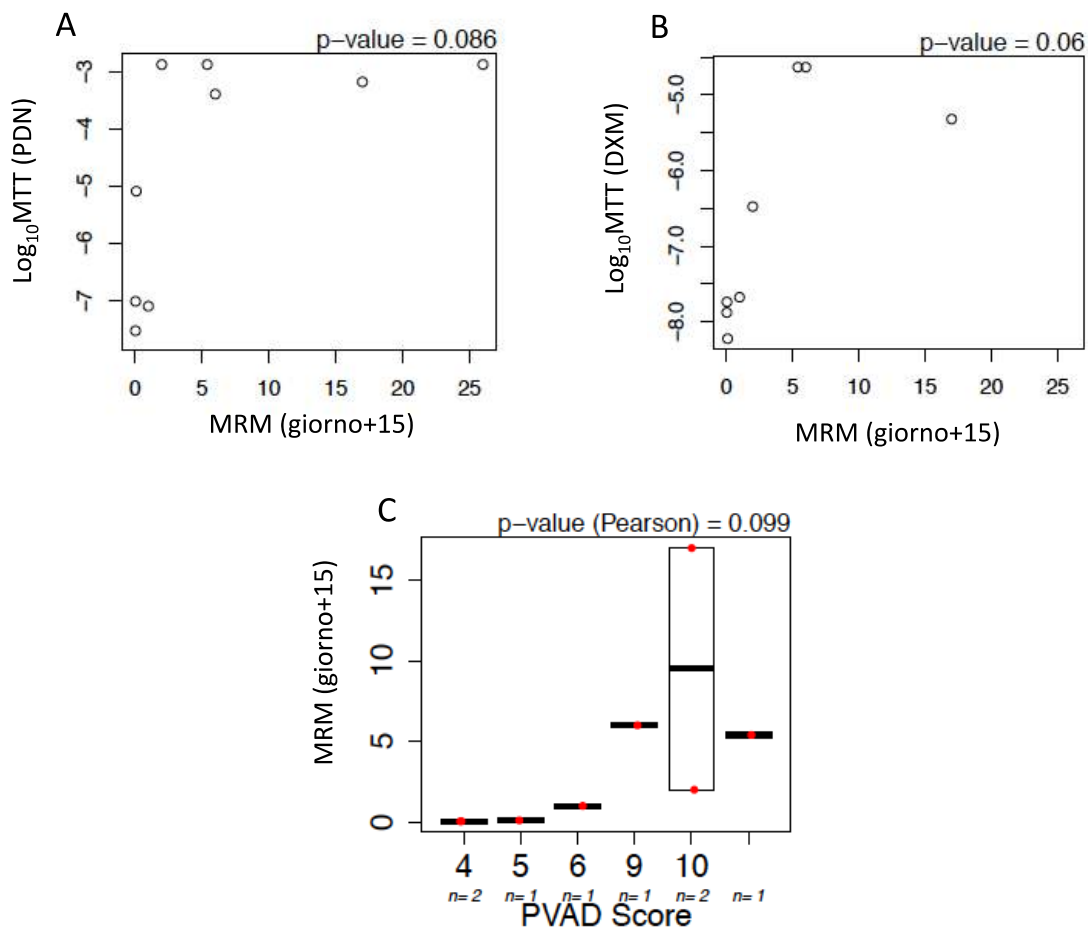
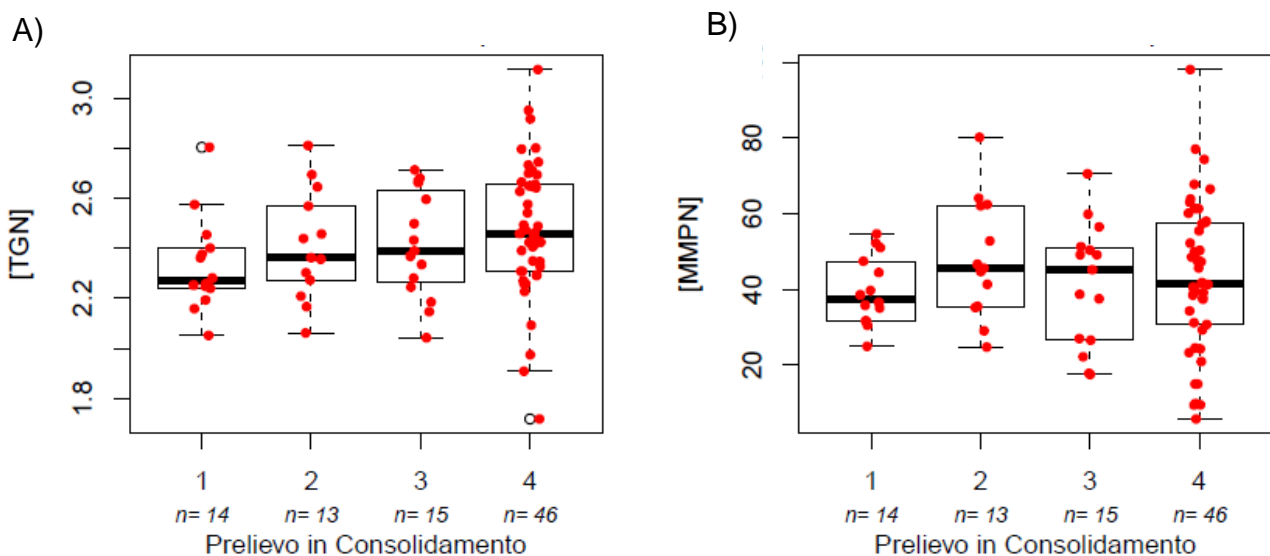


Figura 7: Correlazione tra MRM al giorno +15 e la citotossicità *in vitro* a **A)** PDN, **B)** DXM e **C)** PVAD score.

## ANALISI DEI METABOLITI DELLA MERCAPTOPURINA

I livelli dei metaboliti TGN e MMPN delle tiopurine sono stati valutati in 88 prelievi da 48 pazienti, raccolti durante la fase di consolidamento della terapia, in occasione di ciascuna della quattro

infusioni bisettimanali di metotressato ad alte dosi (Figura 8). La concentrazione dei metaboliti TGN è risultata in aumento durante i 4 prelievi, in quanto vista l'emivita molto lunga di questi metaboliti (circa 1 mese), raggiungono lo stato stazionario solo al termine delle quattro infusioni (Figura 8A).



*Figura 8: concentrazione dei metaboliti della mercaptopurina TGN (A) e MMPN (B) in prelievi raccolti durante la fase di consolidamento, contestualmente a ciascuna delle quattro infusioni di metotressato ad alte dosi. Per i metaboliti TGN si osserva un'associazione significativa tra il numero di prelievo e la concentrazione di TGN ( $p$ -value regressione lineare = 0,044).*

Ci si è dunque concentrati sui 48 prelievi di altrettanti pazienti, raccolti durante la fase di consolidamento, ciascuno contestualmente all'ultima (IV) infusione di metotressato ad alte dosi.

Per quanto riguarda la raccolta dei prelievi in mantenimento invece, i livelli dei metaboliti della mercaptopurina sono quindi stati valutati

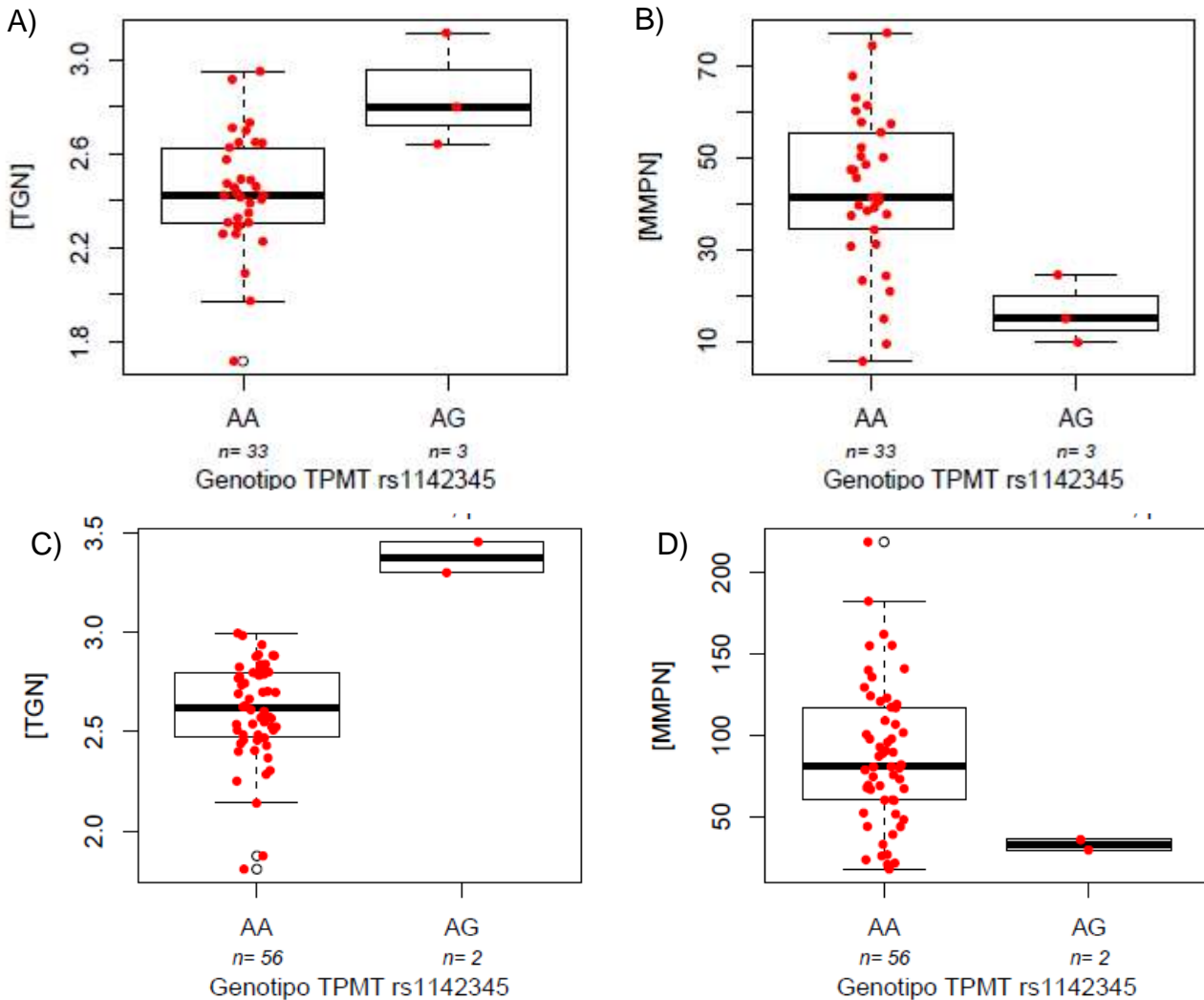
in 61 prelievi di 44 pazienti. I valori medi dei metaboliti attivi TGN e di quelli MMPN ottenuti nelle analisi sono riportati in Tabella 7.

	METABOLITI (pmol/8x10 <sup>8</sup> eritrociti)			
	CONSOLIDAMENTO (n = 48 prelievi/pazienti)		MANTENIMENTO (n = 61 in 44 pazienti)	
	TGN	MMPN	TGN	MMPN
<b>Mediana (range interquartile)</b>	287,8 (205,6 – 450,6)	1731,5 (948,1 – 9640,7)	462,6 (305,0 – 667,0)	6502,7 (2754,4 – 11916,2)
	<b>p-value (regressione)</b>	<b>p-value (regressione)</b>	<b>p-value (modelli lineari ad effetto misto)</b>	<b>p-value (modelli lineari ad effetto misto)</b>
<b>Età (anni)</b>	0,11	0,055	1,00	0,35
<b>Sesso</b>	<b>0,021</b>	0,23	<b>0,031</b>	0,45
<b>TPMT rs1142345</b>	<b>0,010</b>	<b>0,011</b>	<b>0,0007</b>	0,21
<b>TPMT rs1800460</b>	<b>0,014</b>	<b>0,017</b>	<b>0,0011</b>	0,20
<b>ITPA rs1127354</b>	NA	NA	0,27	0,38
<b>ITPA rs7270101</b>	0,33	0,57	0,13	0,79
<b>ITPA rs6051702</b>	NA	NA	0,89	0,15
<b>PACSIN2 rs2413739</b>	0,94	0,68	0,62	0,67

Tabella 7: Tabella riassuntiva dei dosaggi dei metaboliti della mercaptopurina e p-value di associazione ottenuti con regressione lineare (consolidamento) o modelli lineari ad effetto misto (mantenimento) che valutano l'associazione fra i livelli di metaboliti TGN e MMPN misurati nelle fasi di consolidamento o mantenimento e le caratteristiche demografiche (età e sesso) ed i genotipi candidati considerati.

Dalla Tabella 7 e dalla Figura 9, si può notare un'associazione significativa tra i livelli plasmatici dei metaboliti della mercaptopurina TGN e MMPN misurati sia durante la fase di consolidamento che di mantenimento ed i polimorfismi di TPMT: la concentrazione di TGN è più elevata nei pazienti con genotipo variante di TPMT (sia rs1142345 che rs1800460), anche per i metaboliti MMPN, i polimorfismi di TPMT hanno un effetto significativo: i pazienti con

varianti di TPMT (sia rs1142345 che rs1800460) presentano una riduzione nella concentrazione di questi metaboliti.



**Figura 9:** Associazioni statisticamente significative tra la concentrazione dei metaboliti della mercaptopurina ed i genotipi candidati considerati durante la fase di consolidamento (A: concentrazione TGN vs rs1142345, p-value regressione lineare= 0,010; B: concentrazione MMPN vs TPMT rs1142345, p-value regressione lineare = 0,011) e mantenimento (C: concentrazione TGN vs TPMT rs1142345, p-value modelli lineari ad effetto misto = 0,0007; concentrazione MMPN vs TPMT rs1142345, p-value modelli lineari ad effetto misto = 0,0011). Valori simili sono stati osservati per rs1800460 (dati non mostrati). Ciascun punto rappresenta un valore misurato in un totale di 36 pazienti in consolidamento (pannelli A e B) e 30 pazienti in mantenimento (pannelli C e D).

I genotipi considerati di ITPA e PACSIN2 non hanno invece rivelato associazioni statisticamente significative, ma soprattutto per le

varianti di ITPA il numero di pazienti arruolato è al momento limitato.

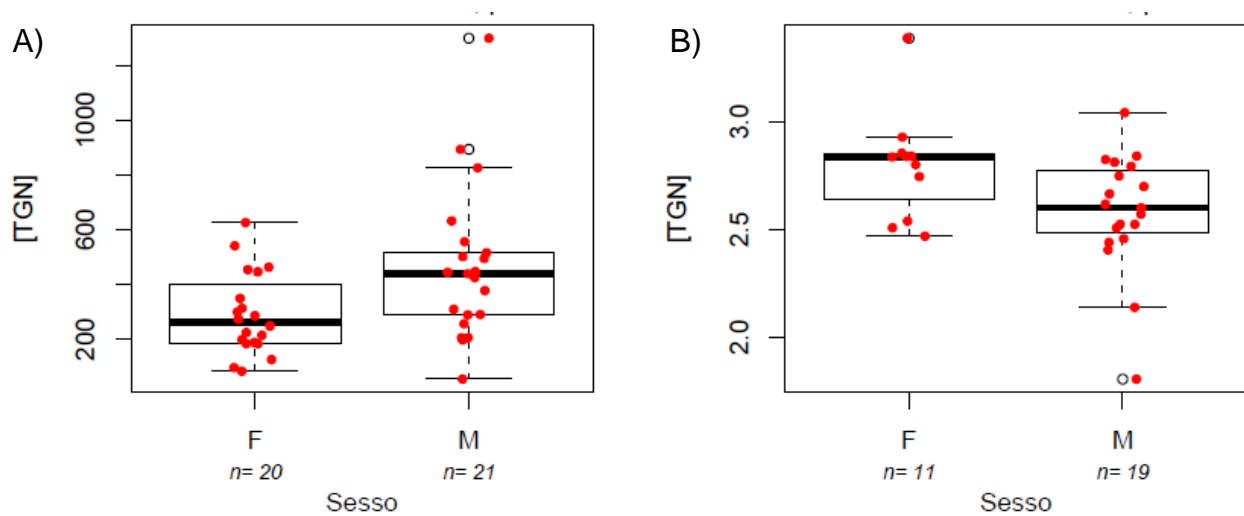


Figura 10: associazione fra sesso e metaboliti TGN della mercaptopurina durante la fase di consolidamento (A:  $p$ -value regressione lineare = 0,021) e mantenimento (B:  $p$ -value modelli lineari ed effetto misto = 0,031). Ogni punto rappresenta la media dei valori osservati in ciascun paziente, per un totale di 41 prelievi in consolidamento e 54 prelievi in mantenimento.

E' stato rilevato un effetto statisticamente significativo anche del sesso sulla concentrazione dei metaboliti TGN sia durante la fase di consolidamento, che quella di mantenimento (Figura 10). La direzione dell'effetto è però opposta nelle due fasi: durante il consolidamento, i pazienti di sesso femminile hanno una concentrazione più bassa dei metaboliti TGN, mentre durante la fase di mantenimento presentano una concentrazione più elevata.

## ANALISI DELL'ATTIVITA' ENZIMATICA DI ITPA

L'attività enzimatica di ITPA è stata valutata tramite HPLC in 43 prelievi di 24 pazienti diversi, raccolti durante le fasi di

mantenimento. Dei pazienti rimanenti non abbiamo ottenuto il materiale necessario all'analisi. I valori mediani dell'attività enzimatica di ITPA e i p-values di associazione con le caratteristiche demografiche (età, sesso) ed i genotipi di interesse sono riportati in Tabella 8.

	<b>ATTIVITA' ITPA (<math>\mu\text{mol/gHb}\cdot\text{h}</math>)</b>
<b>Mediana (range interquartile)</b>	163,5 (49,2 – 525,3)
	<b>p-value (modelli lineari ad effetto misto)</b>
<b>Età</b>	<b>0,012</b>
<b>Sesso</b>	<b>0,0079</b>
<b>ITPA rs1127354</b>	<b>0,032</b>
<b>ITPA rs720101</b>	<b>0,017</b>
<b>ITPA rs605172</b>	<b>0,0027</b>
<b>PACSIN2 rs2413739</b>	0,20

*Tabella 8: Tabella riassuntiva dell'attività enzimatica di ITPA e dei p-values di associazione con le variabili demografiche ed i genotipi di interesse ottenuti con i modelli lineari ad effetto misto.*

Come illustrato in Tabella 8 e in Figura 11, si può notare un'associazione fra l'età dei pazienti e l'attività di ITPA, aumentata nei pazienti più vecchi e nei maschi rispetto alle femmine. Tutte le varianti di ITPA considerate dimostrano una riduzione significativa nell'attività dell'enzima. Per quanto riguarda il genotipo di PACSIN2 rs2413739, invece, non si osservano al momento effetti statisticamente significativi sull'attività di ITPA.

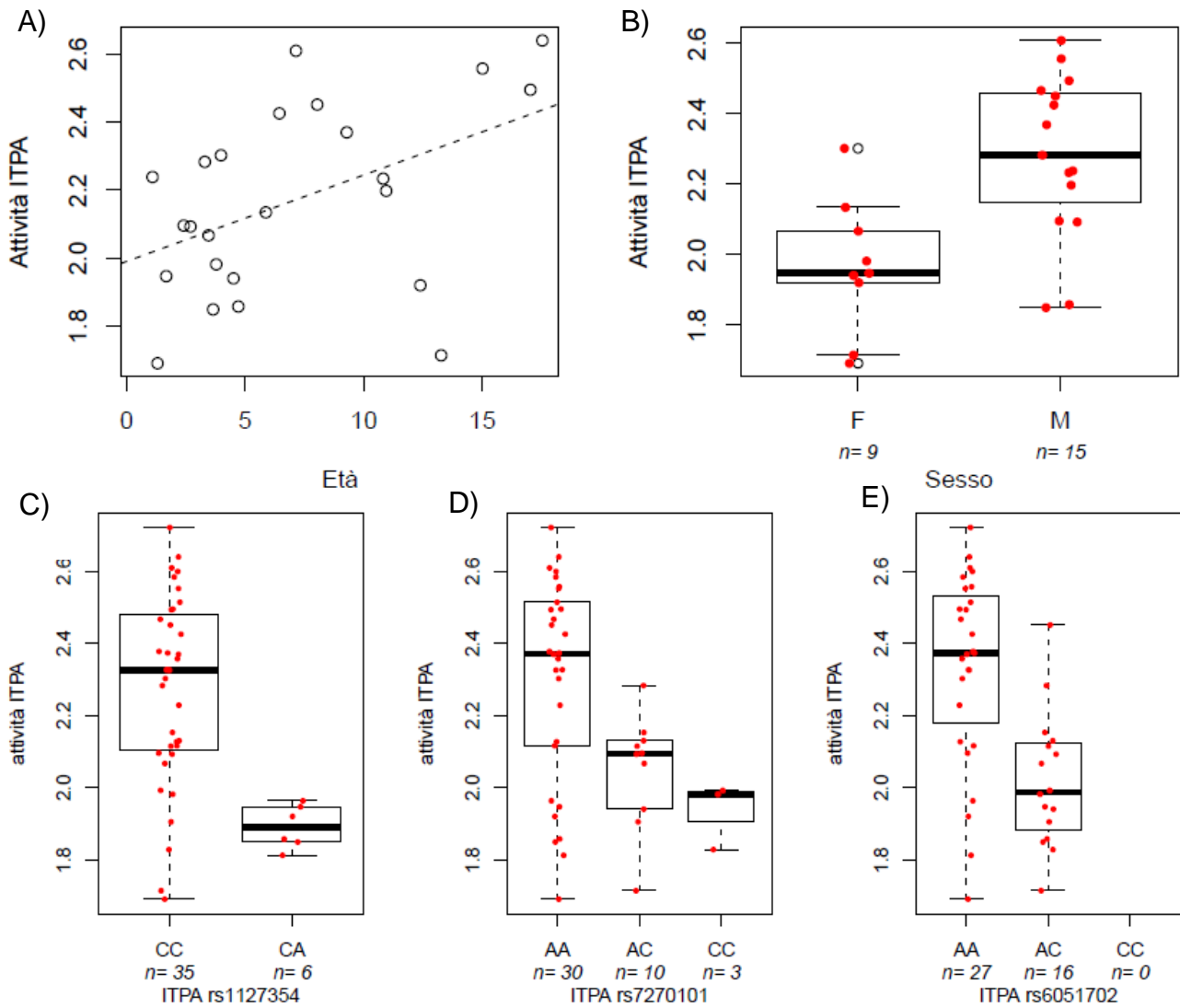


Figura 11: Associazione (modelli lineari ad effetto misto) dei livelli di attività enzimatica per ITPA con: A) età ( $p$ -value = 0,012), B) sesso ( $p$ -value = 0,0079) e genotipi ITPA C) rs1127354 ( $p$ -value = 0,032), D) rs7270101 ( $p$ -value = 0,017), E) rs6051702 ( $p$ -value = 0,0027). Per età e sesso, ogni punto rappresenta la media dei valori osservati in ciascun paziente ( $n = 26$ ), per un totale di 43 prelievi; per i genotipi di ITPA, ciascun punto rappresenta un valore di attività misurato in un totale di 24 pazienti.

## ANALISI DELL'ATTIVITA' ENZIMATICA DI TPMT

L'attività enzimatica di TPMT è stata valutata tramite HPLC in 38 prelievi di 21 pazienti diversi, raccolti durante la fase di mantenimento. I valori mediani dell'attività enzimatica di TPMT e i p-values di associazione con le variabili demografiche (età, sesso) ed i genotipi sono riportati in Tabella 9.

	ATTIVITA' ITPA ( $\mu\text{mol/gHb}\cdot\text{h}$ )
<b>Mediana</b> <b>(range interquartile)</b>	456,3 (375,3 – 544,1)
	<b>p-value</b>
<b>Età</b>	<b>0,044</b>
<b>Sesso</b>	0,42
<b>TPMT</b> <b>rs1142345</b>	<b>0,021</b>
<b>TPMT</b> <b>rs1800460</b>	<b>0,021</b>
<b>PACSIN2</b> <b>rs2413739</b>	<b>0,023</b>

*Tabella 9: Tabella riassuntiva dell'attività enzimatica di ITPA e dei p-values di associazione (modelli lineari ad effetto misto) con le variabili demografiche età e sesso ed i genotipi di interesse.*

Come illustrato in Tabella 11 e in Figura 12, si può notare un'associazione significativa fra l'età dei pazienti e l'attività di TPMT che risulta essere più alta nei pazienti più vecchi. Non è presente invece un effetto significativo del sesso. Per quanto riguarda i genotipi candidati considerati per i geni TPMT e PACSIN2, gli alleli varianti sono associati ad una riduzione nell'attività dell'enzima.

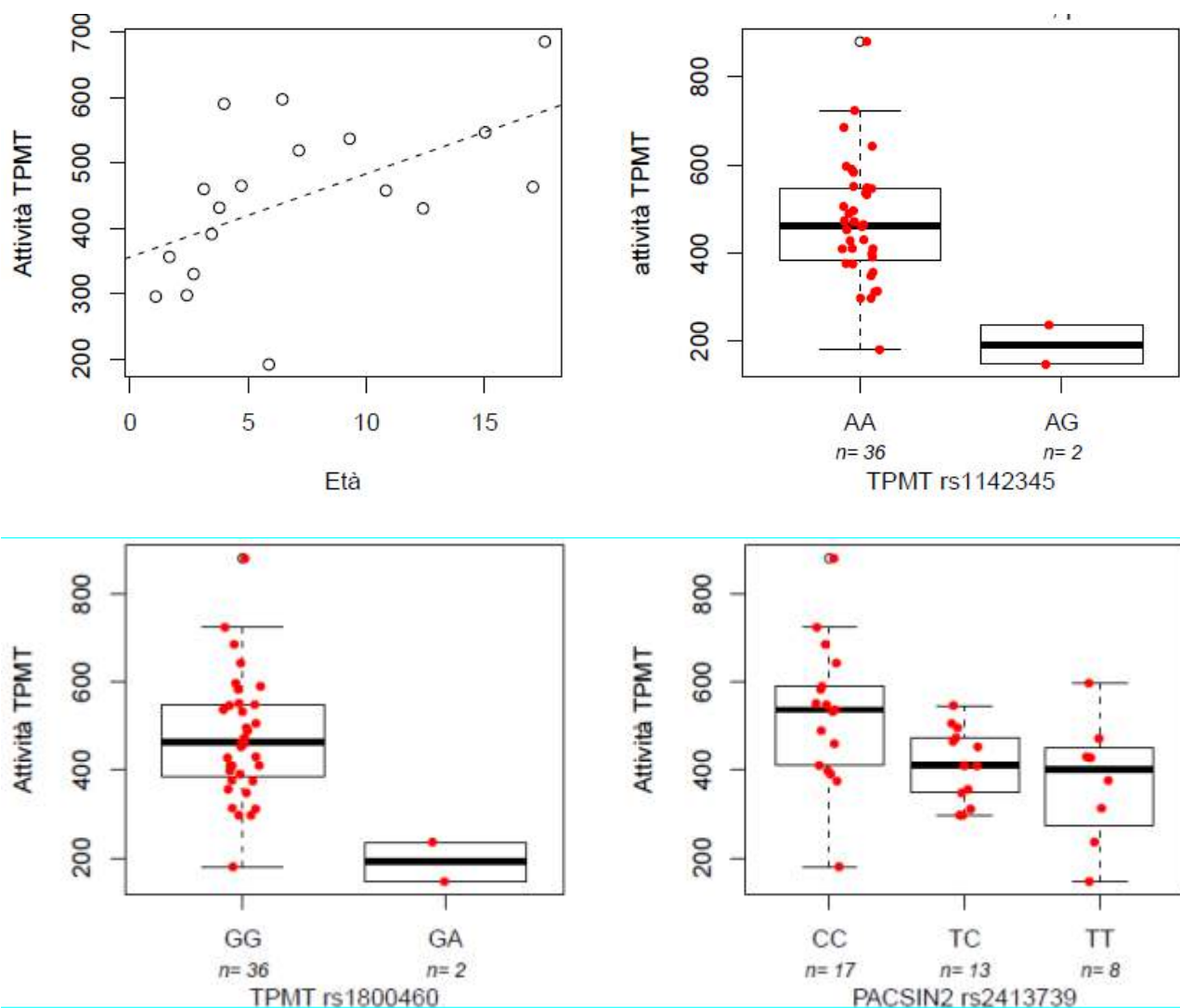
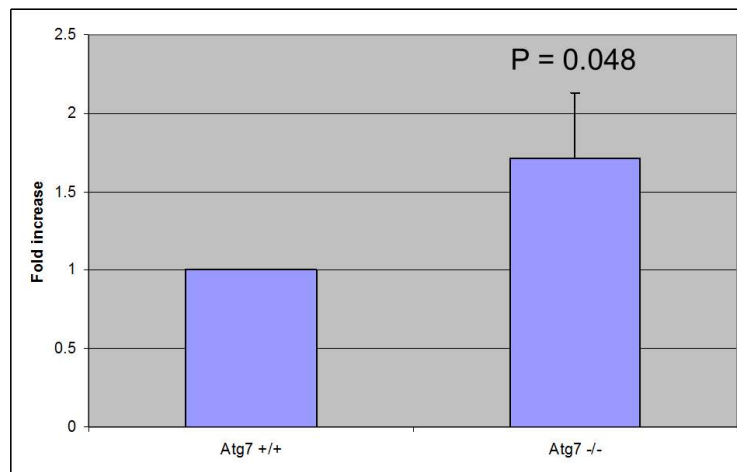


Figura 12: Associazione (modelli lineari ad effetto misto) dei livelli di attività enzimatica per TPMT con: A) età ( $p\text{-value} = 0,044$ ), genotipi TPMT C) rs1142345 ( $p\text{-value} = 0,021$ ), D) rs1800460 ( $p\text{-value} = 0,021$ ) e genotipo PACSIN2 rs2413739 E) ( $p\text{-value} = 0,023$ ). Per l'età e sesso, ogni punto rappresenta la media dei valori osservati in ciascun paziente ( $n = 38$ ), per un totale di 48 prelievi; per i genotipi, ciascun punto rappresenta un valore di attività misurato in un totale di 21 pazienti.

## ANALISI IN VITRO: PACSIN2 E L'AUTOFAGIA

### Le cellule con alterazione dell'autofagia accumulano PACSIN2

Le cellule con meccanismi alterati di autofagia esprimono livelli più alti di PACSIN2 endogeno. La concentrazione della proteina PACSIN2 è stata misurata mediante western blotting, utilizzando un anticorpo specifico per PACSIN2, in fibroblasti di embrioni di topo (mouse embryonic fibroblasts, MEFs) da animali ingegnerizzati per disattivare il gene dell'autofagia ATG7. I livelli di PACSIN2 sono stati quantificati mediante densitometria normalizzata per GAPDH. Nelle MEFs con l'inattivazione di ATG7, quindi con autofagia non funzionale, la concentrazione di PACSIN2 è risultata circa due volte quella dei MEFs di controllo con autofagia funzionale (Figura 13).



Rapporto in 5 esperimenti misurati su cellule confluenti, p-value da t-test.

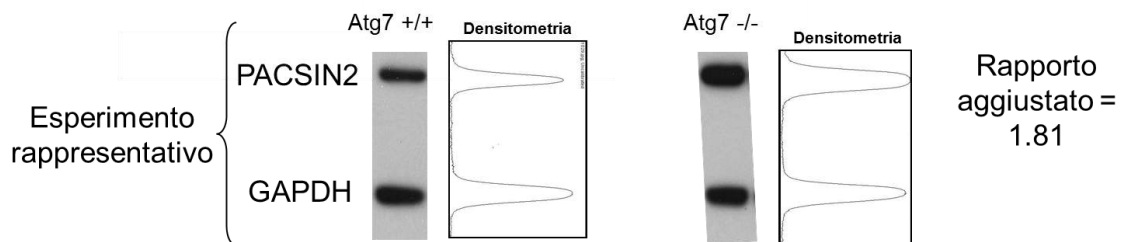


Figura 13: Le cellule con meccanismo di autofagia alterato esprimono costitutivamente livelli più alti di PACSIN2. La misurazione della concentrazione di PACSIN2 è stata effettuata mediante western blot, utilizzando un anticorpo specifico per PACSIN2, su MEFs confluenti in 5 esperimenti indipendenti.

## L'induzione di autofagia mediante trattamento con rapamicina nelle cellule MEFs riduce la produzione di PACSIN2

La rapamicina ha indotto una degradazione di PACSIN2 dipendente dall'autofagia (Figura 14). Atg7<sup>-/-</sup> MEFs sono stati messi in coltura in presenza dell'induttore dell'autofagia rapamicina (50 µg / ml) e cicloesimide (CHX, 5 µg / ml) per 0, 4, 8 and 12 ore. I lisati cellulari sono stati analizzati mediante western blotting, andando a quantificare la riduzione nella concentrazione di PACSIN2. I livelli di PACSIN2 sono stati quantificati mediante densitometria normalizzata per GAPDH.

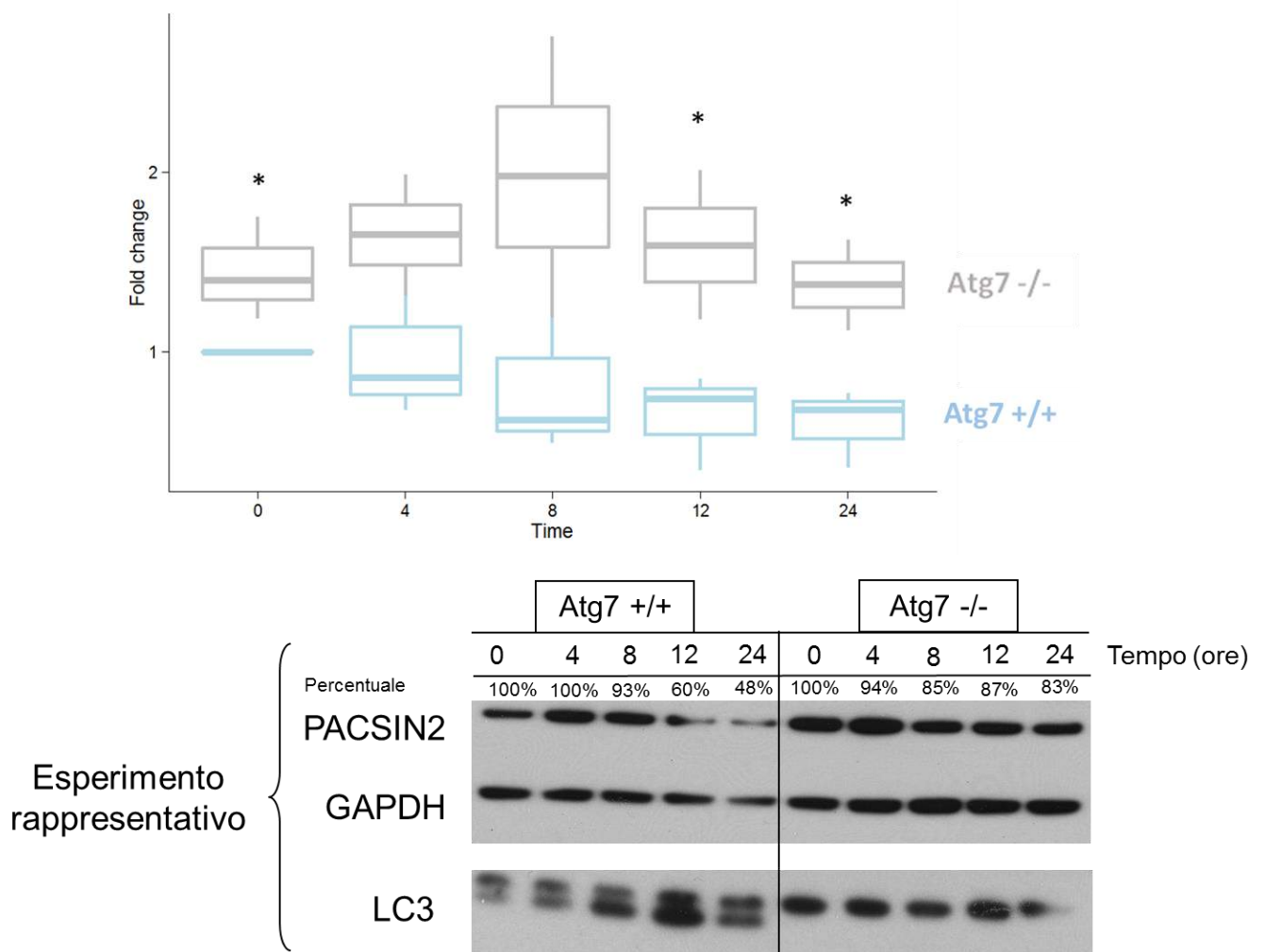


Figura 14: L'induzione di autofagia mediante la rapamicina in MEFs con autofagia funzionale riduce il livello di PACSIN2. La misurazione del livello di PACSIN2 è stata effettuata mediante western blot utilizzando un anticorpo specifico per PACSIN2, su MEFs confluenti in 3 esperimenti indipendenti.

## La perdita di PACSIN2 incrementa il livello di autofagia

### LC3-II western blotting

In conseguenza del silenziamento (“knock-down”) di PACSIN2, il livello basale di LC3-II è aumentato nelle cellule NALM6 e HeLa con espressione forzata of LC3-GFP (HeLa LC3-GFP) (Figura 15). I lisati cellulari delle NALM6 ed HeLa LC3-GFP che esprimono stabilmente shRNA contro PACSIN2 sono stati analizzati mediante western blotting per la perdita di PACSIN2 e per la concentrazione di LC3-II; è stato effettuato un confronto con lisati cellulari provenienti da colture che esprimono shRNA di controllo non specifici.

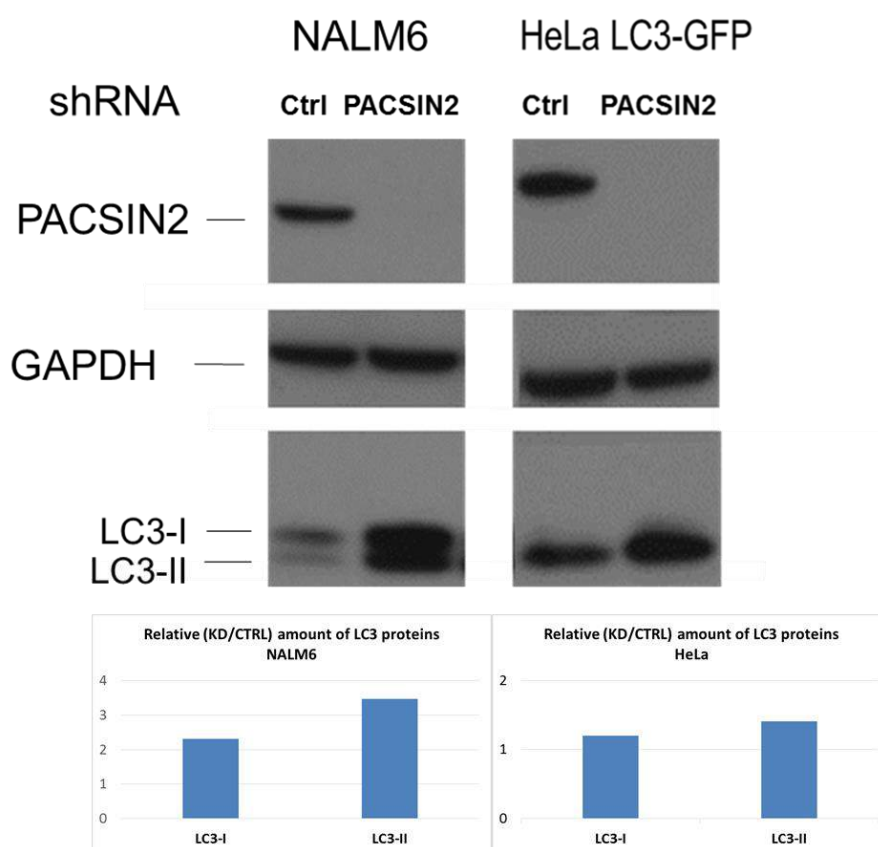


Figura 15: La concentrazione basale di LC3-II è aumentata dopo knock-down di PACSIN2. La misurazione di LC3-I, LC3-II e PACSIN2 è stata effettuata mediante western blot di cellule confluenti. La quantità relativa di proteine è stata calcolata fra i knock-downs di PACSIN2 (KD, trasfettati con shRNAs specifici di PACSIN2) e i controlli (CTRL, trasfettati con scramble shRNAs).

### *Punctate LC3-GFP staining*

In conseguenza di una ridotta espressione (“knock-down”) di PACSIN2, il numero basale di LC3-GFP punctae per cell è aumentato nei Raw 264.7 (macrofagi di topo) e nelle cellule HeLa con espressione forzata di LC3-GFP (Figura 16). Cellule che esprimono stabilmente LC3-GFP sono state messe in coltura e la percentuale di cellule autofagiche, dimostrate dal numero di cellule con LC3-GFP dots signal ( $\geq 5$  dots/cell) sul numero totale di cellule GFP-positive nello stesso campo, è stato determinato usando il microscopio confocale.

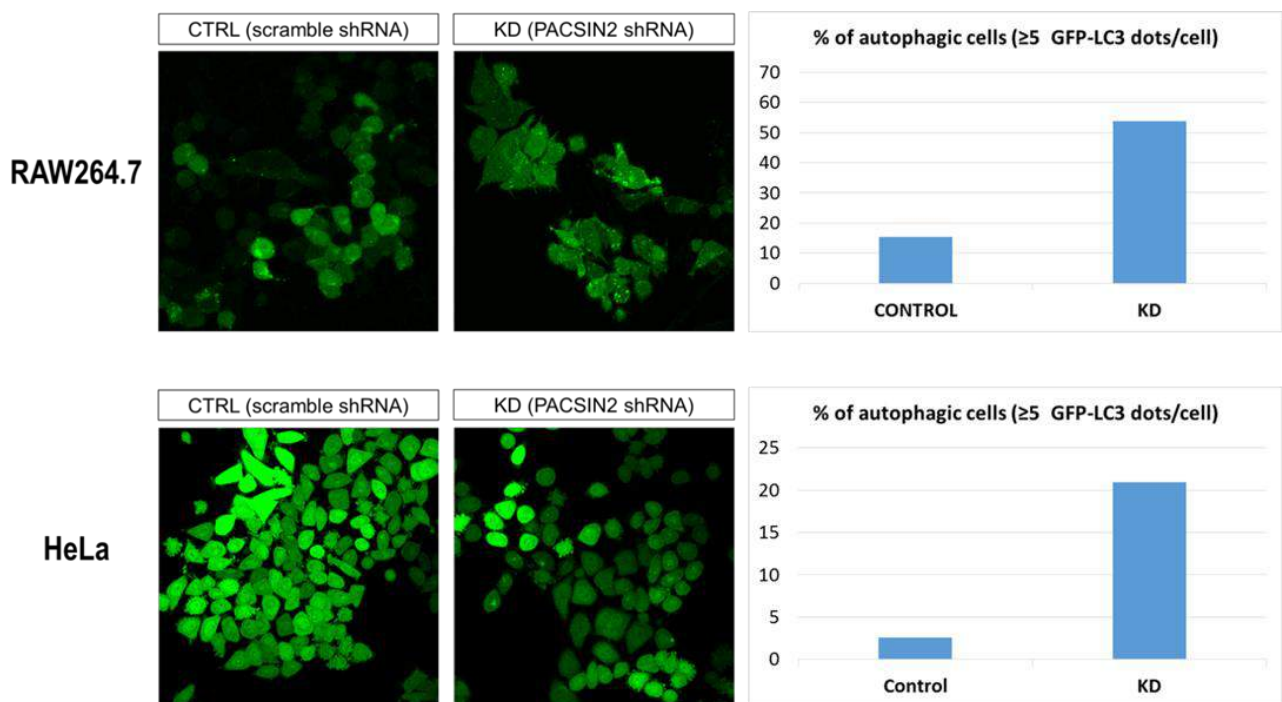


Figura 16: La colorazione Punctate LC3-GFP è aumentata dopo knock-down di PACSIN2. La misurazione di LC3-GFP punctate è stata effettuata mediante microscopio confocale su cellule confluenti.

### **La sequenza primaria di PACSIN2 contiene due siti di legame per LC3**

Il dominio proteico T-X-X-L è stato segnalato legarsi ad una tasca idrofobica altamente conservata (Atg8 omologa) (Nakatogawa et al., 2007)

al., Nat Rev Mol Cell Biol 2009); anche il motivo DDD è stato riportato legarsi a LC3 (è presente per esempio nella proteina p62) (Figura 17). L'esame della struttura primaria di PACSIN2 mostra che entrambi i motivi sono presenti in PACSIN2, supportando la teoria dell'interazione fisica con LC3, proteina che ha un ruolo importante nella formazione dell'autofagosoma.

*N terminal -*

MSVTYDDSVGVEVSSDSFWEVGNKYKRTVVKRIDDGHRLCSDLMNCLHERARIEK  
AYAQQLTEWARRWRQLVEKGPQYGTVEKAWMAFMSEAERVSELHLEVKASL  
MNDDFEKIKNWQKEAFHKQMMGGFKETKEAEDGFRKAQKPWAKKLKEVEAA  
KKAHHAACKEEKLAISREANSKADPSLNPEQLKKLQDKIEKCKQDVLKTKEKYE  
KSLKELDQGTPQYMENMEQVFEQCQQFEKRLRFFREVLLEVQKHLDSLNVAG  
YKAIYHDLEQSIRAADAVEDLRWFRANHGPGMAMNWPQFEEWSADLNRTLSR  
REKKKATDGVTLTGINQTGDQSLPSKPSSTLNVPSNPAQSAQSQSSYNPFEDDD  
TGSTVSEKDDTKAKNVSSYEKTQSYPTDWSDDDESNNPFSSTDANGDSNPFDDDA  
TSGTEVRVRALYDYEGQEHDELSFKAGDELTKMEDEDEQGWCKGRLDNGQVG  
LYPANYVEAIQ

*- C terminal*

Figura 17: La sequenza proteica di PACSIN2 contiene due siti di legame per LC3: la sequenza T-X-X-L si lega ad una tasca idrofobica, altamente conservata negli omologhi Atg8; la sequenza DDD è presente in proteine come p62.

## **Interazione di PACSIN2 con LC3 endogeno nelle linee cellulari NALM6**

PACSIN2 interagisce con LC3 nelle cellule in condizioni basali di crescita e durante il trattamento con cloroquina (Figura 18). I lisati di cellule NALM6 sono stati immunoprecipitati con anti-PACSIN2, anti-LC3 o IgG di coniglio, a cui è seguito immunoblotting con anticorpi contro PACSIN2 and LC3. I lisati sono stati preparati da cellule NALM6 non trattate o esposte alla cloroquina, sostanza che

induce l'accumulo di autofagosomi a livello intracellulare, alla concentrazione 50  $\mu$ M per tre ore.

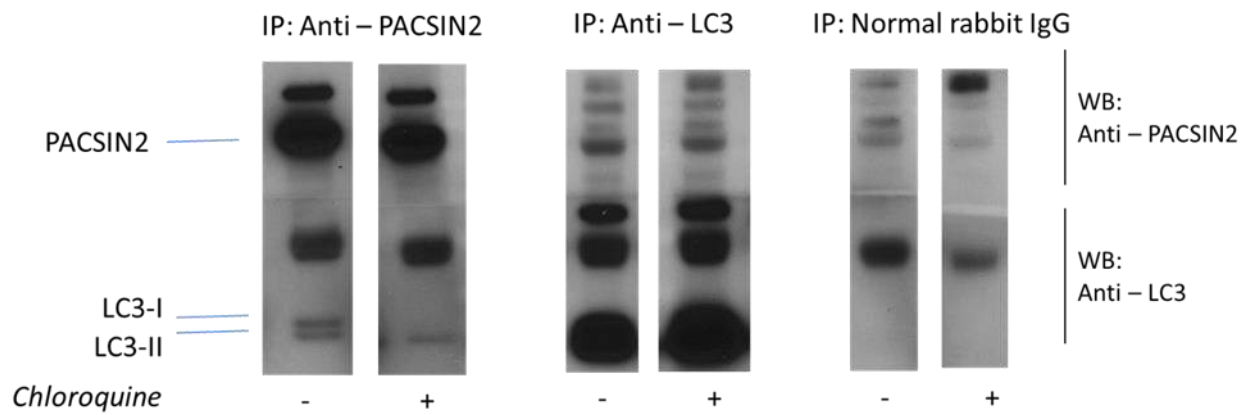


Figura 18: PACSIN2 interagisce con LC3 endogeno nelle NALM6. Lisati di NALM6 sono stati immunoprecipitati con anticorpi anti-PACSIN2, anti-LC3 o immunoglobuline di coniglio IgG, seguito da immunoblotting con anticorpi contro PACSIN2 e LC3. Gli esperimenti sono stati ripetuti tre volte ottenendo risultati riproducibili.

## **DISCUSSIONE**

Il trattamento della LLA nei soggetti in età compresa tra 1 e 18 anni in Italia è standardizzato secondo il protocollo AIEOP LLA 2009. La terapia della LLA è molto complessa, comprende più fasi terapeutiche e ha una durata complessiva di circa 2 anni, in cui è previsto l'utilizzo di vari farmaci antitumorali con meccanismi d'azione diversi, combinati tra loro in regimi terapeutici di differente intensità. La caratterizzazione citogenetica, immunofenotipica e molecolare dei blasti all'esordio e una precoce risposta positiva al trattamento, ha permesso di stratificare i pazienti in gruppi a differente rischio di ricaduta e di elaborare degli approcci polichemioterapici adattati al gruppo di rischio. A questo proposito, già nel precedente protocollo AIEOP LLA 2000 (identificativo NCT00613457, <http://clinicaltrials.gov>), in vigore dal 2000 fino al 2006, era stata introdotta la MRM ai giorni +33 e +78 come fattore prognostico (Van Dongen et al., 1998). Nell'ambito di questo protocollo, Conter e colleghi hanno studiato una coorte di 3184 pazienti affetti da LLA con immunofenotipo pre-B del gruppo di studio congiunto AIEOP e BFM (Berlino-Francoforte-Monaco,

l'associazione per le linee guida per la cura della LLA in Germania, Austria e Svizzera), dimostrando la superiorità della MRM come fattore prognostico rispetto a quelli convenzionali (conta cellulare, età, risposta precoce al prednisone e genotipo) con un netto aumento della sopravvivenza libera da malattia per i pazienti con MRM negativa ( $< 10^{-4}$ ) al giorno +33 (Conter et al., 2010; Paganin et al., 2014). Questo risultato è stato studiato anche su pazienti affetti da LLA con immunofenotipo T, ottenendo le stesse conclusioni (Schrappe et al., 2011). Basso e colleghi hanno in seguito analizzato 815 pazienti, dimostrando che la valutazione della MRM in citofluorimetria al giorno +15 era ancora più predittiva di ricaduta, suggerendo quindi l'utilità di quest'analisi per la stratificazione dei pazienti (Basso et al., 2009). Nell'attuale protocollo AIEOP LLA 2009 la MRM viene valutata ai giorni +15 mediante citofluorimetria e ai giorni +33 e +78 mediante PCR su sangue midollare.

Nonostante le alte percentuali di successo raggiunte dalla terapia, si verificano ancora delle risposte terapeutiche inattese, sia in

senso di ridotta efficacia che di tossicità. Su 1999 pazienti arruolati nel protocollo AIEOP LLA 2000, 306 sono ricaduti. Di questi, 28 pazienti rientravano nella fascia di rischio standard mentre 186 nella fascia del medio rischio (aggiornamento aprile 2008), rivelando quindi che la maggior parte dei bambini recidivati presentava caratteristiche prognostiche favorevoli ed era quindi stata curata con regimi terapeutici meno intensi. Questi dati sottolineano la necessità di individuare criteri aggiuntivi per perfezionare la stratificazione del trattamento, in particolare per quei pazienti attualmente considerati a basso rischio. Inoltre, su 785 pazienti arruolati in 13 centri italiani, il 63% aveva sviluppato episodi di tossicità acuta (grado III-IV) nella fase di induzione 1A (dati non pubblicati). La ricerca quindi di fattori dal valore prognostico o predittivo di tossicità è utile al fine di aumentare la risposta e ridurre gli effetti avversi. A tale scopo, il progetto proposto dal Burlo e in cui si inserisce questa tesi, propone delle strategie farmacogenetiche, farmacocinetiche e farmacodinamiche per l'ottimizzazione delle varie fasi dell'attuale protocollo AIEOP LLA 2009.

La personalizzazione della terapia sulla base delle caratteristiche

genetiche del paziente è di grande rilevanza per il miglioramento del trattamento con farmaci che presentano una significativa variabilità interindividuale nei profili di risposta (Stocco et al., 2013). Nel nostro studio per la valutazione delle variabili farmacogenomiche, le genotipizzazioni vengono effettuate sia sul DNA tumorale dei blasti alla diagnosi che su quello germinale estratto dal sangue periferico del paziente in remissione o dal tampone buccale. Questo viene fatto perché i dati genetici ottenuti dai due tipi di DNA potrebbero non coincidere, limitando l'uso della farmacogenetica in clinica per la cura dei tumori. Ad oggi, questa ipotesi è stata smentita in vari studi riassunti nel lavoro di McWhinney e McLeod che indicano una concordanza quasi completa tra le varianti nei geni farmacogenetici esaminati nel DNA germinale rispetto a quello tumorale (McWhinney et al., 2009).

Nel nostro studio, in tutti i pazienti analizzati finora per i 7 polimorfismi d'interesse, non sono state trovate differenze tra il genotipo leucemico e quello germinale dello stesso individuo. Tra i polimorfismi studiati sono presenti le varianti più comuni di TPMT, enzimi chiave nel metabolismo delle tiopurine. Questi polimorfismi

sono stati definiti come marker farmacogenetici importanti dal punto di vista clinico, tanto che di recente sono state pubblicate le linee guida per l'aggiustamento dei dosaggi di mercaptopurina e 6TG sulla base del genotipo di TPMT (Relling et al., 2013). È ormai noto infatti, che soggetti eterozigoti e omozigoti mutati per il gene TPMT presentano rispettivamente un'attività ridotta e nulla dell'enzima, con conseguenti livelli dei nucleotidi metilati MMPN più bassi e TGN più elevati che comportano un aumentato rischio di tossicità ematologica (Relling et al., 2013). L'aggiustamento dei dosaggi dei farmaci tiopurinici in base al genotipo di TPMT è stato suggerito anche per pazienti affetti da malattie infiammatorie (Schedel et al., 2006; Ansari et al., 2008; Benkov et al., 2013) ed è già in uso nei protocolli per la cura della LLA proposti dal St. Jude Children's Research Hospital di Memphis (USA) (Protocollo Total XV, (identificativo NCT00137111, <http://clinicaltrials.gov>). Nel protocollo AIEOP LLA 2009 tutti i pazienti all'esordio sono studiati per difetti nei geni che codificano per TPMT (presso l'Ospedale San Gerardo di Monza). La genotipizzazione viene eseguita mediante amplificazione e sequenziamento delle varianti alleliche \*2, \*3A,

\*3C e \*9. L'esito delle analisi viene comunicato ai Centri entro 30 giorni dall'esordio solo in caso di riscontro di difetto genetico in omozigosi. Per i pazienti che presentano riduzione dell'attività per difetti genetici in eterozigosi, non è prevista attualmente dal protocollo AIEOP LLA 2009 alcuna riduzione di dosaggio dei farmaci analoghi purinici. Per i pazienti che presentano attività TPMT assente per difetti genetici in omozigosi, si prevede quanto segue: mercaptopurina durante Protocollo IB: somministrare una dose pari al 25% della dose standard; mercaptopurina durante Protocollo M (terapia di consolidamento): somministrare una dose pari al 50% della dose standard; tioguanina durante Protocollo IIB/IIIB: somministrare una dose pari al 25% della dose standard. Tra i pazienti finora arruolati nel presente studio, circa il 6% dei soggetti è risultato eterozigote per gli SNP rs1142345 o rs1800460, frequenza pari a quella attesa in letteratura per la popolazione caucasica; non sono stati invece individuati al momento pazienti omozigoti per le varianti più frequenti di TPMT, come atteso sulla base della frequenza di queste varianti nella popolazione (circa 1 su 300). L'analisi dell'attività di TPMT ha confermato un'associazione

significativa delle varianti del gene con una riduzione dell'attività dell'enzima misurata negli eritrociti dei pazienti.

Le quantificazioni dei metaboliti tiopurinici sono attualmente disponibili per un sottogruppo dei pazienti genotipizzati e confermano un aumento della concentrazione dei metaboliti TGN ed una riduzione dei metaboliti MMPN nei pazienti con alleli varianti, sia durante la fase di consolidamento che durante quella di mantenimento. Invece nessuno degli altri polimorfismi candidati, in ITPA o PACSIN2 ha dimostrato al momento associazioni significative con la concentrazione dei metaboliti delle tiopurine. Studi precedenti in pazienti LLA e trattati secondo il protocollo EORTC58951 (identificativo NCT00003728, <http://clinicaltrials.gov>), hanno indicato che soggetti portatori dello SNP funzionale rs1127354 di ITPA variante presentavano una concentrazione di 6-MMP più elevata rispetto ai wild-type (de Beaumais et al., 2010). Anche Stocco e colleghi (2009), nell'ambito del protocollo Total XIIIB (St. Jude Children's Research Hospital di Memphis, USA), hanno visto che soggetti che ereditano varianti inattive di questo enzima, presentano un accumulo dei nucleotidi MMPN ed una

maggiore incidenza di effetti avversi come neutropenia severa con febbre durante la fase di consolidamento della terapia in cui il dosaggio della mercaptopurina era aggiustato sulla base di TPMT (Stocco et al., 2009). Un recente studio di farmacogenetica effettuato dal nostro gruppo di ricerca sui pazienti pediatrici del protocollo AIEOP LLA 2000 ha riscontrato una correlazione tra il genotipo variante di ITPA e un'aumentata probabilità di tossicità gastrointestinale e neurologica durante la fase di induzione 1A del trattamento. Il genotipo variante è stato associato alla tossicità gastrointestinale anche nella fase di consolidamento (lavoro in preparazioni). Uno studio di Wan Rosalina e colleghi ha dimostrato inoltre che pazienti malesi affetti da LLA che presentavano l'allele variante ITPA 94A erano più a rischio di sviluppare febbre e tossicità epatica durante il trattamento con mercaptopurina (Wan Rosalina et al., 2012). È interessante notare la diversa distribuzione dei genotipi di ITPA tra le varie etnie: lo SNP ha una frequenza del 19% negli Asiatici contro il 5% riscontrato nei Caucasici. E' stato quindi ipotizzato che i pazienti della popolazione asiatica siano più suscettibili all'insorgenza di tossicità da tiopurine legate alla

presenza di varianti del gene ITPA rispetto ai caucasici, in cui gli effetti avversi sono invece associabili prevalentemente al genotipo di TPMT la cui frequenza è del 5% nei Caucasici ed 1% negli Asiatici (Marsh et al., 2009). Recentemente è stato dimostrato che i pazienti asiatici sono più sensibili alle tiopurine: ciò potrebbe essere legato alla presenza negli asiatici di varianti del gene NUDT15, una pirofosfatasi come ITPA, che sono assenti nella popolazione caucasica (Yang et al., 2015). Questi risultati confermano che soggetti con un'attività ridotta di pirofosfatasi come ITPA o NUDT15 possono essere più sensibili al trattamento con tiopurine.

Per quanto riguarda ITPA, oltre alla misurazione dei livelli di metaboliti tiopurinici, per l'analisi delle variabili farmacocinetiche si è proceduto anche alla quantificazione dell'attività enzimatica di ITPA. Come atteso e in accordo con la letteratura (Sumi et al., 2002; Heller et al., 2004), è stata osservata una diminuzione consistente dell'attività enzimatica di ITPA in pazienti con genotipi varianti dei polimorfismi di ITPA considerati. Shipkova e collaboratori hanno studiato la correlazione tra i genotipi di ITPA (SNP rs1127354 e rs7270101) e il fenotipo attività enzimatica nei donatori sani

caucasici, mostrando che pazienti eterozigoti per lo SNP rs1127354 hanno un'attività di ITPA ridotta al 25% e i mutati un'attività nulla rispetto ai wild-type. Lo SNP rs7270101 ha un'azione meno marcata rispetto all'alterazione C94A, infatti è stato dimostrato che pazienti eterozigoti per questa variante presentano valori di attività enzimatica pari al 61% rispetto a quella dei controlli wild-type, mentre nei soggetti omozigoti mutati si riduce ulteriormente al 30%. Anche nella nostra popolazione di pazienti, la frequenza di soggetti con alleli varianti degli SNPs rs1127354 ed rs7270101 di ITPA è risultata pari al 18% in soggetti con genotipo wild-type AA per rs6051702, mentre è risultata del 74% in soggetti con almeno un allele variante C (p-value test di Fisher =  $1,33 \times 10^{-5}$ ), confermando l'effetto di questo SNP intronico, dovuta al fatto che esso rappresenta due varianti funzionalmente rilevanti (Fellay et al., 2010).

L'età è associata all'attività sia di ITPA che di TPMT: pazienti più giovani hanno un'attività più bassa di questi enzimi. Questa osservazione sembra indicare l'importanza di fattori legati allo sviluppo ed alla crescita dei pazienti anche per le caratteristiche

farmacocinetiche (van den Anker et al., 2011). Tuttavia al momento, l'età ha un effetto solo marginale sulla concentrazione dei metaboliti attivi della mercaptopurina. Per quanto riguarda gli effetti del sesso dei pazienti sulle caratteristiche farmacocinetiche, il presente studio ha confermato un'attività più bassa di ITPA in soggetti di sesso femminile precedentemente descritta (Shipkova et al., 2006) mentre per TPMT non sono stati rilevati effetti statisticamente significativi. E' interessante notare che attualmente il sesso dei pazienti presenta un effetto statisticamente significativo anche sulla concentrazione dei metaboliti TGN sia durante la fase di consolidamento che durante quella di mantenimento, tuttavia in direzione contraria, ovvero le femmine presentano una concentrazione più bassa durante il consolidamento e più alta durante il mantenimento. Il ruolo di età e sesso sulla farmacocinetica andrà dunque chiarito arruolando un numero più grande di pazienti e completando le associazioni con la risposta clinica dei pazienti.

Per quanto riguarda la variante di PACSIN2 considerata, rs2413739, i dati del nostro studio confermano un effetto del

genotipo variante T sull'attività di TPMT: pazienti con questa variante presentano un'attività ridotta in maniera significativa. L'effetto registrato è piccolo ed inferiore a quello delle varianti di TPMT, tuttavia è comparabile a quello osservato da uno studio precedente su una popolazione di pazienti con LLA trattati presso il St. Jude Children's Research Hospital (Stocco et al., 2012). Inoltre, lo stesso studio ha messo in luce che lo SNP rs2413739 (allele T) è associato ad una maggiore incidenza di tossicità gastrointestinale grave durante il consolidamento in pazienti leucemici trattati con il protocollo Total XIII (St. Jude Children's Research Hospital), dato confermato anche per il protocollo AIEOP LLA 2000 (Stocco et al., 2012).

Su queste basi si è cercato di chiarire il meccanismo molecolare con cui PACSIN2 può influenzare l'attività di TPMT. Studi precedenti di espressione genica hanno identificato i geni la cui espressione è cambiata significativamente in cellule umane di leucemia nelle quali il gene PACSIN2 è stato silenziato, mediante array Affymetrix GeneChip, rivelando che l'autofagia era fra i processi alterati. Per dimostrare il contributo di PACSIN2

nell'autofagia sono stati eseguiti una serie di esperimenti *in vitro*. Inizialmente si è verificato il livello di PACSIN2 in cellule in cui il meccanismo autofagico è stato interrotto artificialmente, in particolare fibroblasti murini embrionali di topi knock-out per Atg7. E' noto infatti che proteine importanti per i processi autofagici si accumulano in cellule prive di autofagia: Mathew e colleghi hanno descritto livelli costitutivamente più alti di p62 (SQSTM1), proteina che interagisce con le proteine strutturali degli autofagosomi (come LC3) ed è importante per la degradazione delle proteine mediante autofagia, in cellule prive di autofagia, ovvero da topo knock-out per Atg5 (Mathew et al., Cell 2009). I dati descritti in questa tesi dimostrano che PACSIN2 si accumula in cellule prive di autofagia, proprio come accade per p62. E' stato dimostrato che l'induzione dell'autofagia porta alla degradazione di proteine che interagiscono con gli autofagosomi, come ATG16L1. Cadwell e colleghi infatti hanno dimostrato che l'induzione dell'autofagia mediante trattamento con rapamicina induce la degradazione di ATG16L1 in cellule con autofagia funzionale (in particolare fibroblasti embrionali murini da topi di controllo), ma non in quelle prive di autofagia,

ovvero da topi knock-out per ATG5 (Cadwell et al., 2008). Un esperimento simile ha dimostrato che il trattamento con rapamicina non induce la degradazione di PACSIN2 in cellule con autofagia interrotta (fibroblasti embrionali murini da topi knock-out ATG7), degradazione che avviene invece con lo stesso trattamento in cellule di controllo con autofagia funzionale. Per chiarire ulteriormente il ruolo di PACSIN2 nell'autofagia, si è passati a verificare l'effetto del silenziamento di PACSIN2 sul numero di autofagosomi mediante imaging di cellule esprimenti LC3-GFP: infatti uno studio precedente di screening di una libreria di siRNA per il chinoma umano, ha identificato fra 10 siRNA in grado di aumentare il numero di autofagosomi in cellule MCF-7, quello diretto verso PACSIN1, proteina strutturalmente molto simile a PACSIN2 e che si differenzia da quest'ultima per un'espressione confinata nel sistema nervoso centrale. Nel nostro gli esperimenti di imaging hanno rivelato, in due modelli cellulari diversi (cellule RAW ed HeLa), un aumento del numero basale di autofagosomi dopo silenziamento di PACSIN2. L'analisi della sequenza della proteina ha rivelato l'esistenza di sequenze di amminoacidi che conferiscono

la capacità di interagire con LC3 e che sono presenti anche in una proteina come p62 (Nakatogawa et al., 2009). PACSIN2 potrebbe essere quindi un inibitore della formazione di autofagosomi, potenzialmente interagendo con LC3. Esperimenti di co-immunoprecipitazione di PACSIN2 e LC3 dimostrano l'interazione delle due proteine nelle linee cellulari leucemiche NALM6. Su queste basi, dunque, PACSIN2 è un inibitore dell'autofagia, probabilmente mediante interazione con LC3.

La valutazione delle variabili farmacodinamiche sembra suggerire invece che ad un'aumentata resistenza *in vitro* agli steroidi (PDN e DXM, ordine di grandezza  $10^{-3}$ ) corrisponde un aumento della percentuale residua di blasti nel midollo valutata al giorno +15. Questo risultato preliminare sembra incoraggiante soprattutto considerando che a due settimane dall'esordio, i pazienti leucemici sono stati trattati principalmente con prednisone ad alte dosi assunto quotidianamente per via orale e che la valutazione della MRM al giorno +15 si è rilevata particolarmente importante nella predizione dell'outcome (Basso et al., 2009). Le sensibilità *in vitro*

potrebbero aiutare a definire selettivamente i farmaci per cui i blasti mostrano resistenza, una discriminazione che la MRM al giorno +15 non consente. I risultati del test *in vitro* sono disponibili entro una settimana dalla diagnosi, fornendo delle indicazioni tempestive che potrebbero evitare l'uso di chemioterapici inadeguati, dosaggi inappropriati e probabili tossicità. Diversi studi sono stati svolti per valutare se i saggi di sensibilità *in vitro* sui blasti isolati alla diagnosi potessero essere in grado di fornire informazioni sulla risposta alla terapia e quindi essere usati per la stratificazione dei pazienti. Il gruppo olandese di Den Boer ha testato tramite saggio MTT la citotossicità dei chemioterapici in una coorte di pazienti trattati secondo il protocollo DCLSG (Dutch Childhood Leukemia Study Group) LLA VII/VIII ed i risultati ottenuti hanno mostrato che la combinazione dei profili di resistenza *in vitro* di glucocorticoidi, VCR, e ASP (PVA score) è un fattore prognostico indipendente per l'outcome (Den Boer et al., 2003). Escherich e colleghi hanno valutato prospettivamente il valore del test MTT in 202 pazienti trattati secondo il protocollo CoALL. In questo studio il PVA score è stato applicato prospettivamente per la stratificazione del rischio

nell'ambito del protocollo CoALL 06-97, riscontrando una sopravvivenza a lungo termine più alta per i pazienti con un profilo PVA sensibile rispetto a pazienti con profili di PVA intermedi o resistenti. Il PVA score non correlava però con la MRM al termine dell'induzione *in vivo* e il suo uso è stato sospeso nei protocolli successivi. In questo lavoro si è voluto riproporre questo approccio sul protocollo AIEOP LLA 2009, indagando in particolar modo sulla correlazione con la MRM al giorno +15 (Escherich et al., 2011).

In conclusione, questo lavoro di dottorato ha consentito di mettere a punto le metodiche necessarie allo svolgimento del progetto proposto dal IRCSS Burlo Garofolo di Trieste per lo sviluppo di strategie farmacologiche utili alla personalizzazione della cura della LLA secondo il protocollo AIEOP LLA 2009. E' ovviamente indispensabile ampliare la casistica considerata e completare i dati clinici, prima di delineare conclusioni di rilevanza clinica. Risultati promettenti sono però stati riscontrati per i saggi di sensibilità *in vitro* sui blasti isolati all'esordio della malattia e la loro correlazione con la risposta *in vivo* del paziente al giorno +15 (ottimizzazione della fase d'induzione) e sugli effetti dei genotipi candidati

considerati sui parametri misurabili in laboratorio (ottimizzazione dell'uso delle tiopurine nelle fasi di consolidamento e mantenimento).

Inoltre in questo lavoro è stata validata per la prima volta in una coorte indipendente di pazienti l'osservazione iniziale che polimorfismi di PACSIN2 influenzino l'attività dell'enzima TPMT. Gli studi *in vitro* eseguiti hanno chiarito che PACSIN2 è un gene coinvolto nell'autofagia: rimane da dimostrare che l'alterazione di PACSIN2, con meccanismi che coinvolgano direttamente l'autofagia, modula la stabilità della proteina TPMT.

## Bibliografia

- Anglicheau D, Sanquer S, et al. (2002) Thiopurine methyltransferase activity: new conditions for reversed-phase high-performance liquid chromatographic assay without extraction and genotypic-phenotypic correlation. *J Chromatogr B Analyt Technol Biomed Life Sci* 773(2):119-27.
- Ansari A., M. Arenas, et al. (2008) Prospective evaluation of the pharmacogenetics of azathioprine in the treatment of inflammatory bowel disease. *Aliment Pharmacol Ther* 28(8): 973-983.
- Basso G., M. Veltroni, et al. (2009) Risk of relapse of childhood acute lymphoblastic leukemia is predicted by flow cytometric measurement of residual disease on day 15 bone marrow. *J Clin Oncol* 27(31): 5168-5174.
- Behrends, C., Sowa, M.E., Gygi, S.P. and Harper, J.W. (2010) Network organization of the human autophagy system. *Nature* 466, 68–76.
- Benkov K., Y. Lu, et al. (2013) Role of thiopurine metabolite testing and thiopurine methyltransferase determination in pediatric IBD. *J Pediatr Gastroenterol Nutr* 56(3): 333-340.
- Cadwell K, Liu JY et al. (2008) A key role for autophagy and the autophagy gene *Atg16l1* in mouse and human intestinal Paneth cells. *Nature* 456 (7219), 259-63.

- Cheok MH, Evans WE. (2006) Acute lymphoblastic leukaemia: a model for the pharmacogenomics of cancer therapy. *Nat Rev Cancer* 6(2):117-29.
- Conter V., C. R. Bartram, et al. (2010) Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. *Blood* 115(16): 3206-3214.
- De Beaumais A. T., M. Fakhoury, et al. (2011). "Determinants of mercaptopurine toxicity in paediatric acute lymphoblastic leukemia maintenance therapy." *Br J Clin Pharmacol* 71(4): 575-584.
- De Vita V.T. Jr, H. S., Rosenberg S.A. (1997) *Cancer, principles and practice of oncology*.
- Den Boer M. L., D. O. Harms, et al. (2003) Patient stratification based on prednisolonevincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *J Clin Oncol* 21(17): 3262-3268.
- Escherich G, Tröger A, et al. (2011) The long-term impact of in vitro drug sensitivity on risk stratification and treatment outcome in acute lymphoblastic leukemia of childhood (CoALL 06-97). *Haematologica* 96(6):854-62.
- Fellay J, Thompson AJ, et al. (2010) ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. *Nature* 464(7287), 405-8.

- Fullmer A., S. O'Brien, et al. (2009) Novel therapies for relapsed acute lymphoblastic leukemia. *Curr Hematol Malign Rep* 4(3):148-156.
- Gajjar A., R. Ribeiro, et al. (1995) Persistence of circulating blasts after 1 week of multiagent chemotherapy confers a poor prognosis in childhood acute lymphoblastic leukemia." *Blood* 86(4): 1292-1295.
- Heller T., M. Oellerich, et al. (2004) Rapid detection of ITPA 94C>A and IVS2 + 21A>C gene mutations by real-time fluorescence PCR and in vitro demonstration of effect of ITPA IVS2 + 21A>C polymorphism on splicing efficiency. *Clin Chem* 50(11): 2182-2184.
- Jones, T.S., Yang, W. et al. (2007) Using HapMap tools in pharmacogenomic discovery: the thiopurine methyltransferase polymorphism. *Clin. Pharmacol. Ther* 81, 729–734.
- Kerst G., H. Kreyenberg, et al. (2005) Concurrent detection of minimal residual disease (MRD) in childhood acute lymphoblastic leukaemia by flow cytometry and real-time PCR. *Br J Haematol* 128(6): 774-782.
- Kessels, M.M. and Qualmann, B. (2004) The syndapin protein family: linking membrane trafficking with the cytoskeleton. *Cell Sci* 117, 3077–3086.
- Li, F., Wang, L., Burgess, et al., (2008) Thiopurine S-methyltransferase pharmacogenetics: autophagy as a

- mechanism for variant allozyme degradation. *Pharmacogenet. Genomics* 18, 1083–1094.
- Liliemark J., B. Pettersson, et al. (1992) On the biochemical modulation of 6-mercaptopurine by methotrexate in murine WEHI-3b leukemia cells in vitro. *Leuk Res* 16(3): 275-280.
  - Marsh S, Van Booven DJ. (2009) The increasing complexity of mercaptopurine pharmacogenomics. *Clin Pharmacol Ther* 85(2):139-41.
  - McWhinney S. R. and H. L. McLeod (2009) Using germline genotype in cancer pharmacogenetic studies. *Pharmacogenomics* 10(3): 489-493.
  - Mathew R., Karp C.M., et al. (2009) Autophagy suppresses tumorigenesis through elimination of p62. *Cell* 12;137(6):1062-75.
  - Meng H., Tian, L. et al. (2011) PACSIN2 represses cellular migration through direct association with cyclin D1 but not its alternate splice form cyclin D1b. *Cell Cycle* 10, 73–81.
  - Modregger, J., Ritter, B. et al., (2000) All three PACSIN isoforms bind to endocytic proteins and endocytosis. *J Cell Sci* 113(Pt 24), 4511–4521.
  - Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65(1-2): 55-63

- Nakatogawa H, Suzuki K et al. (2009) Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol*, 10(7), 458-67.
- Paganin M, Fabbri G, et al. (2014) Postinduction minimal residual disease monitoring by polymerase chain reaction in children with acute lymphoblastic leukemia. *J Clin Oncol* 2014 32(31):3553-8.
- Peters, E.J., Kraja, A.T. Et al. (2009) Association of thymidylate synthase variants with 5-fluorouracil cytotoxicity. *Pharmacogenet. Genomics* 19, 399-401.
- Pui C. H. and W. E. Evans (2006) Treatment of acute lymphoblastic leukemia. *N Engl J Med* 354(2): 166-178.
- Pui C. H., D. Campana, et al. (2009) Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N Engl J Med* 360(26): 2730-2741.
- Relling MV, Gardner EE, Sandborn WJ, Schmiegelow K, Pui CH, Yee SW, Stein CM, Carrillo M, Evans WE, Hicks JK, Schwab M, Klein TE. Clinical pharmacogenetics implementation consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing: 2013 update. *Clin Pharmacol Ther.* 2013 Apr;93(4):324-5.
- Sanjuan MA, Dillon CP, et al. (2007) Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* 450(7173):1253-7.

- Schrappe M., M. G. Valsecchi, et al. (2011) Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. *Blood* 118(8): 2077-2084.
- Schedel J., A. Godde, et al. (2006) Impact of thiopurine methyltransferase activity and 6-thioguanine nucleotide concentrations in patients with chronic inflammatory diseases. *Ann N Y Acad Sci* 1069: 477-491.
- Shipkova M, Lorenz K, et al. (2006) Measurement of erythrocyte inosine triphosphate pyrophosphohydrolase (ITPA) activity by HPLC and correlation of ITPA genotype-phenotype in a Caucasian population. *Clin Chem* 52(2): 240-7.
- Szyniarowski, P., Corcelle-Termeau, E. et al. (2011) A comprehensive siRNA screen for kinases that suppress macroautophagy in optimal growth conditions. *Autophagy* 7, 892–903.
- Stocco C, Cheok M, et al., (2009) Genetic polymorphism of inosine triphosphate pyrophosphatase is a determinant of mercaptopurine metabolism and toxicity during treatment for acute lymphoblastic leukemia. *Clin Pharmacol Ther* 85(2): 164-172.
- Stocco G, Yang W, et al., (2012) PACSIN2 polymorphism influences TPMT activity and mercaptopurine-related gastrointestinal toxicity. *Hum Mol Genet* 21(21):4793-804.

- Stocco G, Franca R, et al., (2013) Pharmacogenomic approaches for tailored anti-leukemic therapy in children. *Curr Med Chem* 20(17):2237-53.
- Sumi S., A. M. Marinaki, et al. (2002). Genetic basis of inosine triphosphate pyrophosphohydrolase deficiency. *Hum Genet* 111(4-5): 360-367.
- Taherbhoy AM, Tait SW, et al. (2011) Atg8 transfer from Atg7 to Atg3: a distinctive E1-E2 architecture and mechanism in the autophagy pathway. *Mol Cell* 44(3):451-61.
- Takahashi, Y., Coppola, D. et al., (2007) Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* 9, 1142–1151.
- Takahashi, Y., Meyerkord, C.L. Et al., (2011) Bif-1 regulates Atg9 trafficking by mediating the fission of Golgi membranes during autophagy. *Autophagy* 7, 61–73.
- van den Anker JN, Schwab M, Kearns GL. (2011) Developmental pharmacokinetics. *Handb Exp Pharmacol* 2011;205:51-75.
- Van Dongen J. J., T. Seriu, et al. (1998) Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. *Lancet* 352(9142): 1731-1738.
- Wan Rosalina W. R., L. K. Teh, et al. (2012) Polymorphism of ITPA 94C>A and risk of adverse effects among patients with acute lymphoblastic leukaemia treated with 6- mercaptopurine. *J Clin Pharm Ther* 37(2): 237-241.

- Wang, Q., Navarro, M.V. Et al., (2009) Molecular mechanism of membrane constriction and tubulation mediated by the F-BAR protein Pacsin/Syndapin. Proc Natl Acad Sci U. S. A. 106, 12700–12705
- Weinshilboum R. (2001) Thiopurine pharmacogenetics: clinical and molecular studies of thiopurine methyltransferase. Drug Metab Dispos Apr;29(4 Pt 2):601-5.
- Yang J., Landier W., et al., (2015) Inherited NUDT15 Variant Is a Genetic Determinant of Mercaptopurine Intolerance in Children With Acute Lymphoblastic Leukemia. J Clin Oncol 2015 Jan 26. [Epub ahead of print]

## **Ringraziamenti**

Ringrazio la Prof. Giuliana Decorti per avermi permesso di svolgere questo lavoro di dottorato e per il suo sostegno.

Ringrazio il Dr. Marco Rabusin dell'Oncoematologia pediatrica dell'ospedale Burlo Garofolo e tutti i centri AIEOP che collaborano allo studio per l'arruolamento dei pazienti ed il follow-up clinico.

Ringrazio il Prof. Alessandro Ventura della Clinica Pediatrica dell'IRCCS Burlo Garofolo per i suoi consigli ed il suo sostegno.

Ringrazio i Dr.i Raffaella Franca, Alessandra Zanut, Diego Favretto, Noelia Malusà e tutto il laboratorio di Farmacogenomica dell'Università di Trieste per le analisi farmacocinetiche, farmacodinamiche e farmacogenetica.

Ringrazio il Prof. William Evans del St.Jude Children's Research Hospital di Memphis (USA) per la possibilità di svolgere il lavoro in vitro su PACSIN2 e autofagia.

Curriculum vitae di Margherita Londero, nata a Udine il 19/08/80

Telefono: 3280322871

Mail: margheritalondero@yahoo.it

Diploma di Maturità Classica presso il Liceo Classico Jacopo Stellini di Udine nel mese di Luglio 1998 con votazione 89/100.

Laurea in Medicina e Chirurgia presso la Facoltà di Udine conseguita il giorno 20/10/2005 con la votazione di 110 e lode su 110.

Da segnalare durante i 6 anni del corso di laurea:

- Tirocinio presso l'Istituto di Genetica del Policlinico Universitario di Udine dal mese di Luglio al mese di Settembre 2003 per un totale di 185 ore.
- Tirocinio presso il laboratorio del Dipartimento di Biologia Molecolare della Facoltà di Medicina Charles University di Praga dal Novembre 2003 a Maggio 2004 per un totale di 216 ore.
- Partecipazione al Corso di Primo Soccorso svoltosi nel Marzo 2005 presso la sede della Croce Rossa di Udine.
- Internato pre-tesi presso la Clinica Pediatrica del Policlinico Universitario di Udine dal 1 febbraio 2005 al 15 ottobre 2005.
- Tesi sperimentale dal titolo: "Correlazione tra espressività clinica ed istopatologica di Malattia Celiaca e Genotipo HLA".

Immatricolazione presso la Scuola di Specializzazione in Pediatria del Burlo Garofolo di Trieste nell'Agosto 2006

**Attività formativa ,assistenziale e scientifica svolta nel quinquennio di specializzazione in pediatria.**

**Formazione e attività assistenziale.**

**1. Frequenza e attività assistenziale presso Unità Operative dell'IRCCS Burlo**

**Garofolo:**

-Clinica Pediatrica dal 1 Gennaio 2006 al 31 Marzo 2006 e dal 1 Aprile al 30 Giugno 2008

- Neonatologia e TIN dal 1/05 al 30/07 2007 e dal 1 Aprile al 30 Giugno 2010
- SAPS-I ACC : dal 1 Luglio al 30 Settembre 2008
- NPI dal 1 Ottobre al 31 Ottobre 2009
- Gastroenterologia dal 1 Maggio al 30 Settembre 2009
- Numero di turni di guardia diurna in autonomia in PS –I ACC (30) per un totale di 180 ore circa
- Numero di guardia notturna in autonomia in PS-ACC (10) per un totale di 120 ore

## **2.Frequenza di altre strutture pediatriche in Italia**

Tirocinio di 18 mesi (2006-2007) presso la Pediatria dell'Ospedale Santa Maria dei Battuti di Conegliano

## **3. Stage all'estero**

Tirocinio di tre mesi (Nov 2009- Gen 2010) presso l'Ospedale Oncologico Pediatrico St. Jude Children's Research Hospital di Memphis finalizzato all'apprendimento del saggio dell'MTT e alla collaborazione nell'esecuzione di uno studio di ricerca sperimentale.

## **4. Attivita' Scientifica**

### **Pubblicazioni in riviste con I.F.**

- Zemira Cannioto, Margherita Londero. Archives of Diseases in Childhood, 2006;91:116 Lettere elettroniche: "Lumbar discitis: think Bartonella"
- Londero M, Pastore S, Zanazzo GA, Bruno I, Ventura A. *A child with pain after mild trauma.* J Pediatr. 2010 Oct;157(4):693.
- Pastore S, Londero M, Gortani G, Abate MV, Marchetti F, Di Leo G, Ventura A. *Infliximab related vasculitis in patients affected by ulcerative colitis.*J Pediatr Gastroenterol Nutr. 2010 Aug;51(2):226-8
- De Iudicibus S, Stocco G, Martelossi S, Londero M, Ebner E, Pontillo A, Lionetti P, Barabino A, Bartoli F, Ventura A, Decorti G. *"Genetic predictors of glucocorticoid response in pediatric patients with inflammatory bowel diseases"*J Clin Gastroenterol. 2010 Aug 6
- Stocco G, Londero M, Campanozzi A, Martelossi S, Marino S, Malusa N, Bartoli F, Decorti G, Ventura A. *Usefulness of the measurement of azathioprine*

*metabolites in the assessment of non-adherence*. Journal of Crohn's and Colitis (2010) 4, 599–602

- Pastore S, Londero M, et al. *Refractory iron deficiency anemia in a child with portal cavernoma* Gut. 2010 Nov 4.
- Inaba H, Londero M, Maurer S, Onciu M, Ge Y, Taub J. *Acute megakaryoblastic leukemia without the GATA1 mutation following transient myeloproliferative disorder in an infant without Down syndrome*. Journal of Clinical Oncology

### **Publicazioni in riviste peer-reviewed italiane**

- Margherita Londero, Stefano Parlamento. *Ecografia polmonare nel neonato... mito o realtà?* Medico e Bambino, pagine elettroniche (Febbraio 2008)
- Matteo Bramuzzo, Margherita Londero, Gabriele Cont, Stefano Martelossi, Marzia Lazzarini. *Malattie infiammatorie croniche intestinali: epidemiologia e presentazione clinica*. Medico e bambino (Aprile 2009)

### **Abstract a congressi nazionali**

- “Ruolo di polimorfismi genetici nella risposta clinica ai glucocorticoidi in pazienti pediatriche con malattia infiammatoria cronica intestinale” presentato alla Maratona del giovane ricercatore, 23/06/2008, Trieste

### **Altro**

- Corso Teorico-Pratico di Rianimazione in Sala Parto e stabilizzazione del neonato in attesa di trasferimento. (Conegliano, 14-15 maggio 2007)
- Congresso “Cure intensive del neonato”, Verona 29 febbraio/1 marzo 2008
- Corso residenziale “attualità cliniche e terapia biologica nelle malattie infiammatorie croniche intestinali in età pediatrica”, Montecatini Terme, 19-20 Giugno
- PBLS (2009) e PALS (2009)

### **Attività formativa e scientifica svolta durante il periodo del dottorato**

#### **Corsi e congressi**

- 10° corso di formazione avanzata “Ricerca traslazionale in ematologia/oncologia”, Pavia, 16-20 maggio 2011

- Convegno: Personalizzare la terapia farmacologia, ovvero farmacogenetica: dalla teoria alla pratica, Trieste, 7 ottobre 2011
- III Convegno Monotematico SIF “Farmacogenetica e Cancro: dal Laboratorio alla Clinica” Grado, 8 ottobre 2011

### ***Pubblicazioni durante il dottorato***

- Stocco G, Cuzzoni E, De Iudibus S, Franca R, Favretto D, Malusà N, Londero M et al. *Deletion of Glutathione-S-Transferase M1 Reduces Azathioprine Metabolite Concentrations in Young Patients With Inflammatory Bowel Disease*. J Clin Gastroenterol. 2014 Jan;48(1):43-51.
- Stocco G, Franca R, Londero M, Decorti G. DMET™ Plus array delivers results in good concordance with those of several lower-throughput genotyping methods in patient samples. Pharmacogenomics. 2013 Feb;14(3):238-9.
- Stocco G, Franca R, Londero M, Decorti G. *ITPA genetic polymorphism is possibly associated with survival rate in Korean children with acute lymphoblastic leukemia*. Pharmacogenomics. 2013 Feb;14(3):237-8.
- Stocco G, Franca R, Londero M, Decorti G. *Processes for incorporation of pharmacogenetic tests and interpretation in medical records for clinical practice*. Pharmacogenomics. 2013 Feb;14(3):236-7.
- Stocco G, Franca R, Londero M, Decorti G. *Systematic identification of host genomic variation related to treatment outcome of childhood acute lymphoblastic leukemia*. Pharmacogenomics. 2013 Feb;14(3):235-6
- Stocco G, Franca R, Verze gnassi F, Londero M, Rabusin M, Decorti G. Pharmacogenomic approaches for tailored anti-leukemic therapy in children. Curr Med Chem. 2013;20(17):2237-53.
- Stocco G, Franca R, Verze gnassi F, Londero M, Rabusin M, Decorti G. *Multilocus genotypes of relevance for drug metabolizing enzymes and therapy with thiopurines in patients with acute lymphoblastic leukemia*. Front Genet. 2012;3:309.
- Stocco G, Yang W, Crews KR, Thierfelder WE, Decorti G, Londero M, Franca R, Rabusin M, Valsecchi MG, Pei D, Cheng C, Paugh SW, Ramsey LB, Diouf B, McCorkle JR, Jones TS, Pui CH, Relling MV, Evans WE. *PACSIN2 polymorphism influences TPMT activity and mercaptopurine related gastrointestinal toxicity*. Hum Mol Genet. 2012 Nov 1;21(21):4793-804.

- Pastore S, Londero M, Barbieri F, Di Leo G, Papparazzo R, Ventura A. Treatment with pamidronate for osteoporosis complicating long-term intestinal failure. *J Pediatr Gastroenterol Nutr.* 2012 Nov;55(5):615-8.
- Amaddeo A, Ventura A, Marchetti F, Benettoni A, Londero M. *Should cardiac involvement be included in the criteria for diagnosis of Churg Strauss syndrome?* *J Pediatr.* 2012 Apr;160(4):707.
- Inaba H, Londero M, Maurer SH, Onciu M, Ge Y, Taub JW, Rubnitz JE, Raimondi SC. *Acute megakaryoblastic leukemia without GATA1 mutation after transient myeloproliferative disorder in an infant without Down syndrome.* *J Clin Oncol.* 2011 Mar 20;29(9):e230-3.
- Stocco G, Londero M, Campanozzi A, Martelossi S, Marino S, Malusà N, Bartoli F, Decorti G, Ventura A. *Usefulness of the measurement of azathioprine metabolites in the assessment of non-adherence.* *J Crohns Colitis.* 2010 Nov;4(5):599-602.
- Pastore S, Londero M, Cont G, Di Leo G, Ventura A. *Refractory iron deficiency in a child with portal cavernoma.* *Gut.* 2011 Mar;60(3):317, 377.
- De Iudicibus S, Stocco G, Martelossi S, Londero M, Ebner E, Pontillo A, Lionetti P, Barabino A, Bartoli F, Ventura A, Decorti G. *Genetic predictors of glucocorticoid response in pediatric patients with inflammatory bowel diseases.* *J Clin Gastroenterol.* 2011 Jan;45(1).

## Acute Megakaryoblastic Leukemia Without *GATA1* Mutation After Transient Myeloproliferative Disorder in an Infant Without Down Syndrome

### Case Report

A 7-week-old full-term infant girl with unremarkable perinatal history was referred to our hospital for a 5-day history of hematochezia and mild epistaxis. Physical examination showed hepatosplenomegaly, scattered petechiae, and grossly bloody stool. No phenotypic features of Down syndrome (DS) were noted. CBC showed elevated WBC ( $51.2 \times 10^9/L$ ) with 10% blasts, anemia ( $7.1 \text{ g/dL}$ ), and thrombocytopenia ( $54 \times 10^9/L$ ). Bone marrow aspirate (BMA) showed hypercellularity, increased megakaryoblasts (15%), and dysplastic megakaryocytes (Fig 1A). Flow cytometric analysis

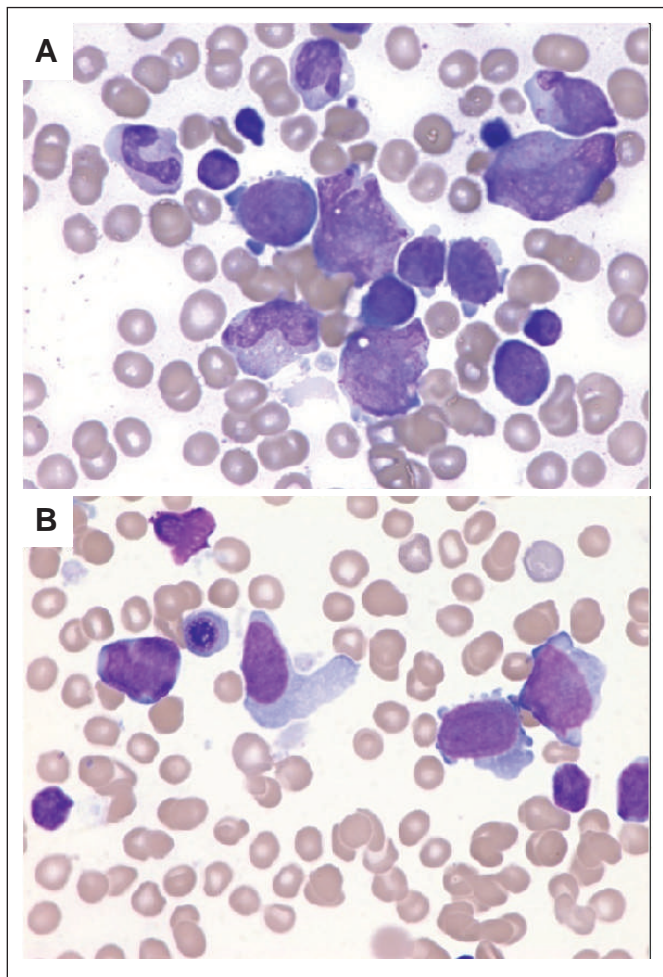


Fig 1.

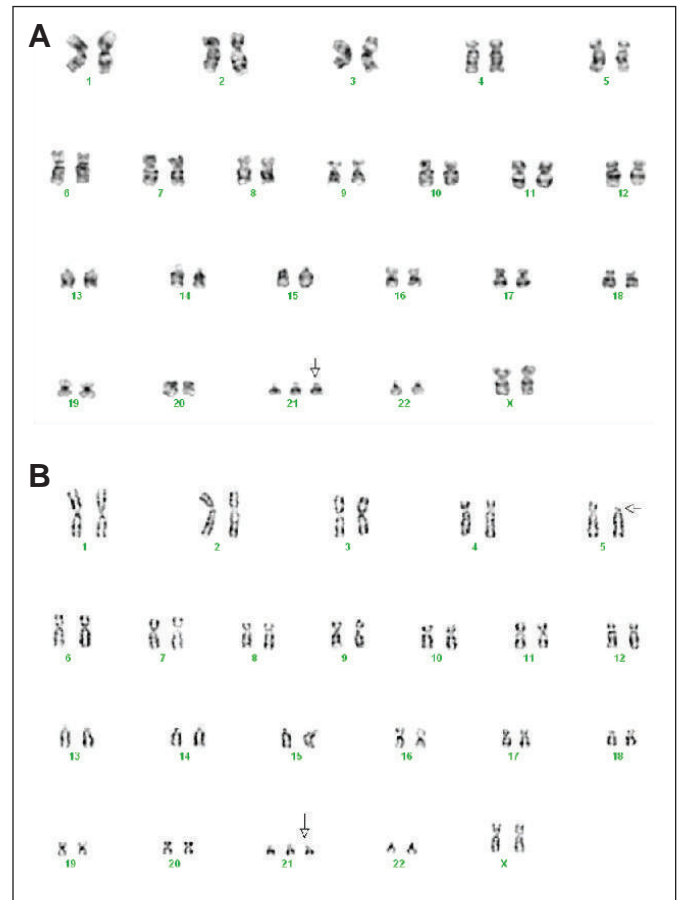


Fig 2.

showed expression of megakaryocyte-specific membrane markers (CD41, CD42b, and CD61) but not myeloperoxidase in blasts. Cytogenetic analysis revealed karyotype 47,XX,+21[16]/46,XX[4] (Fig 2A, arrow indicates trisomy 21). Fluorescence in situ hybridization using *ETO-AML1* probes showed a normal pattern of signal distribution in buccal epithelial cells, without evidence of an extra *AML1* signal. There was a single base pair deletion of G at nucleotide 150 (relative to ATG) in *GATA1* exon 2 in megakaryoblasts (Fig 3, arrow). The patient was diagnosed with transient myeloproliferative disorder (TMD) and became transfusion independent 1 month later. Peripheral blood blasts disappeared within 3 months without administration of chemotherapy.

At 7 months of age (5 months postpresentation), the patient again developed hematochezia and thrombocytopenia ( $10 \times 10^9/L$ ). WBC was  $9.7 \times 10^9/L$  with 1% circulating blasts, and hemoglobin was  $10.5 \text{ g/dL}$ . BMA revealed marked megakaryocytic dysplasia and increased megakaryoblasts (34%; Fig 1B). The blast population expressed megakaryocyte-associated antigens similar to those seen in the TMD sample but lost expression of CD56 and CD8 and showed

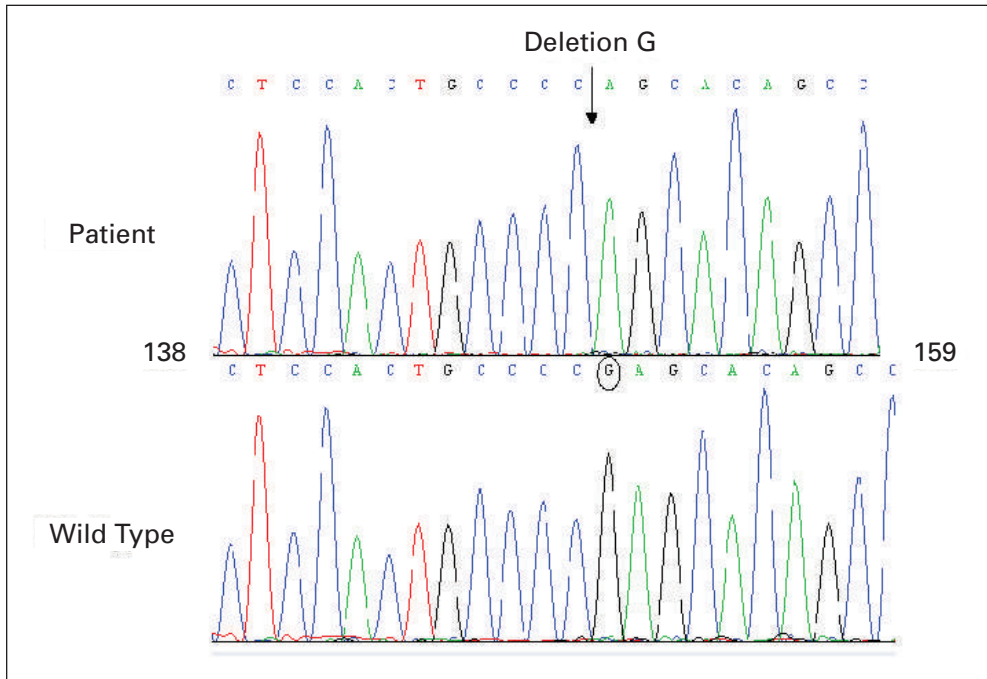


Fig 3.

weaker CD117 expression. Cytogenetic analysis revealed karyotype 47,XX,+21[14]/47,idem,del(5)(p13) [3]/46,XX [8] (Fig 2B, vertical arrow indicates trisomy 21 and horizontal arrow shows a deletion of 5p). FISH using a 5p subtelomeric probe showed an interstitial deletion [del(5)(p13p15)] (arrow, Fig 4). Given these clinical and laboratory findings, the patient was diagnosed with acute megakaryoblastic leukemia (AMKL).

The patient was enrolled onto the multi-institutional protocol AML02 (A Collaborative Trial for the Treatment of Patients With

Newly Diagnosed Acute Myeloid Leukemia or Myelodysplasia).<sup>1</sup> Therapy consisted of standard doses of two courses of remission induction (cytarabine, daunomycin, and etoposide) and two courses of consolidation (cytarabine and mitoxantrone, and cytarabine and L-asparaginase). She had a complete response (negative minimal residual disease) to the first course of remission induction and tolerated chemotherapy well. The third dose of consolidation therapy was not administered because of refractoriness to platelet transfusions associated with development of platelet antibodies. She has been off therapy

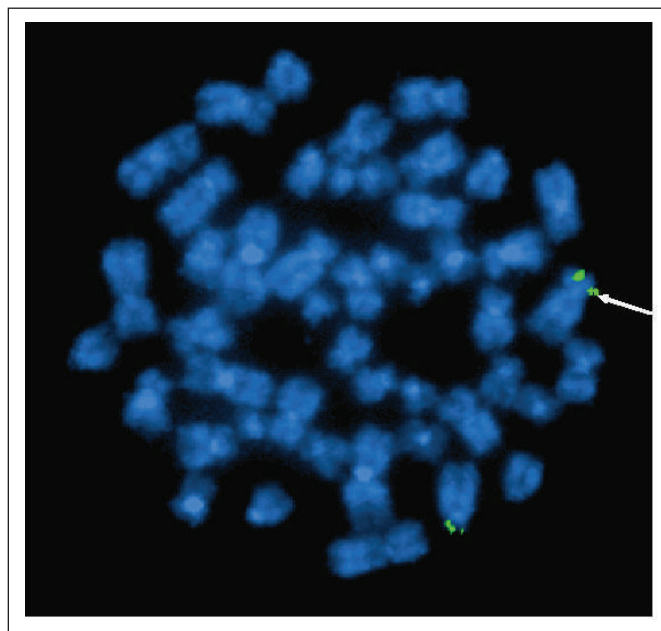


Fig 4.

for 18 months and remains in complete remission. Cytogenetic analysis in remission marrow showed 46,XX. Retrospective analysis of *GATA1* gene using a BMA sample at onset of AMKL did not show any mutations with sequence of 30 clones.

## Discussion

TMD, characterized by clonal proliferation of megakaryoblasts, develops almost exclusively in patients with DS during the neonatal period.<sup>2,3</sup> The reported incidence of TMD in patients with DS is 10% but may actually be higher because some affected fetuses die in utero. TMD usually resolves spontaneously within 2 to 3 months, but 20% to 30% of patients develop overt AMKL 1 to 30 months post-TMD. TMD also occurs in children without DS who have either mosaicism for trisomy 21 or normal karyotype.<sup>4</sup> The frequency and pathogenesis of TMD and subsequent AMKL in these children is not well known because TMD may go undetected.

Classic TMD in DS is characterized by elevated WBC (median,  $47 \times 10^9/L$ ; range, 5 to  $380 \times 10^9/L$ ) with varying percentages of circulating blasts.<sup>2,3</sup> BMA usually reveals dysplastic megakaryocytes and megakaryoblasts that are typically negative for myeloperoxidase and express megakaryocytic markers (CD41, CD42b, and CD61). In both patients with DS and those who are phenotypically normal, blast cells show trisomy 21. Hematopoietic cells in TMD also have acquired mutations in transcription factor gene *GATA1* (Xp11.23), which controls erythropoiesis and megakaryopoiesis.<sup>5</sup> The mutation, seen exclusively in exon 2, leads to truncated protein GATA1s, which lacks the N-terminal transactivation domain but is not leukemogenic in the absence of trisomy 21.<sup>6</sup> The combination of GATA1s and trisomy 21 seems to confer a selective advantage to blasts. Mortality rate from TMD- and DS-associated complications (eg, liver failure, congestive heart failure, renal failure, disseminated intravascular coagulation, hyperleukocytosis, and/or sepsis) can be 10% to 20%.<sup>2,3</sup> Our patient did not have a DS phenotype, and bleeding tendency was controlled by platelet transfusions only. The initial sole cytogenetic abnormality of trisomy 21 in leukemic blasts prompted us to analyze the *GATA1* mutation, which confirmed diagnosis of TMD. Cytogenetic analysis of somatic cells (eg, buccal mucosa and skin fibroblasts) is necessary to rule out DS as well as its mosaicism; in our patient, analysis of buccal mucosa and remission marrow ruled out mosaicism.

WBCs in patients with DS with AMKL and history of TMD (median,  $10 \times 10^9/L$ ; range, 1.8 to  $40.6 \times 10^9/L$ ) are lower than in those presenting with TMD, but BMA examinations are indistinguishable, and *GATA1* mutations are seen in both cases.<sup>2,3</sup> Because not all patients with TMD progress to development of AMKL, additional genetic or epigenetic events are likely required for progression to overt leukemia. Altered telomerase activity, *TP53* mutations, and additional acquired karyotype abnormalities (eg, +8, -7, and -5/5q-) have been reported.<sup>7</sup> A retrospective review reported that five of 16 patients with TMD without DS developed subsequent leukemia, three developed AMKL, and two developed non-AMKL acute myeloid leukemia, but patients were not checked for the presence of *GATA1* mutations.<sup>4</sup>

Our patient developed AMKL subsequent to TMD. She had high WBC with TMD, spontaneous remission, and reappearance of megakaryoblasts with lower WBC. Acquisition of an additional karyotypic abnormality—del(5p)—and development of several immunophenotypic shifts with progression to AMKL suggest that a subclone of TMD

cells evolved and acquired selective advantage. Most likely, *GATA1* mutation was lost with AMKL.

Event-free survival is more than 80% for patients with DS with AMKL but less than 50% in patients without DS with AMKL.<sup>2,3</sup> In *in vitro* studies, AMKL blasts from patients with DS are more sensitive to chemotherapeutic agents, especially cytarabine, than those from patients without DS because of overexpression of the cystathionine  $\beta$ -synthase gene (localized to 21q22.3) and low expression of cytidine deaminase with *GATA1* mutation.<sup>8,9</sup> Conventional AMKL therapy in patients with DS is associated with high treatment-related mortality.<sup>10</sup> Thus, several collaborative study groups have adapted their standard AMKL protocol for patients with DS by reducing chemotherapy doses or prolonging intervals between courses. Our patient received standard doses of chemotherapy because we initially considered that the regimen would be well tolerated in a patient with no evidence of constitutional chromosomal abnormalities and efficacious against leukemia cells with acquired +21. This regimen seemed appropriate because *GATA1* mutation was absent in AMKL sample. Although such cases are rare, our case illustrates that the treatment regimen for AMKL preceded by TMD in non-DS children must be carefully selected. Efficacy of a DS-based reduced-intensity regimen for those who retain *GATA1* mutation remains to be determined.

### Hiroto Inaba

St Jude Children's Research Hospital, Memphis, TN

### Margherita Londero

St Jude Children's Research Hospital, Memphis, TN, and University of Trieste, Trieste, Italy

### Scott H. Maurer and Mihaela Onciu

St Jude Children's Research Hospital, Memphis, TN

### Yubin Ge and Jeffrey W. Taub

Wayne State University School of Medicine, Detroit, MI

### Jeffrey E. Rubnitz and Susana C. Raimondi

St Jude Children's Research Hospital, Memphis, TN

## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

## REFERENCES

1. Rubnitz JE, Inaba H, Dahl G, et al: Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: Results of the AML02 multicentre trial. *Lancet Oncol* 11:543-552, 2010
2. Xavier AC, Ge Y, Taub JW: Down syndrome and malignancies: A unique clinical relationship—A paper from the 2008 William Beaumont Hospital symposium on molecular pathology. *J Mol Diagn* 11:371-380, 2009
3. Gamis AS, Hilden JM: Transient myeloproliferative disorder, a disorder with too few data and many unanswered questions: Does it contain an important piece of the puzzle to understanding hematopoiesis and acute myelogenous leukemia? *J Pediatr Hematol Oncol* 24:2-5, 2002
4. Apollonsky N, Shende A, Ouansafi I, et al: Transient myeloproliferative disorder in neonates with and without Down syndrome: A tale of 2 syndromes. *J Pediatr Hematol Oncol* 30:860-864, 2008
5. Wechsler J, Greene M, McDevitt MA, et al: Acquired mutations in *GATA1* in the megakaryoblastic leukemia of Down syndrome. *Nat Genet* 32:148-152, 2002
6. Hollanda LM, Lima CS, Cunha AF, et al: An inherited mutation leading to production of only the short isoform of *GATA-1* is associated with impaired erythropoiesis. *Nat Genet* 38:807-812, 2006
7. Roy A, Roberts I, Norton A, et al: Acute megakaryoblastic leukaemia (AMKL) and transient myeloproliferative disorder (TMD) in Down syndrome: A multi-step model of myeloid leukaemogenesis. *Br J Haematol* 147:3-12, 2009

## Diagnosis in Oncology

8. Taub JW, Huang X, Matherly LH, et al: Expression of chromosome 21-localized genes in acute myeloid leukemia: Differences between Down syndrome and non-Down syndrome blast cells and relationship to in vitro sensitivity to cytosine arabinoside and daunorubicin. *Blood* 94:1393-1400, 1999

9. Ge Y, Stout ML, Tatman DA, et al: GATA1, cytidine deaminase, and the high cure rate of Down syndrome children with acute megakaryocytic leukemia. *J Natl Cancer Inst* 97:226-231, 2005

10. Lange BJ, Kobrin N, Barnard DR, et al: Distinctive demography, biology, and outcome of acute myeloid leukemia and myelodysplastic syndrome in children with Down syndrome: Children's Cancer Group Studies 2861 and 2891. *Blood* 91:608-615, 1998

DOI: 10.1200/JCO.2010.32.3634; published online ahead of print at [www.jco.org](http://www.jco.org) on January 4, 2011

---

## Acknowledgment

We acknowledge the expertise of Vani J. Shanker, PhD, ELS, in the editorial review of the manuscript. This research was supported in part by Cancer Center Support Grant No. CA21765 from the National Institutes of Health and the American Lebanese Syrian Associated Charities.

# Genetic Predictors of Glucocorticoid Response in Pediatric Patients With Inflammatory Bowel Diseases

Sara De Iudicibus, PharmD,\*† Gabriele Stocco, PhD,\* Stefano Martellosi, MD,†  
 Margherita Londero, MD,† Egle Ebner, PharmD,\* Alessandra Pontillo, PhD,†  
 Paolo Lionetti, MD,‡ Arrigo Barabino, MD,§ Fiora Bartoli, MD,†  
 Alessandro Ventura, MD,† and Giuliana Decorti, MD\*

**Background:** Glucocorticoids (GCs) are used in moderate-to-severe inflammatory bowel diseases (IBD) but their effect is often unpredictable.

**Aim:** To determine the influence of 4 polymorphisms in the GC receptor [nuclear receptor subfamily 3, group C, member 1 (*NR3C1*)], interleukin-1 $\beta$  (*IL-1 $\beta$* ), and NACHT leucine-rich-repeat protein 1 (*NALP1*) genes, on the clinical response to steroids in pediatric patients with IBD.

**Methods:** One hundred fifty-four young IBD patients treated with GCs for at least 30 days and with a minimum follow-up of 1 year were genotyped. The polymorphisms considered are the *BcII* in the *NR3C1* gene, C-511T in *IL-1 $\beta$*  gene, and Leu155His and rs2670660/C in *NALP1* gene. Patients were grouped as responder, dependant, and resistant to GCs. The relation between GC response and the genetic polymorphisms considered was examined using univariate, multivariate, and Classification and Regression Tree (CART) analysis.

**Results:** Univariate analysis showed that *BcII* polymorphism was more frequent in responders compared with dependant patients ( $P=0.03$ ) and with the combined dependant and resistant groups ( $P=0.02$ ). Moreover, the *NALP1* Leu155His polymorphism was less frequent in the GC responsive group compared with resistant ( $P=0.0059$ ) and nonresponder ( $P=0.02$ ) groups. Multivariate analysis comparing responders and nonresponders confirmed an association between *BcII* mutated genotype and steroid response ( $P=0.030$ ), and between *NALP1* Leu155His mutant variant and nonresponders ( $P=0.033$ ). An association between steroid response and male sex was also observed ( $P=0.034$ ). In addition, Leu155His mutated genotype was associated with steroid resistance ( $P=0.034$ ). Two CART analyses supported these findings by

showing that *BcII* and Leu155His polymorphisms had the greatest effect on steroid response (permutation  $P$  value = 0.046). The second CART analysis also identified age of disease onset and male sex as important variables affecting response.

**Conclusions:** These results confirm that genetic and demographic factors may affect the response to GCs in young patients with IBD and strengthen the importance of studying high-order interactions for predicting response.

**Key Words:** glucocorticoids, inflammatory bowel disease, genetic polymorphisms, CART analysis

(*J Clin Gastroenterol* 2011;45:e1–e7)

The incidence of inflammatory bowel disease (IBD), Crohn's disease (CD), and ulcerative colitis (UC), has been increasing in recent years, in particular in children and adolescents,<sup>1</sup> and it is currently estimated that 20% to 30% of patients experience onset of their symptoms below the age of 20 years.<sup>2</sup>

Glucocorticoids (GCs) are effective in inducing remission in patients with moderate-to-severe IBD, and have been considered the standard for treatment,<sup>3</sup> however, interindividual differences in the efficacy of these medications have been observed: up to 90% of pediatric patients have a rapid improvement of symptoms if treated with GCs at dose equivalent to 1 to 2 mg/kg/d of prednisone.<sup>4</sup> However, after 1 year, only 55% of these patients are still in remission and are deemed steroid-responsive. Around 40% of patients are not able to discontinue the therapy, experiencing an increase in disease activity when the dose is reduced and are considered steroid-dependant; 7% of subjects are resistant and do not respond to GC therapy.<sup>5,6</sup>

Given the high incidence of suboptimal response, associated with a significant number of side effects, the identification of subjects who are most likely to respond poorly to GCs is extremely important, as these subjects could be considered for treatment with other immunosuppressants. The molecular mechanisms of steroid resistance and/or dependence are, however, poorly understood<sup>7</sup> and there is presently no clinical, pharmacologic, or genetic assay that allows predicting GC response in patients with IBD.

GCs exert their biological effects through binding to the GC receptor, which regulates either positively or negatively, the expression of its target genes.<sup>8–10</sup> Polymorphisms of the GC receptor gene (*NR3C1*; nuclear receptor subfamily 3, group C, member 1) have been described within the normal population and have been related to increased<sup>11</sup> or reduced<sup>12</sup> sensitivity to cortisol. In addition, the *BcII* polymorphism of

Received for publication November 18, 2009; accepted May 18, 2010.

From the \*Department of Life Sciences, University of Trieste;

†Department of Reproductive and Developmental Sciences and IRCCS, Istituto per l'Infanzia Burlo Garofolo, Clinica Pediatrica, Trieste; ‡Department of Pediatrics, University of Florence, Ospedale Meyer, Florence; and §Gastroenterology Unit, Research Children's Hospital "Gaslini," Genoa, Italy.

This research was supported by a grant from Istituto per l'Infanzia Burlo Garofolo, Trieste, Italy (RC 2007). Dr Sara De Iudicibus and Dr Alessandra Pontillo are recipients of a research fellowship from IRCCS Burlo Garofolo, Trieste. Dr Gabriele Stocco is recipient of a postdoctoral fellowship from the University of Trieste.

Sara De Iudicibus and Gabriele Stocco contributed equally to the article.

Conflict of interest: none declared.

Reprints: Giuliana Decorti, MD, Department of Life Sciences, University of Trieste, Via L. Giorgieri 7, 34127 Trieste, Italy (e-mail: decorti@units.it).

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Website (www.jcge.com).

Copyright © 2010 by Lippincott Williams & Wilkins

*NR3C1* gene has been related, in a recent study conducted in our laboratory, to an increased response to GCs in pediatric patients with IBD.<sup>13</sup>

GC resistance in IBD has been also related to increased levels of proinflammatory cytokines,<sup>7,14</sup> possibly through modifications in GC effects because of interference with the GC receptor signalling.<sup>15</sup> The high interindividual variation in GC response could even depend on alteration in cytokine production and therefore on the inflammation severity.<sup>16</sup> Interleukin-1 $\beta$  (IL-1 $\beta$ ) is the proinflammatory cytokine with the highest level of expression in the intestinal mucosa of both healthy individuals and IBD patients, and is a central element in the regulation of inflammation in IBD.<sup>17,18</sup> An association of IL-1 $\beta$  production with IL-1 $\beta$  single nucleotide polymorphisms (SNPs) has been recently suggested; in particular, SNPs at position -511 and -31 in the promoter region and at position +3954 in exon 5<sup>19</sup> have been related to the course and severity of IBD.<sup>20</sup>

IL-1 $\beta$  is produced as an inactive cytoplasmic precursor (proIL-1 $\beta$ , p35) that must be cleaved to generate the mature active form (p17); caspase-1, also known as IL-1 $\beta$ -converting enzyme, is required for this cleavage.<sup>21</sup> Caspase-1 is part of multiprotein cytoplasmic complexes, called NACHT leucine-rich-repeat protein 1 and 3 (NALP1 and NALP3) inflammasomes. Jin et al<sup>22,23</sup> have recently shown that variants of NALP1 are associated with the incidence of different autoimmune diseases and have suggested that mutations in *NALP1* gene (NLRP1: NLR family, pyrin domain containing 1) may result in a deregulated secretion of IL-1 $\beta$ .

The primary aim of this study was hence to determine the influence of genetic polymorphisms of *NR3C1*, *IL-1 $\beta$* , and *NALP1* genes in the clinical response to GCs in pediatric patients with IBD. In addition, the secondary aim was to evaluate the high order gene-gene and gene-demographic interactions, using the Classification and Regression Tree (CART) methodology.

## METHODS

### Subjects

This study included 154 young patients (73 female and 81 male; mean age at disease onset  $\pm$  SD;  $11.9 \pm 5.05$  y). All the patients (82 CD and 72 UC) were newly diagnosed and enrolled between July 2000 and March 2008 in the Gastroenterology Clinics of Children's Hospitals of Trieste, Firenze, and Genoa, Italy. The study included all consecutive patients who had been treated with GCs for at least 30 days and had a minimum of 1 year of follow-up evaluation. The subjects gave their written consent to participate in the study, which received the approval by the local ethics committee. CD and UC were diagnosed by histologic, radiologic, endoscopic, and clinical criteria. Clinical data were obtained retrospectively and were analyzed blindly from the genotype results. Therapeutic treatment for IBD consisted of a first course of oral steroid therapy (prednisone 1 to 2 mg/kg/d) for 2 to 4 weeks, and subsequently dose tapering every week.<sup>5,6</sup>

Patients were divided in 3 groups: therapeutic success was identified with GC discontinuation without steroid requirements during at least 1 year (responders) and GC-dependency was defined by relapse upon dose reduction, impeding steroid discontinuation.<sup>24</sup> Patients who did not respond after initial 30 days of GC treatment were considered resistant.<sup>25</sup> Nonresponder patients included the dependant and resistant groups. Response to steroid was

determined on the basis of clinical and physical parameters and laboratory data: abdominal pain, stool pattern, rectal bleeding, general well-being, presence of extraintestinal manifestations, and laboratory data<sup>26,27</sup> were recorded.

### Genetic Analysis

Venous blood (2.0 mL) was drawn into vacutainer tubes containing ethylenediaminetetraacetic acid and DNA was obtained by a standard phenol/chloroform extraction procedure and the Leu155His (rs12150220/A) polymorphisms in *NALP1* gene and *BclI* in *NR3C1* gene were determined by polymerase chain reaction-restriction fragment length polymorphism assays. DNA amplification and restriction were carried out for *BclI* polymorphism as described earlier<sup>28</sup>; for *NALP1* polymorphism, primers were designed using a web utility (<http://seq.yeastgenome.org/cgi-bin/web-primer>). Primers, restriction enzymes, and conditions used are shown in supplemental Table, Supplemental Digital Content 1, <http://links.lww.com/JCG/A17>. Genotyping for the rs2670660/C polymorphism in the *NALP1* gene and the C-511 T polymorphism (rs16944/T) in the *IL-1 $\beta$*  gene was carried out using TaqMan genotyping technologies (Applied Biosystems, UK) on a ABI7900 HT sequence detection system device. All genotyping assays were prevalidated by the suppliers.

### Statistical Analysis

Statistical analysis was carried out using R software (version 2.9.1). Any possible association between response to GCs and polymorphisms in each gene was investigated calculating odds ratio (OR) and 95% confidence intervals (CI) from contingency tables and using 2-sided Fisher exact test. To control for confounding variables, multivariate logistic regression was performed with a model considering the response to GCs as the dependant variable and patients' age at the onset of the disease, sex, diagnosis, and genotypes, as the independent variables.

### CART Analysis

CART analysis was performed to take into account the multiple possible important factors influencing response to steroids in IBD, and possible interactions between them. CART analysis is a form of binary recursive partitioning.<sup>29</sup> The analysis was carried out with the rpart R package of the software R, using the standard parameters implemented in the rpart function.<sup>30</sup> To find the best variable, the software checks all possible splitting variables, and possible values of the variable to be used to split the node. In choosing the best splitter, the program seeks to maximize the average "homogeneity" of the 2 daughter nodes. The process of node splitting, followed by the assignment of a predicted class (in this case "response" or "no response" to GCs) to each node, is repeated for each daughter node and continued recursively until it is impossible to proceed because of small number of cases in each daughter node. Therefore, associations determined by terminal nodes can be clearly visualized and associated to risk assessment. Misclassification error was estimated through 10-fold cross-validation; the misclassification rate is a measure of heterogeneity in the subsets, estimated from the proportion of patients who are in the minority "GCs response" group within each subset. The best splits were assigned by the 1-SE rule: the split selected is the one with the largest cross-validation error within 1 SD of the minimum cross-validation error observed.<sup>30</sup> Permutation testing (1000 permutations) allowed to determine the

**TABLE 1.** Characteristic of All the Patients Divided Considering the Response to Treatment With GCs in Responders (GC Withdrawal Without Steroid Requirements During at Least 1 y), Dependants (Relapse Upon Dose Reduction and Impeding Steroid Discontinuation), and Resistant (no Response After Initial 30 d of GC Treatment)

	GC- Responders (n = 84)	GC- Dependants (n = 55)	GC- Resistant (n = 15)
Age in years at disease onset (mean ± SD)	12.4 ± 5.5	11.05 ± 4.8	12.5 ± 3.3
Sex			
Male n; (%)	50; (59.5)	24; (43.6)	7; (46.7)
Female n; (%)	34; (40.5)	31; (56.4)	8; (53.3)
Diagnosis			
CD n; (%)	49; (58.3)	26; (47.3)	7; (46.7)
UC n; (%)	35; (41.7)	29; (52.7)	8; (53.3)

CD indicates Crohn's disease; GC, glucocorticoid; UC, ulcerative colitis.

significance of the final model, relative to a model without any splits. We fit a logistic regression model to compare with the CART results and to allow us to determine *P* values for each covariate in the full model.

Two sets of CART were built: the first one was built starting only with the significant genetic predictors of response to GCs, identified by the univariate analysis<sup>31</sup>; the second one was built using all the available covariates.<sup>29</sup>

*P* values lower than 0.05 were considered statistically significant.

**RESULTS**

One hundred fifty-four IBD patients, who required treatment with GCs, were included in the study and were

divided into 3 groups on the basis of steroid response: 84 patients were GC responders, 55 GC dependants, and 15 were considered resistant. The demographic and clinical details of all the patients are summarized in Table 1. There was no significant difference in age, sex, and type of IBD between the 3 groups.

The frequency of 4 polymorphisms in 3 different genes was evaluated and is reported in Table 2: all the considered polymorphisms were in Hardy-Weinberg equilibrium.

The *BclII* mutated genotype in intron 2 of *NR3C1* gene was significantly more frequent in patients who responded to GCs (21.4%) compared with combined steroid dependant and resistant groups (7.1%; OR = 0.28, 95% CI = 0.09-0.80, *P* = 0.02); this polymorphism was significantly more frequent in responders compared with steroid dependants (7.3%; OR = 0.29, 95% CI = 0.09-0.90, *P* = 0.03) (Table 2).

The *IL-1β* gene polymorphism, C-511 T, and *NALP1* promoter polymorphism (rs2670660/C) were equally frequent in all groups of patients (Table 2).

In contrast, the homozygous mutated genotype for the Leu155His SNP in *NALP1* gene, was significantly less frequent in the responder (17.8%) compared with the resistant group (53.3%; OR = 0.19, 95% CI = 0.05-0.71, *P* = 0.0059). A significant difference was observed also between responders and combined dependant and resistant groups (nonresponders) (34.3%; OR = 2.38, 95% CI = 1.07-5.45, *P* = 0.02; Table 2).

The results of the logistic regression analysis are reported in Table 3. This analysis, performed between responders and nonresponders (Table 3A), confirmed an independent significant association between the *BclII* mutated genotype and steroid response (adjusted OR = 0.29, 95% CI = 0.09-0.089, *P* = 0.030), and between *NALP1* Leu155His mutant variant and nonresponse (adjusted OR = 3.12, 95%

**TABLE 2.** Univariate Analysis (Fisher Exact Test) and Genotype Distribution of *NR3C1*, *IL-1β*, and *NALP1* Gene Polymorphisms in IBD Patients Divided in Responders (GC Withdrawal Without Steroid Requirements During at Least 1 y), Dependants (Relapse Upon Dose Reduction and Impeding Steroid Discontinuation), and Resistant (no Response After Initial 30 d of GC Treatment)

	Genotype			OR (95% CI)	<i>P</i>
	WT (%)	HET (%)	MUT (%)	MUT Versus NON MUT	
<b>NR3C1 <i>BclII</i></b>					
Responders (84)	35 (41.7)	31 (36.9)	18 (21.4)	—	—
Dependants (55)	22 (40.0)	29 (52.7)	4 (7.3)	0.29 (0.09-0.90)	0.03
Resistant (15)	9 (60.0)	5 (33.3)	1 (6.7)	0.27 (0.03-2.13)	0.29
Nonresponders (70)	31 (44.3)	34 (48.6)	5 (7.1)	0.28 (0.09-0.80)	0.02
<b>IL-1β C-511T</b>					
Responders (79)	39 (49.4)	34 (43.0)	6 (7.6)	—	—
Dependants (54)	28 (51.9)	24 (44.4)	2 (3.7)	0.47 (0.04-2.76)	0.47
Resistant (14)	6 (42.9)	6 (42.9)	2 (14.2)	0.49 (0.07-5.60)	0.34
Nonresponders (68)	34 (50.0)	30 (44.1)	4 (5.9)	0.76 (0.15-3.37)	0.75
<b>NALP1 rs2670660/C</b>					
Responders (77)	21 (27.3)	36 (46.7)	20 (26.0)	—	—
Dependants (54)	16 (29.6)	21 (38.9)	17 (31.5)	1.30 (0.56-3.01)	0.55
Resistant (13)	1 (7.6)	6 (46.2)	6 (46.2)	0.41 (0.10-1.68)	0.18
Nonresponders (67)	17 (25.4)	27 (40.3)	23 (34.3)	1.48 (0.68-3.25)	0.28
<b>NALP1 Leu155His</b>					
Responders (84)	13 (15.5)	56 (66.7)	15 (17.8)	—	—
Dependants (55)	16 (29.1)	23 (41.8)	16 (29.1)	1.69 (0.77-4.57)	0.146
Resistant (15)	1 (6.7)	6 (40.0)	8 (53.3)	0.19 (0.05-0.71)	0.0059
Nonresponders (70)	17 (24.3)	29 (41.4)	24 (34.3)	2.38 (1.07-5.45)	0.02

Nonresponders include dependants and resistant.

CI indicates confidence intervals; GC, glucocorticoid; HET, heterozygous; IBD, inflammatory bowel disease; IL-1β, interleukin-1β; MUT, mutated; *NALP1*, NACHT leucine-rich-repeat protein 1; *NR3C1*, nuclear receptor subfamily 3, group C, member 1; OR, odds ratio; WT, wild type.

**TABLE 3.** Logistic Regression Model for the Response to Steroid Therapy (OR With 95% CI and *P* Values for the Independent Variables)

Variable	OR (CI)	<i>P</i>
Nonresponders (n = 70) versus Responders (n = 84)		
Age of disease onset (each year)	0.96 (0.89-1.04)	0.33
Sex (male vs. female)	0.46 (0.22-0.94)	0.034
IBD (UC vs. CD)	1.51 (0.74-3.12)	0.26
NR3C1 <i>BcII</i> (MUT vs. NON MUT)	0.29 (0.09-0.89)	0.030
IL-1 $\beta$ C-511T (MUT vs. NON MUT)	0.84 (0.20-3.54)	0.81
NALP1 rs2670660/C (MUT vs. NON MUT)	0.85 (0.31-2.34)	0.76
NALP1 Leu155His (MUT vs. NON MUT)	3.12 (1.10-8.90)	0.033
Resistant (n = 15) versus Responders (n = 84)		
Age at the onset of the disease (each year)	0.99 (0.85-1.16)	0.95
Sex (male vs. female)	0.58 (0.14-2.40)	0.45
IBD (UC vs. CD)	1.26 (0.30-5.32)	0.75
NR3C1 <i>BcII</i> (MUT vs. NON MUT)	0.34 (0.03-3.26)	0.35
IL-1 $\beta$ C-511T (MUT vs. NON MUT)	2.41 (0.31-18.60)	0.40
NALP1 rs2670660/C (MUT vs. NON MUT)	0.82 (0.11-6.14)	0.85
NALP1 Leu155His (MUT vs. NON MUT)	8.25 (1.18-57.96)	0.034

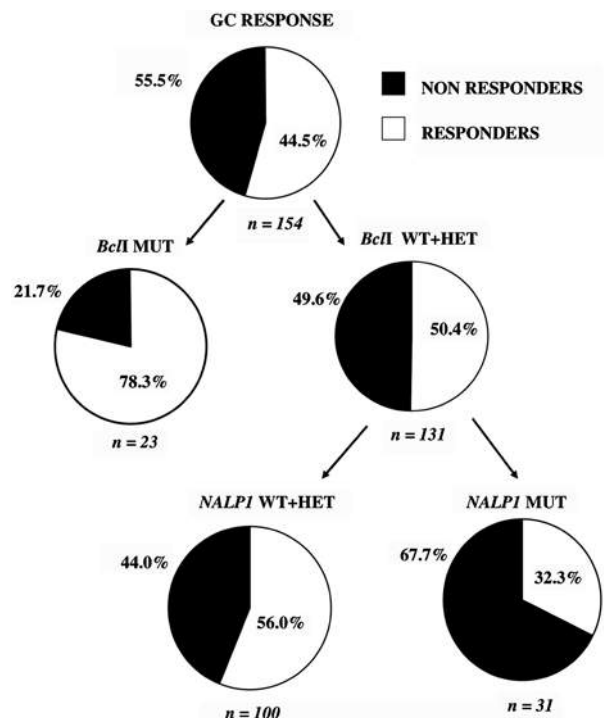
CD indicates Crohn's disease; CI, confidence intervals; IBD, inflammatory bowel disease; IL-1 $\beta$ , interleukin-1 $\beta$ ; MUT, mutated; NALP1, NACHT leucine-rich-repeat protein 1; NR3C1, nuclear receptor subfamily 3, group C, member 1; OR, odds ratio; UC, ulcerative colitis.

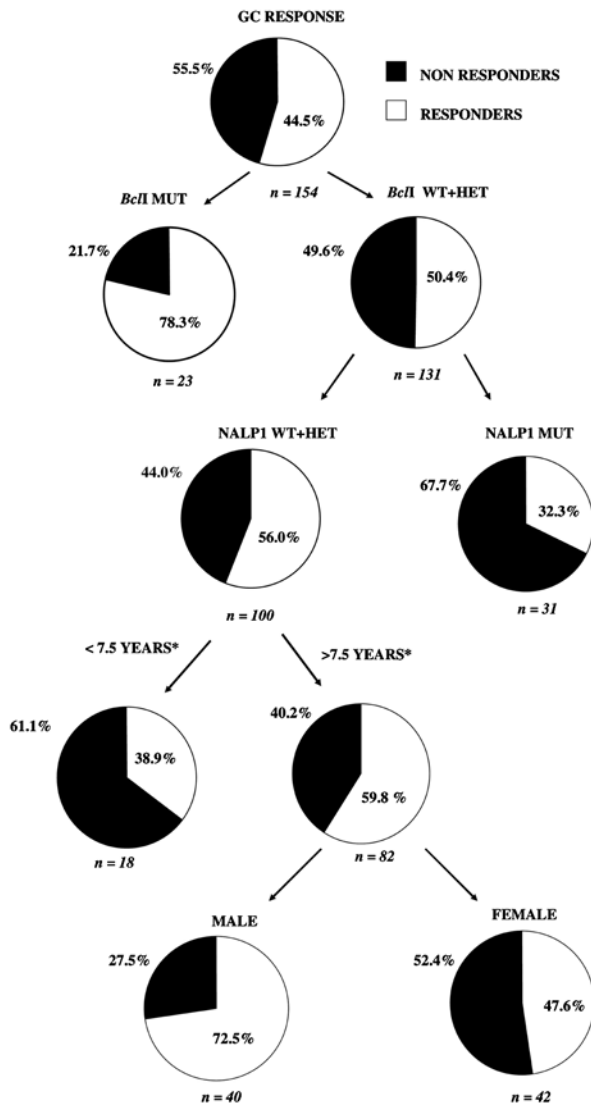
CI = 1.10-8.90, *P* = 0.033). In addition, a significant association was observed between steroid response and male sex (adjusted OR = 0.46, 95% CI = 0.22-0.94, *P* = 0.034). Multivariate analysis comparing resistant patients to responders (Table 3B), showed that NALP1 Leu155His mutated genotype was associated with GC resistance (adjusted OR = 8.25, 95% CI = 1.18-57.96, *P* = 0.034). All other variables were not significantly associated.

Two types of CART analysis were performed: the first one explored the interaction among the significant genetic factors for GC response (Fig. 1, permutation *P* value for the CART = 0.046). This analysis revealed that the *BcII* polymorphism was the characteristic that best distinguished patients who responded to GC therapy: the presence of the mutated genotype for this SNP conferred a higher probability of a good GC response (78.3%) in comparison with patients who are not carriers of this SNP (50.4%, *P* = 0.018). Among the 131 patients nonmutated for the *BcII* polymorphism, the most important feature to distinguish GC response was the NALP1 Leu155His polymorphism: 67.7% of the mutated patients for this SNP, but only 44% of subjects with a nonmutated NALP1 genotype did not respond to GC therapy (*P* = 0.023).

The second CART analysis performed starting from all the independent variables considered in this study (ie, age at disease onset, sex, IBD diagnosis, and the considered *IL-1 $\beta$* , *NALP1*, and *NR3C1* gene polymorphisms) (Fig. 2), confirmed a significant independent effect of *BcII* and NALP1 polymorphisms, as described above. Moreover, this analysis indicated that age at disease onset and male sex are important variables affecting response. Among the 100 patients nonmutated both for the *BcII* and for the NALP1 Leu155His polymorphisms, the most important feature to distinguish GC response was age at disease onset, with 61.1% of patients with an age lower than 7.5 years not responding to therapy (40.2% were nonresponder among patients with an age above 7.5 y). Among the 82 patients with an age above 7.5 years and nonmutated for both NR3C1 *BcII* and NALP1 Leu155His polymorphisms, the most important feature to distinguish GC response was sex,

with 27.5% of male patients not responding to therapy (52.4% were nonresponder among female patients). Logistic regression in the 100 patients nonmutated for both NR3C1 *BcII* and NALP1 Leu155His polymorphisms, considering response to GCs as the dependant variable and age at disease onset (higher or lower than 7.5) and sex

**FIGURE 1.** Classification and Regression Tree built starting only with the significant genetic predictors of response to glucocorticoids (GCs), identified by the univariate analysis. HET indicates heterozygous; MUT, mutated; NALP1, NACHT leucine-rich-repeat protein 1; WT, wild type.



**FIGURE 2.** Classification and Regression Tree built using all the available covariates (\*: age at the onset of the disease). GC indicates glucocorticoid; HET, heterozygous; MUT, mutated; NALP1, NACHT leucine-rich-repeat protein 1; WT, wild type.

as the nondependent variables, confirmed a significant effect of the interaction term between age and sex (adjusted OR = 0.10, 95% CI = 0.01-0.88,  $P = 0.038$ , Table 4); this logistic regression analysis confirmed a significant reduced probability of nonresponse to therapy for male patients with an age at disease onset above 7.5 years, as indicated in Figure 3. This interaction was not significant considering the whole population ( $P = 0.081$ ), however, it became significant only when the effects of the NR3C1 and NALP1 variants considered were excluded.

**DISCUSSION**

This study, using univariate and multivariate (logistic regression) analysis showed a significant effect of the *BclII* polymorphism in the *NR3C1* gene, and of the NALP1 Leu155His SNP on response to GCs.

**TABLE 4.** Logistic Regression Model for the Response to GC Therapy in the Patients Nonmutated for Both NR3C1 *BclII* and NALP1 Leu155His Polymorphisms (OR With 95% CI and  $P$  Values for the Independent Variables Selected by the CART)

Variable	OR (CI)	$P$
Nonresponders (n = 56) versus Responders (n = 44)		
Age at disease onset ( $\geq 7.5$ vs. $< 7.5$ ) (y)	1.47 (0.29-7.37)	0.64
Sex (male vs. female)	3.56 (0.48-26.28)	0.21
Interaction age: sex	0.10 (0.01-0.88)	0.038

CART indicates Classification and Regression Tree; CI, confidence intervals; GC, glucocorticoid; NALP1, NACHT leucine-rich-repeat protein 1; NR3C1, nuclear receptor subfamily 3, group C, member 1; OR, odds ratio.

To explore the interactions among the genetic and demographic factors important for GC response, 2 types of CART analysis were performed. The first one was built starting only with the significant genetic predictors of response to GCs, identified by the univariate analysis<sup>31</sup>; the second one was built using all the available covariates.<sup>29</sup> In the first CART analysis, only the *BclII* polymorphism in the *NR3C1* gene and the NALP1 Leu155His SNP, earlier identified by the univariate analysis, were considered. The *BclII* SNP was classified as the initial split, and hence represents the best indicator of response to GCs. Patients with the *BclII* mutated genotype had the highest probability of response to GC therapy, and needed less frequently additional courses of this therapy. This result is in line with an earlier study conducted in IBD,<sup>13</sup> and with a number of reports that identified this polymorphism as related to an increased sensitivity to GCs.<sup>32-36</sup>

Among patients with the nonmutated *BclII* genotype, carriers of the NALP1 homozygote Leu155His variant genotype exhibited a higher probability of nonresponse to therapy. NALP1 is a member of the nucleotide oligomerization domain (NOD)-like receptors family; the protein recruits the adapter protein ASC, caspase-1 and caspase-5, to form a complex called NALP1 inflammasome that is central in the activation of proinflammatory IL-1 $\beta$ .<sup>37,38</sup> The nonsynonymous coding SNP rs12150220, earlier reported to confer susceptibility to autoimmune and autoinflammatory diseases,<sup>22,39</sup> results in a Leu > His amino acidic change in position 155, located between the N-terminal pyrin and the NACHT domains of the human NALP1 protein. This region is highly conserved in primates<sup>23</sup> suggesting its critical role in protein function. Mutations in other genes of the NACHT family and in particular in NOD2/CARD15 and *NALP3* genes, have been described and related to IBD and other autoinflammatory conditions.<sup>40-42</sup> In addition, a role for combined polymorphisms in *NALP3* and *CARD8* has been recently suggested in CD.<sup>43</sup> Most of these variant nucleotides are located in the NACHT domain, physically close to the Leu155His SNP, and result in an increased production of proinflammatory mediators. Of particular interest, NALP1 is highly expressed in simple columnar epithelial tissues, such as those lining the digestive tract (stomach, small intestine, and colon), whereas *NALP3* expression has not been showed in cells of the gastrointestinal tract.<sup>44</sup>

The high frequency of variant Leu155His genotype in patients resistant to GC therapy, observed in our study, is possibly related to an activation of pro-IL-1 $\beta$ , mediated by

the inflammasome, and subsequent increased production of mature IL-1 $\beta$ . This cytokine is expressed at high concentrations in the intestinal mucosa of IBD patients<sup>17,18</sup> and has been shown to induce steroid resistance in vitro, in the Caco-2 human colon carcinoma cell line.<sup>45</sup> Genetic polymorphisms of the *IL-1 $\beta$*  gene have been associated with an increased secretion of the cytokine, although data are not univocal<sup>19,20,46–48</sup>; however, in our study the C-511T polymorphism was equally frequent in GC responder patients and in nonresponders.

In the second CART analysis, in which all the variables were considered, in patients with the nonmutated *BclI* and NALP1 genotype, age and sex were taken into account for diagnostic classification by the algorithm. In particular, in male subjects with an age at disease onset above 7.5 years, response to GC therapy was significantly more frequent, and the logistic regression analysis confirmed a significant effect of the interaction term between age and sex when the effects of NR3C1 and NALP1 variants were excluded. This result is in agreement with a recent study that has shown that in CD, young age is predictive for disease recurrence.<sup>49</sup> Accordingly, the influence of female hormonal status in IBD activity has also been recognized, and it has been demonstrated that, in AtT-20 cells, progesterone can enhance the dissociation of GCs from their receptors, resulting in a decreased response.<sup>50</sup> Interestingly, 81% of the 42 female patients in the terminal node had an age at the onset of the disease  $\geq 11$  years.

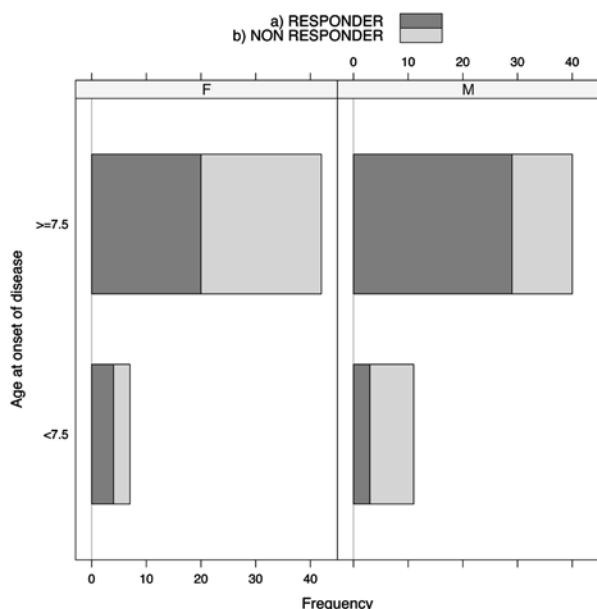
All the effects on response to GCs discussed in this study and revealed by the classification tree (ie, NALP1 Leu155His polymorphism, NR3C1 *BclI* polymorphism, and interaction between age and sex, Figs. 1, 2, 3) are significant even after straightforward logistic regression in the appropriate CART-defined subgroup, as described above; therefore, these effects relate to a true outcome difference in clinical practice for the pediatric population considered in this study. However, these

results are of interest also for adult patients, particularly in acute colitis where steroids may not work, and these patients may be considered for Infliximab as first line therapy or surgery without wasting time for steroids to exert an effect.

In conclusion, the most important finding of this study, conducted in young patients with IBD, is that *BclI* and NALP1 polymorphisms are significantly associated with the response to GCs; moreover, the use of a nonparametric statistic tree-based method allowed identification of significant interactions between response to GCs, genetic variants, and a panel of potentially important demographic factors. Compared with single factor analysis, this approach exhibited greater power to model the modest variations in probability of response to GCs conferred by genetic variants and demographics variables, reflecting the complexity of events potentially involved in GC response. Although these data are of interest, they should be interpreted with caution because of the limited number of subjects in the stratified analyses and in some CART terminal nodes. Future independent validations carried out in a larger study population may shed further light on the mechanisms underlying these complex high-order interactions and clarify which genomic variation affect the probability of a good response to GCs in an individual child with IBD.

## REFERENCES

1. Diefenbach KA, Breuer CK. Pediatric inflammatory bowel disease. *World J Gastroenterol*. 2006;12:3204–3212.
2. Castro M, Papadatou B, Baldassare M, et al. Inflammatory bowel disease in children and adolescents in Italy: data from the pediatric national IBD register (1996–2003). *Inflamm Bowel Dis*. 2008;14:1246–1252.
3. Friedman S. General principles of medical therapy of inflammatory bowel disease. *Gastroenterol Clin North Am*. 2004;33:191–208.
4. Markowitz J, Grancher K, Kohn N, et al. A multicenter trial of 6-mercaptopurine and prednisone in children with newly diagnosed Crohn's disease. *Gastroenterology*. 2000;119:895–902.
5. Hyams J, Markowitz J, Lerer T, et al. The natural history of corticosteroid therapy for ulcerative colitis in children. *Clin Gastroenterol Hepatol*. 2006;4:1118–1123.
6. Markowitz J, Hyams J, Mack D, et al. Corticosteroid therapy in the age of infliximab: acute and 1-year outcomes in newly diagnosed children with Crohn's disease. *Clin Gastroenterol Hepatol*. 2006;4:1124–1129.
7. Barnes PJ, Adcock IM. Glucocorticoid resistance in inflammatory diseases. *Lancet*. 2009;373:1905–1917.
8. Ashwell JD, Lu FW, Vacchio MS. Glucocorticoids in T cell development and function\*. *Annu Rev Immunol*. 2000;18:309–345.
9. De Bosscher K, Vanden Berghe W, Haegeman G. Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. *J Neuroimmunol*. 2000;109:16–22.
10. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev*. 2003;24:488–522.
11. Van Rossum EF, Lamberts SW. Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. *Recent Prog Horm Res*. 2004;59:333–357.
12. Van Rossum EF, Koper JW, Huizenga NA, et al. A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. *Diabetes*. 2002;51:3128–3134.
13. De Iudicibus S, Stocco G, Martelossi S, et al. Association of *BclI* polymorphism of the glucocorticoid receptor gene locus



**FIGURE 3.** Bar plot representing the significant interaction between age at onset of disease and sex in patients nonmutated (wild type + heterozygous) for the *BclI* and Leu155His polymorphisms. F indicates female; M, male.

- with response to glucocorticoids in inflammatory bowel disease. *Gut*. 2007;56:1319–1320.
14. Farrell RJ, Kelleher D. Glucocorticoid resistance in inflammatory bowel disease. *J Endocrinol*. 2003;178:339–346.
  15. Miller AH, Pariante CM, Pearce BD. Effects of cytokines on glucocorticoid receptor expression and function. Glucocorticoid resistance and relevance to depression. *Adv Exp Med Biol*. 1999;461:107–116.
  16. Buttgerit F, Saag KG, Cutolo M, et al. The molecular basis for the effectiveness, toxicity, and resistance to glucocorticoids: focus on the treatment of rheumatoid arthritis. *Scand J Rheumatol*. 2005;34:14–21.
  17. Raddatz D, Bockemuhl M, Ramadori G. Quantitative measurement of cytokine mRNA in inflammatory bowel disease: relation to clinical and endoscopic activity and outcome. *Eur J Gastroenterol Hepatol*. 2005;17:547–557.
  18. Sawa Y, Oshitani N, Adachi K, et al. Comprehensive analysis of intestinal cytokine messenger RNA profile by real-time quantitative polymerase chain reaction in patients with inflammatory bowel disease. *Int J Mol Med*. 2003;11:175–179.
  19. Pociot F, Molvig J, Wogensens L, et al. A TaqI polymorphism in the human interleukin-1 beta (IL-1 beta) gene correlates with IL-1 beta secretion in vitro. *Eur J Clin Invest*. 1992;22:396–402.
  20. Nemetz A, Nosti-Escanilla MP, Molnar T, et al. IL1B gene polymorphisms influence the course and severity of inflammatory bowel disease. *Immunogenetics*. 1999;49:527–531.
  21. Thornberry NA, Bull HG, Calaycay JR, et al. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature*. 1992;356:768–774.
  22. Jin Y, Birlea SA, Fain PR, et al. Genetic variations in NALP1 are associated with generalized vitiligo in a Romanian population. *J Invest Dermatol*. 2007;127:2558–2562.
  23. Jin Y, Mailloux CM, Gowan K, et al. NALP1 in vitiligo-associated multiple autoimmune disease. *N Engl J Med*. 2007;356:1216–1225.
  24. Binder V, Hendriksen C, Kreiner S. Prognosis in Crohn's disease—based on results from a regional patient group from the county of Copenhagen. *Gut*. 1985;26:146–150.
  25. Ardizzone S, Maconi G, Russo A, et al. Randomised controlled trial of azathioprine and 5-aminosalicylic acid for treatment of steroid dependent ulcerative colitis. *Gut*. 2006;55:47–53.
  26. Hyams JS, Ferry GD, Mandel FS, et al. Development and validation of a pediatric Crohn's disease activity index. *J Pediatr Gastroenterol Nutr*. 1991;12:439–447.
  27. Turner D, Hyams J, Markowitz J, et al. Appraisal of the pediatric ulcerative colitis activity index (PUCAI). *Inflamm Bowel Dis*. 2009;15:1218–1223.
  28. Bachmann AW, Sedgley TL, Jackson RV, et al. Glucocorticoid receptor polymorphisms and post-traumatic stress disorder. *Psychoneuroendocrinology*. 2005;30:297–306.
  29. Davies SM, Borowitz MJ, Rosner GL, et al. Pharmacogenetics of minimal residual disease response in children with B-precursor acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Blood*. 2008;111:2984–2990.
  30. Venables W, Ripley B. *Modern Applied Statistics With S*. New York: Springer; 2002:251–269.
  31. Kamdem LK, Hamilton L, Cheng C, et al. Genetic predictors of glucocorticoid-induced hypertension in children with acute lymphoblastic leukemia. *Pharmacogenet Genomics*. 2008;18:507–514.
  32. van Rossum EF, Koper JW, van den Beld AW, et al. Identification of the BclI polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index. *Clin Endocrinol*. 2003;59:585–592.
  33. Ukkola O, Rosmond R, Tremblay A, et al. Glucocorticoid receptor Bcl I variant is associated with an increased atherogenic profile in response to long-term overfeeding. *Atherosclerosis*. 2001;157:221–224.
  34. Buemann B, Vohl MC, Chagnon M, et al. Abdominal visceral fat is associated with a BclI restriction fragment length polymorphism at the glucocorticoid receptor gene locus. *Obes Res*. 1997;5:186–192.
  35. Di Blasio AM, van Rossum EF, Maestrini S, et al. The relation between two polymorphisms in the glucocorticoid receptor gene and body mass index, blood pressure and cholesterol in obese patients. *Clin Endocrinol*. 2003;59:68–74.
  36. Tremblay A, Bouchard L, Bouchard C, et al. Long-term adiposity changes are related to a glucocorticoid receptor polymorphism in young females. *J Clin Endocrinol Metab*. 2003;88:3141–3145.
  37. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell*. 2002;10:417–426.
  38. Martinon F, Hofmann K, Tschopp J. The pyrin domain: a possible member of the death domain-fold family implicated in apoptosis and inflammation. *Curr Biol*. 2001;11:R118–R120.
  39. Magitta NF, Boe Wolff AS, Johansson S, et al. A coding polymorphism in NALP1 confers risk for autoimmune Addison's disease and type 1 diabetes. *Genes Immun*. 2009;10:120–124.
  40. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 2001;411:599–603.
  41. Miceli-Richard C, Lesage S, Rybojad M, et al. CARD15 mutations in Blau syndrome. *Nat Genet*. 2001;29:19–20.
  42. Ting JP, Kastner DL, Hoffman HM. CATERPILLERS, pyrin and hereditary immunological disorders. *Nat Rev Immunol*. 2006;6:183–195.
  43. Schoultz I, Verma D, Halfvarsson J, et al. Combined polymorphisms in genes encoding the inflammasome components NALP3 and CARD8 confer susceptibility to Crohn's disease in Swedish men. *Am J Gastroenterol*. 2009;104:1180–1188.
  44. Kummer JA, Broekhuizen R, Everett H, et al. Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. *J Histochem Cytochem*. 2007;55:443–452.
  45. Raddatz D, Toth S, Schworer H, et al. Glucocorticoid receptor signaling in the intestinal epithelial cell lines IEC-6 and Caco-2: evidence of inhibition by interleukin-1beta. *Int J Colorectal Dis*. 2001;16:377–383.
  46. Kimura R, Nishioka T, Soemantri A, et al. Cis-acting effect of the IL1B C-31T polymorphism on IL-1 beta mRNA expression. *Genes Immun*. 2004;5:572–575.
  47. Wen AQ, Wang J, Feng K, et al. Effects of haplotypes in the interleukin 1beta promoter on lipopolysaccharide-induced interleukin 1beta expression. *Shock*. 2006;26:25–30.
  48. Hall SK, Perregaux DG, Gabel CA, et al. Correlation of polymorphic variation in the promoter region of the interleukin-1 beta gene with secretion of interleukin-1 beta protein. *Arthritis Rheum*. 2004;50:1976–1983.
  49. Romberg-Camps MJ, Dagnelie PC, Kester AD, et al. Influence of phenotype at diagnosis and of other potential prognostic factors on the course of inflammatory bowel disease. *Am J Gastroenterol*. 2009;104:371–383.
  50. Svec F, Yeakley J, Harrison RW III. Progesterone enhances glucocorticoid dissociation from the AtT-20 cell glucocorticoid receptor. *Endocrinology*. 1980;107:566–572.



SHORT REPORT

# Usefulness of the measurement of azathioprine metabolites in the assessment of non-adherence

Gabriele Stocco<sup>a</sup>, Margherita Londero<sup>b</sup>, Angelo Campanozzi<sup>c</sup>,  
Stefano Martellosi<sup>d</sup>, Sara Marino<sup>a,d</sup>, Noelia Malusa<sup>e</sup>, Fiora Bartoli<sup>b</sup>,  
Giuliana Decorti<sup>a,\*</sup>, Alessandro Ventura<sup>b</sup>

<sup>a</sup> Department of Life Sciences, University of Trieste, Italy

<sup>b</sup> Department of Reproductive and Developmental Sciences, University of Trieste, Italy

<sup>c</sup> Unit of Pediatrics, University of Foggia, Italy

<sup>d</sup> IRCCS Burlo Garofolo, Pediatric Clinic, Trieste, Italy

<sup>e</sup> Department of Prevention, Sanitary Services Agency Number 1, Trieste, Italy

Received 15 December 2009; received in revised form 9 April 2010; accepted 9 April 2010

## KEYWORDS

Azathioprine;  
Metabolites;  
Adherence;  
Inflammatory  
bowel disease;  
Autoimmune hepatitis

## Abstract

Azathioprine is a thiopurine immunosuppressive antimetabolite used to chronically treat inflammatory bowel disease and autoimmune hepatitis. Azathioprine treatment is a long-term therapy and therefore it is at risk for non-adherence, which is considered an important determinant of treatment inefficacy. Measurement of 6-thioguanine and 6-methylmercaptapurine nucleotides has been recently suggested as a screener for non-adherence detection.

We describe four young patients in which non-adherence to azathioprine therapy was detected only through the measurement of drug metabolite concentrations, and the criterion for non-adherence was undetectable metabolite levels. After the identification of non-adherence, patients and their families were approached and the importance of a correct drug administration was thoroughly enlightened and discussed; this allowed obtaining a full remission in all subjects.

Our observations support the use of undetectable metabolite levels as indicators of non-adherence to therapy in azathioprine treated patients. The additional level of medical supervision given by this assay allows getting a better adherence to medical treatment, which results in an improvement in the response to therapy; these benefits may justify the costs associated with the assay.

© 2010 European Crohn's and Colitis Organisation. Published by Elsevier B.V. All rights reserved.

## 1. Introduction

Azathioprine is a thiopurine immunosuppressive antimetabolite used to treat immune diseases, among which inflammatory bowel disease and autoimmune hepatitis.<sup>1,2</sup> The compound is a

\* Corresponding author. Department of Life Sciences, University of Trieste, Via L. Giorgieri 7, 9, 34127 Trieste, Italy. Tel.: +39 040 5587949; fax: +39 040 577435.

E-mail address: [decorti@units.it](mailto:decorti@units.it) (G. Decorti).

prodrug of 6-mercaptopurine that is activated after intracellular conversion to thioguanine nucleotides (6-TGN) and methylated metabolites (6-MMPN); these species are formed by a multienzymatic process that comprehends polymorphic thiopurine-S-methyltransferase (TPMT).<sup>3</sup> Like all chronic therapies, azathioprine treatment is at risk for non-adherence.<sup>4-8</sup>

Identification of non-adherence to therapy is often difficult, and subjective and objective measures have been suggested.<sup>7</sup> Bioassays such as 6-TGN and 6-MMPN levels are considered potentially useful.<sup>9,10</sup> In a recent study,<sup>7</sup> this assay has been suggested as a rapid screener for non-adherence in young patients with inflammatory bowel disease; the screening was shown to be reliable for those patients who have undetectable metabolite levels. However, given the small sample size, these findings have been considered quite preliminary.

In the present report, four cases are described in which young patients were not compliant to azathioprine therapy and this was detected only through the measurement of drug metabolite concentrations; these patients were part of a cohort included in a study on the optimization of azathioprine therapy through drug metabolite measurement,<sup>11,12</sup> performed by an HPLC assay,<sup>13</sup> and approval by the Ethic Committee has been obtained for this research. After identification of non-adherence, patients and families were approached about their azathioprine concentrations and the importance of a correct drug administration, and this resulted in better adherence to therapy (Table 1).

## 2. Case report

### 2.1. Case 1

A 4-year-old girl presented in October 2002 with pancolitis characterized by severe diarrhea with bloody stools (more than 6 times a day) and abdominal pain. A liver biopsy, performed because of an elevation of liver enzymes (5 times the normal values), revealed a sclerosing cholangitis that was

successfully treated with ursodeoxycholic acid (15 mg/kg/day).

The patient was treated with methylprednisolone 2 mg/kg/day for one month, then the steroid was tapered, but relapse occurred and azathioprine (3 mg/kg/day) was started together with oral mesalazine (40 mg/kg/day). Therapy was however unsuccessful, and diarrhea with bloody stools persisted, with absence of abdominal pain or other symptoms. A slight elevation of liver enzymes also occurred, and ursodeoxycholic acid was reintroduced at a lower dose (7 mg/kg/day), that the patient is still taking. Treatment with azathioprine and oral mesalazine was anyway continued for three years. However, in November 2005, monitoring of azathioprine metabolites revealed undetectable levels for both 6-TGN and 6-MMPN. Parents, that blamed each other for not giving the medication to the child, were approached and drug metabolite concentrations were discussed; the importance of a correct administration of the drug was again explained and stressed. Afterwards, the patient went into remission and metabolite levels raised for TGN to  $252 \text{ pmol}/8 \times 10^8 \text{ erythrocytes (U)}$  and for 6-MMPN to 4121 U. Liver enzymes also normalized, suggesting the presence of some immune mediated hepatic distress. Genotyping revealed wild-type thiopurine-S-methyltransferase (TPMT) status.

### 2.2. Case 2

The second patient was a young woman, diagnosed in 1999, at the age of 20, with Crohn's disease. She had a history of abdominal pain, diarrhea, bloody stools, weight loss and asthenia, and an esophageal gastroduodenoscopy revealed a gastroduodenal localization of the disease. Remission was obtained with steroid therapy (prednisone 1 mg/kg/day and slow tapering), however, during subsequent years, gastrointestinal symptoms frequently recurred and azathioprine (1.5 mg/kg/day) was finally started in February 2003, but discontinued after 2 months for pregnancy. During pregnancy, the patient was again treated with prednisone (0.8 mg/kg/day for 2 weeks and slow tapering) with persistence of mild symptoms. One month after delivery, in November 2003, the

**Table 1** Demographical and clinical characteristic of the patients.

	Patient #1	Patient #2	Patient #3	Patient #4
Age at diagnosis (years)	4	20	13	9
Gender	F	F	M	F
Disease	Pancolitis/sclerosing colangitis	Crohn's disease	Ulcerative colitis	Autoimmune hepatitis
Azathioprine dose	3 mg/kg/day	1.5 mg/kg/day	0.7 mg/kg/day	2 mg/kg/day
Other drugs	Methylprednisolone 2 mg/kg/day Mesalazine 40 mg/kg/day	Prednisone 0.8 mg/kg/day	Prednisone 1 mg/kg/day	Prednisone 2 mg/kg/day
Azathioprine metabolites (pmol/ $8 \times 10^8$ rbc) first evaluation	Undetectable	Undetectable	Undetectable	Undetectable
Azathioprine metabolites (pmol/ $8 \times 10^8$ rbc) Second evaluation	6–8×TGN: 252	6–8×TGN: 185	6–8×TGN: 94	6–8×TGN: 168
TPMT genotype	6–8×MMPN: 4121 Wild type	6–8×MMPN: 3693 Wild type	6–8×MMPN: 706 TPMT*3A heterozygous	6–8×MMPN: 701 Wild type

patient developed polyarticular arthritis with involvement of the temporomandibular joint. In December 2004, due to a flare of Crohn's disease with severe symptoms, namely, epigastric pain, marked diarrhea (10 stools daily), bloody stools and perianal abscess, prednisone (0.8 mg/kg/day) treatment was again started together with azathioprine (1.5 mg/kg/day). However, this time, therapy was unsuccessful, with persistence of epigastric pain, diarrhea with bloody stools and perianal abscess. Endoscopic and colonoscopic findings in July 2005 showed multiple duodenal erosions, and presence of a perianal abscess. Noncompliance to therapy was suspected, but initially denied by the patient, however, in June 2006, after confirmation of undetectable levels of azathioprine metabolites in her erythrocytes, the patient admitted not taking the immunosuppressant. The importance of being compliant to the administration schedule of the drug was discussed with the patient; periodic reinforcement of information about the importance of adherence was subsequently given at control visits. Therapy with azathioprine 2.3 mg/kg/day was resumed, and a marked improvement of symptoms was then evident, with decrease in defecation frequency, reduction of abdominal pain, and almost complete regression of colonic lesions, with persistence of duodenal signs. Metabolite measurement revealed that 6-TGN and 6-MMPN values had risen to 185 U and 3693 U respectively. TPMT analysis showed a wild-type genotype.

### 2.3. Case 3

A 13-year-old boy was diagnosed in October 2001 with extensive ulcerative colitis presenting with abdominal pain, diarrhea, bloody stools and tenesmus. He was treated unsuccessfully with oral mesalazine and enemas; prednisone therapy (1 mg/kg/day) was therefore started, but steroid dependence developed, and warranted the use of azathioprine (0.7 mg/kg/day) in November 2002. The treatment failed to induce any improvement, with persistence of abdominal pain, bloody stools (from traces of blood to evident hematochezia), and anemia; colonoscopic findings, in February 2004, showed signs of active colitis. In November 2004, laboratory monitoring of azathioprine metabolites revealed that the levels of 6-TGN and 6-MMPN were undetectable. The reason for therapy failure was hence evident, as the patient had not been taking azathioprine for months. Upon this disclosure, his mother, on her own initiative, started administering secretly the drug, by hiding it into the food, and a progressive improvement in clinical conditions was evident, even at relatively low dose of azathioprine (1.5 mg/kg/day); the defecation frequency decreased to normal, rectal blood loss almost completely disappeared, and, at the end of January 2005, colonoscopy evidenced mild signs of the disease only in distal segments. Monitoring of azathioprine metabolites revealed a raise in their concentration (94 U and 706 U for 6-TGN and 6-MMPN respectively), supporting increased adherence to therapy. Genotyping revealed a TPMT genotype heterozygous for the inactive variant allele TPMT\*3A, associated with a reduced inactivation of the drug and higher concentration of active 6-TGN metabolites at standard doses: this could explain the prompt response at the relatively low dose of azathioprine administered.<sup>11</sup>

### 2.4. Case 4

The patient was diagnosed with coeliac disease at the age of 3, with sideropenic anemia, diarrhea, anorexia and deflection from the individual growth curve; the girl scarcely adhered to gluten free diet. In 1999, at the age of 9, she developed autoimmune hepatitis with persistent elevation of serum transaminases, positive smooth muscle antibody test and histologic evidence on hepatic biopsy. After an initial, unsuccessful treatment with cyclosporine (5 mg/kg/day) for 2 months, prednisone (2 mg/kg/day), was started, together with azathioprine (2 mg/kg/day), with an initial very good response that lasted until April 2003. However, during the following years, the girl developed a severe depression, resulting in a suicide attempt in 2005.

A relapse of hepatitis occurred in February 2006 and the absence of detectable azathioprine metabolites, ascertained in March 2006, suggested non-compliance with therapy. The importance of correct drug assumption was clearly explained to the girl, and therapeutic drug monitoring of metabolite concentrations was discussed, and a more cooperative approach between the patient and the physician was established. Azathioprine was then resumed at the dosage previously employed, together with low dose prednisone (2.5 mg/day), and the patient responded well to therapy. After one month, the 6-TGN and 6-MMPN levels were 168 U and 701 U respectively. Repeated measurements during following months always revealed, for both metabolites, values in the therapeutic range. Genotyping demonstrated a wild-type TPMT genotype.

## 3. Discussion

Azathioprine is used to treat immune disorders like inflammatory bowel disease<sup>9</sup> and autoimmune hepatitis,<sup>10</sup> that are chronic relapsing conditions, whose intensity varies in an unpredictable fashion. Chronicity of the disease and irregularity of the symptoms are both characteristics that influence negatively treatment adherence, since patients may not perceive properly the risks related to interrupting the drug administration.<sup>5,6</sup> Moreover, for azathioprine, concern about adverse drug reactions and long-term safety may contribute to therapy non-adherence.<sup>6,14</sup> For the patients described in the present report, other factors may have impacted adherence, such as parent conflict in case 1, oppositional behaviour problems in case 3, and depression in case 4. In young patients, the prevalence of non-adherence to prescribed medications is about 50% in children, and even higher in adolescents<sup>15</sup> and detecting medication non-adherence is often difficult.<sup>7</sup> Measurement of azathioprine metabolites has been suggested as a useful technique to assess non-adherence.

For the patients described in this report, by measuring drug metabolites, whose levels were, in all cases, extremely low or non quantifiable, non-adherence to azathioprine treatment was revealed; after identification of reduced compliance, alerting patients and their families to the importance of therapy adherence, in order to benefit from it, allowed to obtain a full remission. Our study hence confirms the utility of using metabolite levels as complementary adherence assessment,<sup>7</sup> although we show that this method is accurate for patients who have undetectable metabolite levels. It should

however be noted that other factors can contribute to reduced metabolite levels, such as interindividual differences in drug metabolism.

Improving compliance in patients with a chronic disease, such as inflammatory bowel disease or autoimmune hepatitis, has been recognized as a major challenge for physicians<sup>5,6,16</sup>; indeed, patient non-adherence to medication adversely impacts on disease symptoms, and is a major determinant of disease relapse.<sup>6,17</sup> The additional level of medical supervision given by this clinical pharmacology assay can therefore be proposed as rapid and simple screener for verification of patients' compliance, in order to improve the success of therapy.

## Acknowledgments

Dr. Gabriele Stocco is recipient of a postdoctoral fellowship from the University of Trieste. Dr. Sara Marino is recipient of a research fellowship from IRCCS Burlo Garofolo, Trieste.

Statement of authorship: Stocco G, Londero M, Decorti G, Bartoli F, Ventura A designed research; Londero M, Campanozzi A, Martellosi S collected data and performed research; Stocco G, Marino S, Malusà N performed analytical laboratory tests; Stocco G analyzed data; Stocco G, Decorti G, Ventura A conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

## References

1. Barabino A, Torrente F, Ventura A, Cucchiara S, Castro M, Barbera C. Azathioprine in paediatric inflammatory bowel disease: an Italian multicentre survey. *Aliment Pharmacol Ther* 2002;16:1125–30.
2. Maggiore G. Chronic hepatitis in children. *Curr Opin Pediatr* 1995;7:539–46.
3. Elion GB. The purine path to chemotherapy. *Science* 1989;244:41–7.
4. Bokemeyer B, Teml A, Roggel C, Hartmann P, Fischer C, Schaeffeler E, et al. Adherence to thiopurine treatment in outpatients with Crohn's disease. *Aliment Pharmacol Ther* 2007;26:217–25.
5. Osterberg L, Blaschke T. Adherence to medication. *N Engl J Med* 2005;353:487–97.
6. Robinson A. Review article: improving adherence to medication in patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2008;27(Suppl 1):9–14.
7. Hommel KA, Davis CM, Baldassano RN. Objective versus subjective assessment of oral medication adherence in pediatric inflammatory bowel disease. *Inflamm Bowel Dis* 2009;15:589–93.
8. Hommel KA, Mackner LM, Denson LA, Crandall WV. Treatment regimen adherence in pediatric gastroenterology. *J Pediatr Gastroenterol Nutr* 2008;47:526–43.
9. Osterman MT, Kundu R, Lichtenstein GR, Lewis JD. Association of 6-thioguanine nucleotide levels and inflammatory bowel disease activity: a meta-analysis. *Gastroenterology* 2006;130:1047–53.
10. Heneghan MA, Allan ML, Bornstein JD, Muir AJ, Tendler DA. Utility of thiopurine methyltransferase genotyping and phenotyping, and measurement of azathioprine metabolites in the management of patients with autoimmune hepatitis. *J Hepatol* 2006;45:584–91.
11. Stocco G, Martellosi S, Decorti G, Bartoli F, Ventura A. Thiopurine-S-methyltransferase genotype and the response to azathioprine in inflammatory bowel disease. *Aliment Pharmacol Ther* 2007;26:1083–4 author reply 84–5.
12. Stocco G, Martellosi S, Malusa N, Marino S, Decorti G, Bartoli F, et al. Interruption of mesalamine and reduction of the blood concentration of the active metabolites of azathioprine: possible causes of ulcerative colitis relapse. *Dig Dis Sci* 2008;53:3246–9.
13. Dervieux T, Bouliou R. Simultaneous determination of 6-thioguanine and methyl 6-mercaptopurine nucleotides of azathioprine in red blood cells by HPLC. *Clin Chem* 1998;44:551–5.
14. Mantzaris GJ, Roussos A, Kalantzis C, Koilakou S, Raptis N, Kalantzis N. How adherent to treatment with azathioprine are patients with Crohn's disease in long-term remission? *Inflamm Bowel Dis* 2007;13:446–50.
15. Hommel KA, Davis CM, Baldassano RN. Medication adherence and quality of life in pediatric inflammatory bowel disease. *J Pediatr Psychol* 2008;33:867–74.
16. Ooi CY, Bohane TD, Lee D, Naidoo D, Day AS. Thiopurine metabolite monitoring in paediatric inflammatory bowel disease. *Aliment Pharmacol Ther* 2007;25:941–7.
17. Kane S, Huo D, Aikens J, Hanauer S. Medication nonadherence and the outcomes of patients with quiescent ulcerative colitis. *Am J Med* 2003;114:39–43.

# PAC<sub>SIN</sub>2 polymorphism influences TPMT activity and mercaptopurine-related gastrointestinal toxicity

Gabriele Stocco<sup>1,4</sup>, Wenjian Yang<sup>1</sup>, Kristine R. Crews<sup>1</sup>, William E. Thierfelder<sup>1</sup>, Giuliana Decorti<sup>4</sup>, Margherita Londero<sup>5</sup>, Raffaella Franca<sup>5</sup>, Marco Rabusin<sup>5</sup>, Maria Grazia Valsecchi<sup>6</sup>, Deqing Pei<sup>2</sup>, Cheng Cheng<sup>2</sup>, Steven W. Paugh<sup>1</sup>, Laura B. Ramsey<sup>1</sup>, Barthelemy Diouf<sup>1</sup>, Joseph Robert McCorkle<sup>1</sup>, Terreia S. Jones<sup>7</sup>, Ching-Hon Pui<sup>3</sup>, Mary V. Relling<sup>1,7</sup> and William E. Evans<sup>1,7,\*</sup>

<sup>1</sup>Department of Pharmaceutical Sciences, <sup>2</sup>Department of Biostatistics and <sup>3</sup>Department of Oncology, St Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA <sup>4</sup>Department of Life Sciences, University of Trieste, Trieste, Italy, <sup>5</sup>Institute for Maternal and Child Health—IRCCS 'Burlo Garofolo', Trieste, Italy, <sup>6</sup>University Milano Bicocca, Milan, Italy and <sup>7</sup>University of Tennessee Health Sciences Center, Memphis, TN, USA

Received March 26, 2012; Revised June 29, 2012; Accepted July 19, 2012

Treatment-related toxicity can be life-threatening and is the primary cause of interruption or discontinuation of chemotherapy for acute lymphoblastic leukemia (ALL), leading to an increased risk of relapse. Mercaptopurine is an essential component of continuation therapy in all ALL treatment protocols worldwide. Genetic polymorphisms in thiopurine S-methyltransferase (TPMT) are known to have a marked effect on mercaptopurine metabolism and toxicity; however, some patients with wild-type TPMT develop toxicity during mercaptopurine treatment for reasons that are not well understood. To identify additional genetic determinants of mercaptopurine toxicity, a genome-wide analysis was performed in a panel of human HapMap cell lines to identify trans-acting genes whose expression and/or single-nucleotide polymorphisms (SNPs) are related to TPMT activity, then validated in patients with ALL. The highest ranking gene with both mRNA expression and SNPs associated with TPMT activity in HapMap cell lines was *protein kinase C and casein kinase substrate in neurons 2 (PAC<sub>SIN</sub>2)*. The association of a PAC<sub>SIN</sub>2 SNP (rs2413739) with TPMT activity was confirmed in patients and knock-down of PAC<sub>SIN</sub>2 mRNA in human leukemia cells (NALM6) resulted in significantly lower TPMT activity. Moreover, this PAC<sub>SIN</sub>2 SNP was significantly associated with the incidence of severe gastrointestinal (GI) toxicity during consolidation therapy containing mercaptopurine, and remained significant in a multivariate analysis including TPMT and *SLCO1B1* as covariates, consistent with its influence on TPMT activity. The association with GI toxicity was also validated in a separate cohort of pediatric patients with ALL. These data indicate that polymorphism in PAC<sub>SIN</sub>2 significantly modulates TPMT activity and influences the risk of GI toxicity associated with mercaptopurine therapy.

## INTRODUCTION

Approximately 80% of children with acute lymphoblastic leukemia (ALL) can be cured with combination chemotherapy that almost always includes the purine anti-metabolite mercaptopurine (1,2). However, treatment-related toxicity with this medication can be life-threatening and is the primary cause of interruption or discontinuation of chemotherapy, which can increase the risk of disease recurrence. Germline polymorphisms in genes encoding drug-metabolizing enzymes can significantly

influence the efficacy and toxicity of antileukemic therapy (3). For mercaptopurine, methylation of the thiol moiety, which is catalyzed by the enzyme thiopurine S-methyltransferase (TPMT), yields inactive 6-methyl-mercaptopurine (4,5). Although genetic polymorphisms in the gene encoding human TPMT are known to markedly influence mercaptopurine metabolism and toxicity, there are patients with wild-type TPMT who also develop toxicity for reasons that are not fully understood (6,7).

Mercaptopurine is used together with high-dose methotrexate in post-remission consolidation therapy for pediatric

\*To whom correspondence should be addressed. Tel: +1 9015953301; Fax: +19015958600; Email: william.evans@stjude.org

ALL; this treatment regimen is associated with gastrointestinal (GI) toxicity that can be severe and constitutes an important adverse effect of ALL consolidation therapy (2).

In a previous study investigating the genetic basis of inter-individual differences in methotrexate pharmacokinetics in children with ALL, we showed that single-nucleotide polymorphisms (SNPs) in *solute carrier organic anion transporter family, member 1B1 (SLCO1B1)* are associated with methotrexate clearance and GI toxicity during consolidation therapy (8).

Hapmap cell lines (from Utah residents with ancestry from northern and western Europe; CEU) are a model system suitable for genome-wide studies of genetic polymorphisms influencing pharmacological phenotypes that can be measured in cell lines, despite limited statistical power due to the relatively small sample size (9,10). We previously used a genome-wide approach to rank 17 542 genes for their association with TPMT activity in HapMap cells: a TPMT haplotype significantly predicted TPMT phenotype; however, haplotypes of 96 genes ranked higher than TPMT, indicating the possibility that genetic polymorphism in other genes could influence TPMT activity (11). This prior genome-wide SNP analysis provided important insights into trans-acting genes influencing TPMT activity, but it is known that the incorporation of gene expression with genome-wide SNP analyses can reduce false positives and increase the robustness of discoveries (12), consistent with trait-associated SNPs being more likely to be expression quantitative trait loci (13).

Therefore, to identify additional genetic variability influencing TPMT activity, a genome-wide analysis was performed to identify trans-genes whose expression or SNPs are related to TPMT activity in a panel of human HapMap cell lines, with subsequent validation of their effects on TPMT activity and mercaptopurine toxicity in patients receiving mercaptopurine therapy.

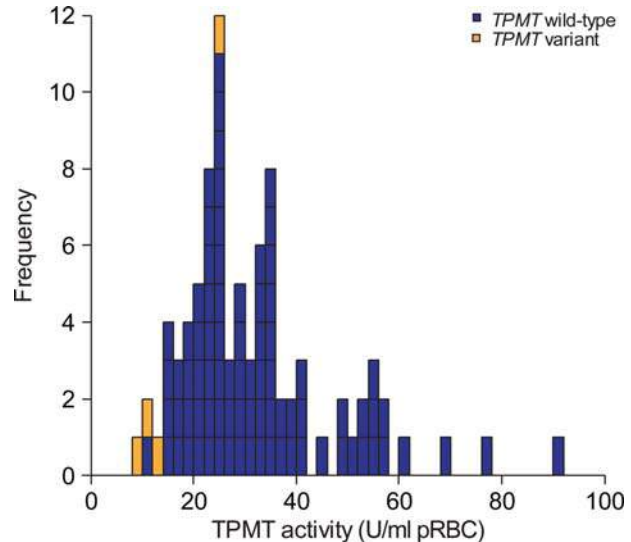
## RESULTS

### TPMT activity in HapMap (CEU) cells

TPMT activity was measured in the 30 HapMap CEU trios (Fig. 1). The median value was 28.5 U/10<sup>9</sup> cells, with a range of 9.1–90.4. Genotype for SNP rs1142345, the most common SNP defining a non-functional TPMT allele in humans (*TPMT*\*3A and *TPMT*\*3C both contain rs1142345), was assessed in all HapMap CEU cell lines considered. As depicted in Figure 1 and as we have previously reported (11), the four cell lines from subjects heterozygous for the rs1142345 SNP (i.e. AG genotype) had significantly lower TPMT activity than the 83 cell lines with the AA wild-type genotype for this SNP (*P*-value Welch test = 0.00026).

### Genes whose expression or SNPs were significantly associated with TPMT activity in HapMap cells

A summary of genes whose mRNA expression or SNPs were associated with TPMT activity in the HapMap cells is depicted in Figure 2. Association between gene expression and TPMT activity was analyzed for 15 661 genes: the expression of 38 genes was significantly correlated with TPMT activity with a

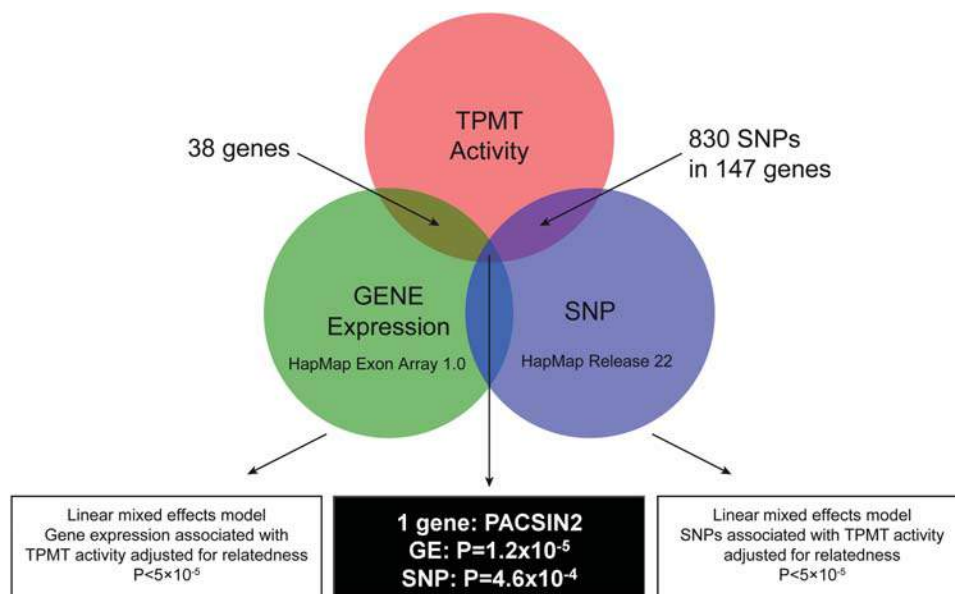


**Figure 1.** Distribution of TPMT activity measured in 87 HapMap CEU cell lines. Each rectangle represents the TPMT activity value measured in one cell line. Blue rectangles represent cell lines from individuals with wild-type *TPMT*, whereas orange rectangles represent cell lines from individuals with variant *TPMT* (rs1142342 AG genotype); the four cell lines from subjects heterozygous for the rs1142345 SNP (i.e. AG genotype) had significantly lower TPMT activity than the 83 cell lines with the AA wild-type genotype for this SNP (*P*-value Welch test = 0.00026) (11).

*P*-value  $< 5 \times 10^{-5}$  (Table 1). Among these 38 genes, only one gene also had a SNP annotated to its sequence that was significantly associated with TPMT activity at a *P*-value  $< 5 \times 10^{-4}$ : *protein kinase C and casein kinase substrate in neurons 2 (PACSN2)* mRNA expression (*P*-value linear model =  $1.20 \times 10^{-5}$ , correlation estimate = 0.36) and SNP rs2413739 (*P*-value linear model =  $4.6 \times 10^{-4}$ ) were both significantly associated with TPMT activity.

### Relation of PACSN2 SNP and TPMT activity in patients

The effect on erythrocyte TPMT activity of the *PACSN2* SNP identified in the HapMap population (rs2413739) was assessed in ALL patients who either had a wild-type *TPMT* genotype or were heterozygous for the most common variant *TPMT* alleles. TPMT activity and genotype was evaluated in 299 patients; the *PACSN2* genotype was successfully determined for 286 of the 299 patients for whom TPMT activity and the genotype were measured (for 13 patients, *PACSN2* genotyping could not be determined because of technical reasons). This analysis showed that both the *TPMT* and *PACSN2* variants were significantly associated with TPMT activity (Fig. 3). Indeed SNPs in both genes had a significant association with TPMT enzyme activity in a univariate analysis (*P*-values from linear model: *TPMT* =  $6.0 \times 10^{-16}$ ; *PACSN2* = 0.027). Moreover, these SNPs (*TPMT*, *PACSN2*) were also significantly related to TPMT activity in a multivariate analysis that combined the two SNPs in a multilocus genotype (*P*-values from linear model, *TPMT* =  $3.0 \times 10^{-16}$  and *PACSN2* = 0.011); this multivariate model explained 22.4% of variability in TPMT activity. Effects of *TPMT* and *PACSN2* genotypes were significant in patients



**Figure 2.** Diagram describing the analysis strategy to identify the most important trans-genes whose expression and SNPs are associated with TPMT activity in the HapMap CEU population. Among the genes whose expression is highly associated with TPMT activity (false discovery rate 1.6%), *PACSIN2* is the only one that has significant SNPs ( $P$ -value  $< 0.0005$ ) associated with TPMT activity.

after adjusting for ethnicity, evaluated as self-reported race, addressing the possibility of a potential confounding effect of ancestry ( $P$ -values from linear model adjusted for ethnicity,  $TPMT = 6.4 \times 10^{-16}$ ;  $PACSIN2 = 0.034$ ). The *PACSIN2* SNP was significantly related to TPMT activity both in patients with the wild-type *TPMT* genotype (explaining 1.8% of variability,  $P$ -value from linear model = 0.031) and in those with the variant *TPMT* genotype (explaining 30.8% of variability,  $P$ -value from linear model = 0.0091); the difference in the median TPMT activity in patients with wild-type versus variant *PACSIN2* genotypes (SNP rs2413739) was 1.2 U/ml packed erythrocytes for patients with wild-type *TPMT* and 2.6 U/ml packed erythrocytes for those with variant *TPMT* (Fig. 3).

#### ***PACSIN2* SNP had a significant association with GI toxicity in patients treated with mercaptopurine**

The entire cohort of Total 13B patients was considered for the genetic association discovery study for GI toxicity in patients: of 247 children with newly diagnosed ALL enrolled on the Total 13B study, 208 were evaluable herein. We excluded one patient who died shortly after diagnosis and did not have a remission DNA sample, five patients with Down syndrome, and one patient with cystic fibrosis because their underlying diseases can influence toxicity; we also excluded 32 patients who came off study for various reasons: induction failure ( $n = 1$ ), ALL relapse ( $n = 12$ ), second cancer ( $n = 3$ ), stem cell transplantation ( $n = 3$ ), non-compliance ( $n = 3$ ), non-GI toxicity ( $n = 3$ ), refused therapy ( $n = 2$ ), death unrelated to disease or therapy ( $n = 2$ ) or other reasons ( $n = 3$ ) (14). Among the 208 children with ALL receiving therapy according to St Jude Total 13B protocol and evaluable for the toxicity during consolidation therapy, 16 (7.7%) developed

severe (Grade 3–4) GI toxicity; the *TPMT* genotype was available for all 208 patients enrolled, whereas *PACSIN2* and *SLCO1B1* genotypes were determined in 189 (for 19 patients, *PACSIN2* and/or *SLCO1B1* could not be determined because of technical reasons). For these 189 children with complete genotyping data for *TPMT*, *SLCO1B1* and *PACSIN2*, the frequency of GI toxicity was 8.5%; among these patients, deficiency in TPMT activity predisposed to an increased incidence of severe GI toxicity during consolidation therapy which comprised methotrexate (2 g/m<sup>2</sup>/week) and mercaptopurine (75 mg/m<sup>2</sup>/day, in all patients during consolidation therapy, regardless of TPMT genotype). Indeed, among nine patients with a variant *TPMT* allele, the frequency of GI toxicity was 33%, compared with 7.2% in patients with wild-type *TPMT* ( $P$ -value from logistic regression model = 0.0058, Fig. 4). *PACSIN2* SNP rs2413739 also had a significant association with GI toxicity during consolidation therapy: the frequency of toxicity was 2.1, 9.1 and 13.2%, respectively, for the CC, CT and TT allele ( $P$ -value from logistic regression model = 0.046, Fig. 4). An *SLCO1B1* SNP (rs11045879) that we have previously reported to be associated with GI toxicity in ALL patients (8) was also associated with the incidence of GI toxicity: none of the patients with the *SLCO1B1* CC or CT genotype had GI toxicity, whereas 11.8% of the patients with the wild-type *SLCO1B1* TT genotype had GI toxicity ( $P$ -value from logistic regression model = 0.012, Fig. 4).

#### ***TPMT*, *SLCO1B1* and *PACSIN2* multi-locus genotype and GI toxicity**

Multivariate logistic regression models along with classification and regression trees analyses (CART) were used to assess the effects of *TPMT*, *SLCO1B1* and *PACSIN2* SNPs and their interaction in predisposing to GI toxicity. As

**Table 1.** Genes whose expression was statistically associated with TPMT activity ( $P$ -value lower than  $5 \times 10^{-5}$ ) in 87 Human HapMap CEU cell lines

Transcript ID	Chromosome	Gene ID	Gene symbol	Reference sequence	Correlation estimate	$P$ -value
3764471	chr17	9110	<i>MTMR4</i>	NM_004687	0.55	1.48E-07
3454006	chr12	91010	<i>FMNL3</i>	NM_198900, NM_175736	0.52	5.27E-07
3496409	chr13	2262	<i>GPC5</i>	NM_004466	0.48	6.91E-07
3290785	chr10	8030	<i>CCDC6</i>	NM_005436	0.49	1.07E-06
3456212	chr12	7786	<i>MAP3K12</i>	NM_006301	0.47	1.35E-06
3403595	chr12	50856	<i>CLEC4A</i>	NM_016184, NM_194450, NM_194447, NM_194448	0.39	1.85E-06
3275729	chr10	3559	<i>IL2RA</i>	NM_000417	0.44	2.46E-06
3662417	chr16	1071	<i>CETP</i>	NM_000078	0.39	2.91E-06
3832256	chr19	10653	<i>SPINT2</i>	NM_021102	0.44	3.20E-06
3063795	chr7	55262	<i>C7orf43</i>	NM_018275	0.46	5.96E-06
3156307	chr8	5747	<i>PTK2</i>	NM_005607, NM_153831	0.43	6.57E-06
3883819	chr20	22839	<i>DLGAP4</i>	NM_183006, NM_001042486, NM_014902	0.40	7.88E-06
3338968	chr11	55191	<i>NADSYN1</i>	NM_018161	0.46	1.08E-05
3426257	chr12	8835	<i>SOCS2</i>	NM_003877	0.44	1.11E-05
3962678	chr22	11252	<i>PACSN2</i>	NM_007229	0.38	1.20E-05
3965784	chr22	5600	<i>MAPK11</i>	NM_002751	0.37	1.42E-05
3621080	chr15	9333	<i>TGM5</i>	NM_201631, NM_004245	0.34	1.44E-05
3608638	chr15	9899	<i>SV2B</i>	NM_014848	0.39	1.47E-05
3802980	chr18	1824	<i>DSC2</i>	NM_004949, NM_024422	0.41	1.50E-05
3470193	chr12	1240	<i>CMKLR1</i>	NM_004072	0.40	1.53E-05
3479438	chr12	55743	<i>CHFR</i>	NM_018223	0.49	1.69E-05
2803329	chr5	10409	<i>BASP1</i>	NM_006317	0.47	1.78E-05
3362263	chr11	23258	<i>RAB6IP1</i>	NM_015213	0.45	2.27E-05
3830484	chr19	2867	<i>FFAR2</i>	NM_005306	0.35	2.32E-05
2766492	chr4	201895	<i>C4orf34</i>	NM_174921	0.39	2.72E-05
3476097	chr12	8099	<i>CDK2AP1</i>	NM_004642	0.40	2.85E-05
3833793	chr19	53916	<i>RAB4B</i>	NM_016154	0.45	3.14E-05
3405032	chr12	2120	<i>ETV6</i>	NM_001987	0.50	3.23E-05
3240987	chr10	1326	<i>MAP3K8</i>	NM_005204	0.48	3.25E-05
2412312	chr1	22996	<i>C1orf34</i>	NM_001080494	0.41	3.52E-05
3802924	chr18	1825	<i>DSC3</i>	NM_001941, NM_024423	0.38	3.65E-05
2485688	chr2	23177	<i>CEP68</i>	NM_015147	0.39	3.77E-05
3662612	chr16	89970	<i>RSPRY1</i>	NM_133368	0.48	3.82E-05
2556752	chr2	200734	<i>SPRED2</i>	NM_181784	0.47	3.84E-05
3753860	chr17	256957	<i>C17orf66</i>	NM_152781	0.31	4.31E-05
2500275	chr2	10018	<i>BCL2L11</i>	NM_138621, NM_006538	-0.40	4.47E-05
3470597	chr12	54434	<i>SSH1</i>	NM_018984	0.38	4.66E-05
3338192	chr11	595	<i>CCND1</i>	NM_053056	0.36	4.72E-05

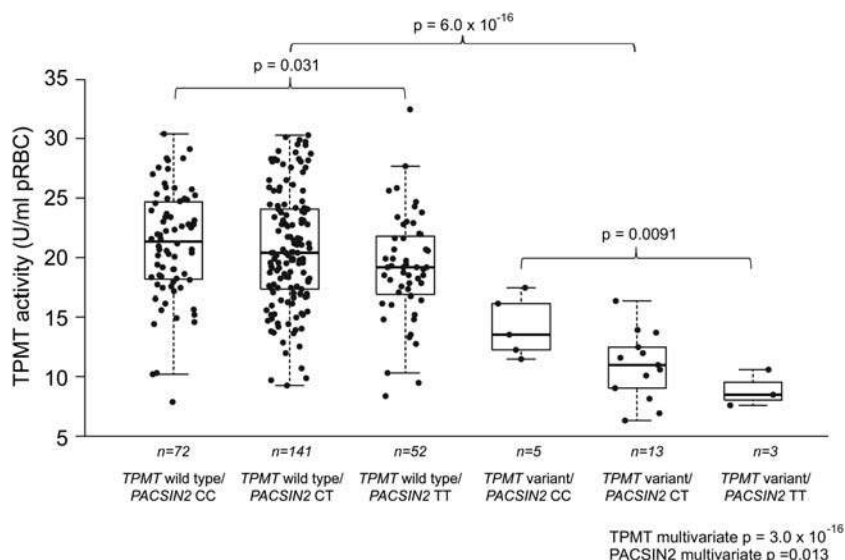
$P$ -values are from linear mixed effects model with formula:  $\log_2(\text{TPMT activity}) \sim \text{gene expression}$ , adjusted for relatedness.

summarized in Table 2, all three SNPs had a significant relation with the GI toxicity in the multivariate analysis; the effect of the considered SNPs on the incidence of GI toxicity was confirmed after adjusting for ethnicity as defined by self-reported race, addressing the possibility of a potential confounding effect determined by ancestry (Table 2). CART analysis to evaluate interactions between the genotypes in reference to their relation to the GI toxicity (Fig. 5) revealed that the *TPMT* genotype best distinguished GI toxicity probability (split 1 on CART, Fig. 5), with the presence of the variant *TPMT* allele conferring an increased probability of GI toxicity (33.3%) compared with patients with wild-type *TPMT* (7.2%,  $P$ -value from logistic regression model on subgroup = 0.0058). Among the nine patients with variant *TPMT*, no additional effect of *PACSN2* and *SLCO1B1* was evident, although the number of these patients was relatively low. Among the 180 patients with wild-type *TPMT*, the most important genotype to distinguish the probability of GI toxicity was the *SLCO1B1* SNP (split 2 on CART, Fig. 5): none of the 52 patients with a C (variant) allele for the *SLCO1B1* SNP developed toxicity, whereas the probability

among the 128 patients with the TT genotype was 10.2% ( $P$ -value from logistic regression model on subgroup = 0.017). Among the 128 patients with a wild-type allele for *TPMT* and TT *SLCO1B1* genotypes (split 3 on CART) the probability of GI toxicity in patients with the *PACSN2* TT allele was two times higher than in those with a C allele (15.8 versus 7.8%), albeit not statistically significant given the small number of patients ( $P$ -value from a logistic regression model on subgroup = 0.17).

#### Validation of *TPMT*, *SLCO1B1* and *PACSN2* SNPs on mercaptopurine-related GI toxicity in an independent cohort of patients

To validate the effect of *TPMT*, *SLCO1B1* and *PACSN2* SNPs on the incidence of severe GI toxicity during consolidation therapy of pediatric ALL, we assessed an independent set of patients who were treated on the Associazione Italiana di Ematologia ed Oncologia Pediatrica (Italy); Berlin-Frankfurt-Münster (Germany, Austria, Switzerland) ALL Study Group (AIEOP-BFM-ALL) 2000 protocol. *PACSN2*



**Figure 3.** The box plot shows TPMT activity in 286 patients with ALL as a function of the *TPMT* rs1142345 [A719G]/*PACSN2* rs2413739 multilocus genotype. There were no GG genotypes for *TPMT* rs1142345 and therefore all variant genotypes for *TPMT* are heterozygous. SNPs in both genes had a significant association with TPMT enzyme activity in a univariate analysis ( $P$ -values from linear model with formula TPMT activity–genotype, considering an additive effect for the genotype: *TPMT* =  $6.0 \times 10^{-16}$ ; *PACSN2*  $P = 0.027$ ). Moreover, these SNPs (*TPMT*, *PACSN2*) were also significantly related to TPMT activity in a multivariate analysis that combined the two SNPs in a multilocus genotype (*TPMT*  $P$ -value from linear model =  $3.0 \times 10^{-16}$  and *PACSN2*  $P$ -value from linear model = 0.011). The *PACSN2* genotype had a significant effect even in the subgroup with the wild-type *TPMT* genotype ( $P$ -value from linear model = 0.031) and in the subgroup with the variant *TPMT* genotype ( $P$ -value from linear model = 0.0091).

SNP rs2413739 also showed a correlation with the incidence of GI toxicity in this cohort; the frequency of cases with GI toxicity was 28, 32 and 51% in the CC, CT and TT genotypes, respectively ( $P$ -value from logistic regression model = 0.032, Fig. 6), as did SNP rs737782, in LD with rs2413739, with frequencies of GI toxicity of 26, 31 and 51% in the CC, CT and GG genotypes, respectively ( $P$ -value from a logistic regression model = 0.015, Fig. 6). However, for SNP rs5996259, not in LD with rs2413739, there was not an association with toxicity ( $P$ -value from a logistic regression model = 0.32). The *TPMT* and *SLCO1B1* SNPs did not show a significant association with the incidence of GI toxicity in the Italian cohort, perhaps due to the lower dosage of mercaptopurine used in this protocol (25 versus 75 mg/m<sup>2</sup>/day).

### Knock-down of *PACSN2* was associated with reduction in TPMT activity and mRNA in a human B-lineage leukemia cell line

Knock-down of *PACSN2* was performed in the NALM6 B-lineage ALL human cell line, heterologously expressing either *TPMT\*1* or *TPMT\*3A*. Through the heterologous expression of *TPMT\*1*, enzymatic activity increased approximately six times in comparison with TPMT activity in native NALM6 (mean activity 176.8 versus 28.9 U/10<sup>9</sup> cells,  $P$ -value Welch test =  $1.1 \times 10^{-6}$ ), whereas *TPMT\*3A* expression lead to only a modest (<2-fold) increase in TPMT activity (41.5 versus 28.9 U/10<sup>9</sup> cells,  $P$ -value Welch  $t$ -test = 0.028), due to the known rapid degradation of TPMT protein encoded by *TPMT\*3A* in comparison with *TPMT\*1* (15).

After knock-down of *PACSN2*, a significant (30%) decrease in TPMT activity was observed in cells heterologously

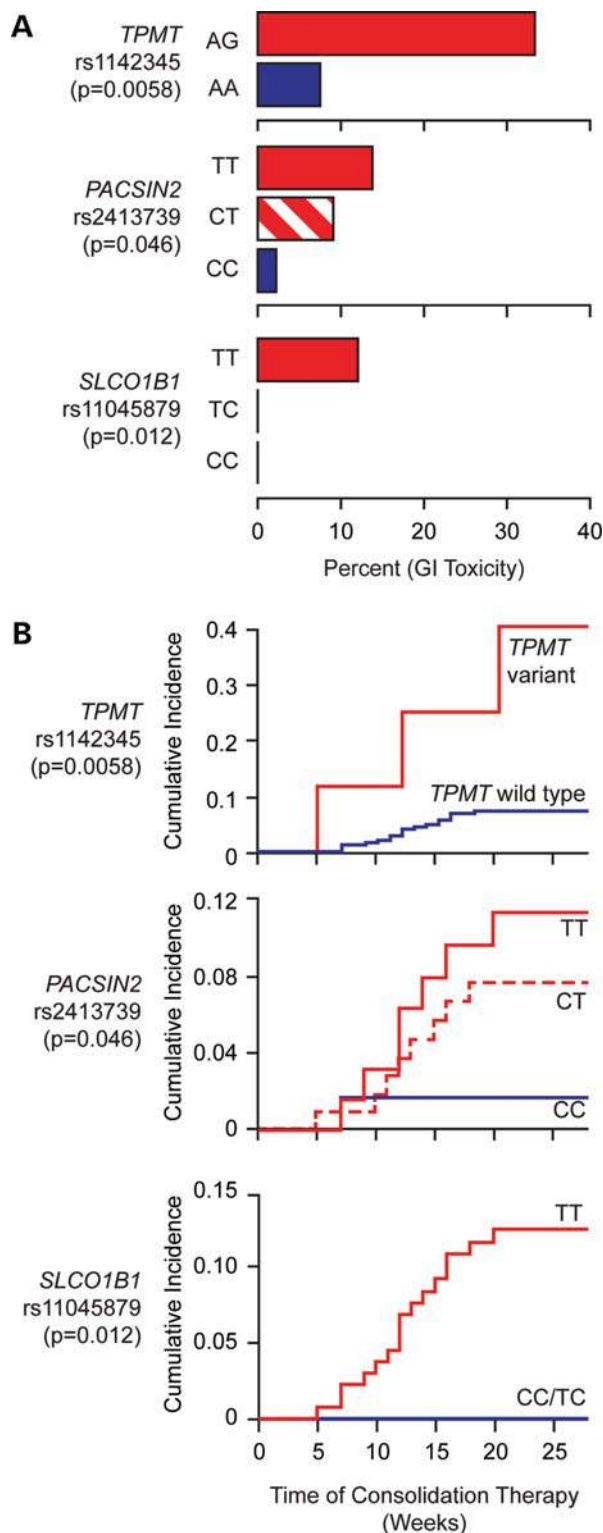
expressing *TPMT\*1* (123.8 U/10<sup>9</sup> cells in *PACSN2* knock-down versus 176.8 U/10<sup>9</sup> in a knock-down scrambled vector control,  $P$ -value Welch  $t$ -test = 0.0036), but less so in cells expressing *TPMT\*3A* (47.3 versus 41.5 U/10<sup>9</sup> cells,  $P$ -value Welch  $t$ -test = 0.090) (Fig. 7).

Measurement of TPMT mRNA in these cells confirmed the presence of the knock-down of *PACSN2* and showed an effect (reduction in 30%) on the expression of *TPMT* mRNA comparable with the effect on TPMT activity (Supplementary Material, Fig. S1).

### Pathway analysis of genes modified after *PACSN2* knock-down identifies autophagy as significant process

For global gene expressions in NALM6 with *PACSN2* knock-down in comparison with a scrambled vector control, 13 990 Affymetrix Human Genome U133 Plus 2.0 Array mRNA probe sets passed the filtering criteria based on signal intensity and probe sets present/absent call. Of these, 20 probe sets changed at least 50% [ $P$ -value LPE test  $< 1 \times 10^{-3}$ , false discovery rate (FDR) = ~65%] (Tables 3). To test the reliability of microarray data, we validated four genes [*PACSN2*, *CSMD1*, *KIAA0226L/protein phosphatase 1, regulatory (inhibitor) subunit 2 pseudogene 4 (PPP1R2P4)*, *SH3-domain GRB2-like endophilin B1 (SH3GLB1)*], by real-time PCR, confirming changes as assessed by microarray analysis (Supplementary Material, Fig. S2).

Enrichment analysis using MetaCore GeneGO revealed that genes within certain functional pathways were over-represented among those differentially expressed after knock-down of *PACSN2* (Table 4): the most highly ranked GeneGo pathways included vesicle formation (rank #1,  $P$ -value =



**Figure 4.** The frequency of severe (Grade 3–4) GI toxicity during consolidation therapy in patients with ALL treated according to the St Jude Total 13B protocol as a function of TPMT rs1142345, PACSIN2 rs2413739, SLCO1B1 rs11045879 genotypes. Panel A shows barplots reporting the percentage of patients developing GI toxicity and the corresponding genotypes. Panel B represents cumulative incidence plots of GI toxicity and the corresponding genotypes for the same patients. There were no GG genotypes for TPMT rs1142345 and therefore all variant genotypes for TPMT are heterozygous.

0.014), osteopontin and granzyme signaling (rank #2,  $P$ -value = 0.022) and autophagy (rank #3,  $P$ -value = 0.023). Highly ranked GeneOntology processes included cellular membrane organization (rank #1,  $P$ -value = 0.00010), membrane organization (rank #2,  $P$ -value = 0.00010) and cellular component organization (rank #3,  $P$ -value = 0.00016). GeneGo biological process networks included proteolysis in cell-cycle and apoptosis (rank #1,  $P$ -value = 0.010) and actin filaments in cytoskeleton (rank #2,  $P$ -value = 0.020); complete results for enrichment analysis are provided in Supplementary Material, Table S1a–c.

## DISCUSSION

This study has documented that *PACSIN2* modulates TPMT activity and that a common SNP in *PACSIN2* is significantly associated with the incidence of mercaptopurine-related GI toxicity in children with ALL. Although the importance of TPMT genetic polymorphisms in determining the mercaptopurine response and hematological toxicity in children with ALL is very well established (mechanistically and clinically) (6,16), this is the first *in vivo* evidence that genetic variation in a second gene (*PACSIN2*) also influences TPMT activity. *PACSIN2* was identified by genome-wide SNP and gene expression analyses of the human HapMap cells (9,10,12,13) for which TPMT activity was also determined (11), and the association was validated in patients. Indeed, the highest ranking gene for association with TPMT activity in the HapMap cell lines based on gene expression and SNPs (after adjusting for TPMT genotype) was *PACSIN2*, which carries a SNP (rs2413739) that is significantly associated with both TPMT activity and severe GI toxicity in children receiving mercaptopurine for the treatment of ALL. Moreover, knock-down of *PACSIN2* mRNA in human leukemia cells (NALM6) heterologously expressing *TPMT\*1* resulted in significantly lower TPMT activity and mRNA.

*PACSIN2*, also called syndapin II, is a member of the ‘protein kinase C and casein kinase substrate in neurons’ family of proteins; they are involved in linking the actin cytoskeleton with vesicle formation by regulating tubulin polymerization and exert their function mainly through protein–protein interactions with different substrates, such as dynamin or N-WASP (17). There are data indicating that *PACSIN2* has a role in various biological processes, including endocytosis (18), cell-cycle control (19) and autophagy (20).

To identify additional genes and biological processes that are potentially altered by *PACSIN2*, we identified genes

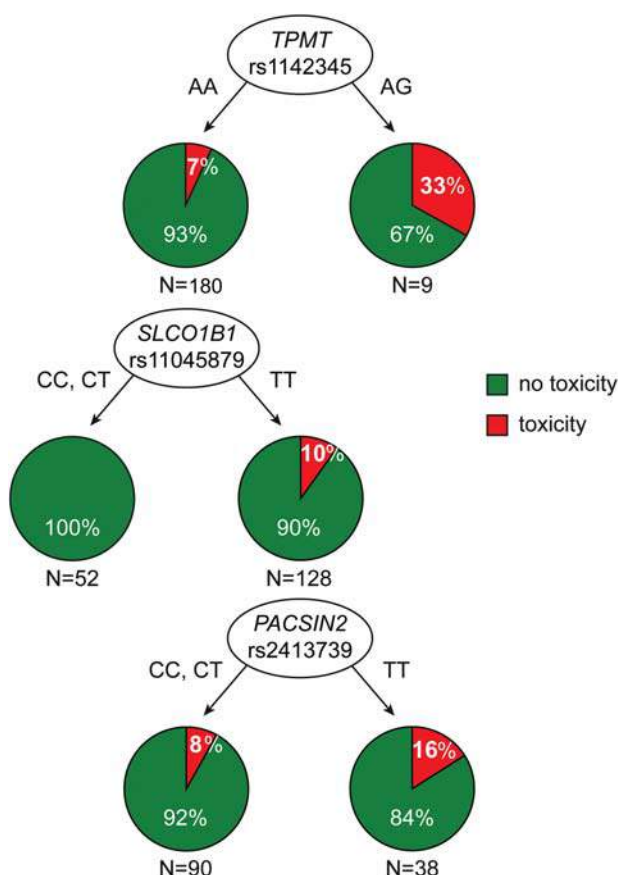
Among nine patients with a variant TPMT allele, the frequency of gastrointestinal toxicity was 33%, compared with 7.2% in patients with the wild-type TPMT ( $P$ -value from logistic regression model = 0.0058); PACSIN2 SNP rs2413739 also had a significant association with GI toxicity during consolidation therapy: the frequency of toxicity was 2.1, 9.1 and 13.2%, respectively, for the CC, CT and TT allele ( $P$ -value from logistic regression model = 0.046). An SLCO1B1 SNP (rs11045879) that we have previously reported to be associated with GI toxicity in ALL patients (9) was also associated with the incidence of GI toxicity: none of the patients with the SLCO1B1 CC or CT genotype had GI toxicity, whereas 11.8% of the patients with the wild-type SLCO1B1 TT genotype had GI toxicity ( $P$ -value from logistic regression model = 0.012).

**Table 2.** Multivariate logistic regression model for the effects of *PACSN2*, *SLCO1B1* and *TPMT* genotypes on the incidence of GI toxicity

	Multivariate logistic regression model for the incidence of GI toxicity			Multivariate logistic regression model for the incidence of GI toxicity, adjusted for ethnicity <sup>a</sup>		
	Effect <sup>b</sup>	95% Confidence interval	P-value	Effect <sup>b</sup>	95% Confidence interval	P-value
<i>PACSN2</i> rs2413739 genotype (T versus C)	2.06	1.01–4.56	0.040	2.09	1.03–4.58	0.037
<i>SLCO1B1</i> rs11045879 genotype (T versus C)	12.61	1.69–1613.81	0.017	11.49	1.56–1466.31	0.0016
<i>TPMT</i> rs1142345 genotype (A versus G)	6.03	1.23–27.26	0.0085	6.58	1.11–33.33	0.045

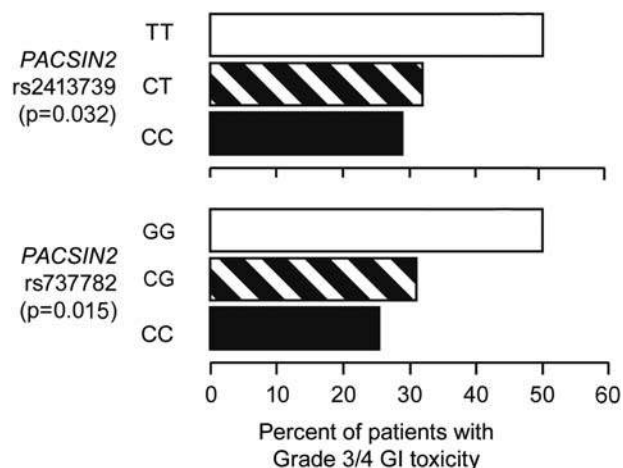
<sup>a</sup>Ethnicity was defined by self-reported race (white for 143 patients, black for 37 patients, Asian for 1 patient and other for 8 patients).

<sup>b</sup>The coefficient or effect size represents the odds ratio for toxicity associated with each variable listed. For example, a patient with a G allele for *TPMT* rs1142345 would have an odd ratio toxicity of 6.03 in comparison with a patient with the A allele.

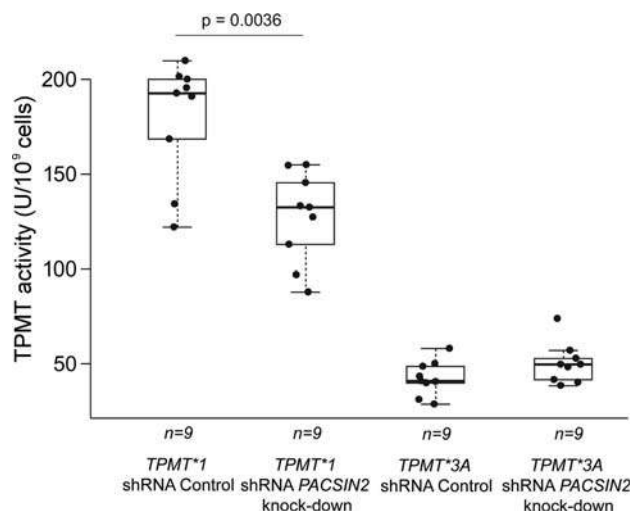


**Figure 5.** Classification and regression tree analysis for genetic predictors influencing those patients at an increased risk for GI toxicity. The three SNPs have independent effects, with *TPMT* being ranked before *SLCO1B1* and *PACSN2*.

whose expression changed significantly in human leukemia cells in which the *PACSN2* had been knocked-down, revealing actin and cytoskeleton (17,21), membrane organization (22) and autophagy (20) as processes/pathways significantly altered by knock-down of *PACSN2*. Identification of autophagy was driven largely by the reduction in expression of *SH3GLB1* by *PACSN2* knock-down. *SH3GLB1*, also known as endophilin B1/BIF-1, interacts with the UVRAG-Beclin 1, and contributes to the activation of autophagy (23); it is



**Figure 6.** The frequency of severe (Grade 3–4) GI toxicity during consolidation therapy in the validation cohort, the Italian AIEOP-BFM-ALL 2000 and genotypes in *PACSN2*; *PACSN2* SNP rs2413739 and rs737782 resulted associated with the incidence of GI toxicity in this cohort (P-values from logistic regression model, 0.032 and 0.015, respectively).



**Figure 7.** *TPMT* activity in NALM6 cells heterologously expressing *TPMT\*1* or *TPMT\*3A* and with knock-down of *PACSN2*. After knock-down of *PACSN2*, a significant (30%) decrease in *TPMT* activity was observed in cells heterologously expressing *TPMT\*1* (P-value Welch t-test = 0.0036).

**Table 3.** Genes whose mRNA was differentially expressed after knock-down of *PAC SIN2* in human NALM6 leukemia cells

Gene name	Gene symbol	Probe set ID	Ratio KD versus Ctrl	P-value	False discovery rate
CUB and Sushi multiple domains 1	<i>CSMD1</i>	231223_at	3.3	1.87E-10	0
Not annotated to a gene	NA	1562093_at	19.2	2.28E-10	0
Protein kinase C and casein kinase substrate in neurons 2	<i>PAC SIN2</i>	201651_s_at	0.4	1.54E-07	7.00E-04
Microtubule-associated protein 2	<i>MAP2</i>	225540_at	9.6	5.72E-07	0.002
Not annotated to a gene	NA	216532_x_at	2.9	1.05E-05	0.0293
Transcription elongation factor A (SII)-like 4	<i>TCEAL4</i>	202371_at	3.2	2.90E-05	0.0674
KIAA0226L/protein phosphatase 1, regulatory (inhibitor) subunit 2 pseudogene 4	<i>KIAA0266L</i> , <i>PPP1R2P4</i>	44790_s_at	0.5	4.13E-05	0.0822
Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	<i>ID2</i>	201565_s_at	2.6	7.61E-05	0.1327
Granzyme K (granzyme 3; tryptase II)	<i>GZMK</i>	206666_at	0.5	8.91E-05	0.1379
Not annotated to a gene	NA	241883_x_at	4.9	0.000117	0.1632
Secreted phosphoprotein 1	<i>SPP1</i>	209875_s_at	2.1	0.000166	0.2107
Coiled-coil domain containing 88A	<i>CCDC88A</i>	219387_at	2.0	0.000205	0.2233
Protein phosphatase 1, regulatory (inhibitor) subunit 2 pseudogene 4	<i>KIAA0266L</i> , <i>PPP1R2P4</i>	219471_at	0.5	0.000208	0.2233
Not annotated to a gene	NA	1560525_at	3.5	0.000331	0.3293
Intersectin 1 (SH3 domain protein)	<i>ITSN1</i>	209298_s_at	3.6	0.000588	0.4962
SH3-domain GRB2-like endophilin B1	<i>SH3GLB1</i>	210101_x_at	0.5	0.000596	0.4962
Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	<i>GZMA</i>	205488_at	0.4	0.00064	0.4962
Zinc finger protein 75a	<i>ZNF75A</i>	227670_at	3.8	0.000641	0.4962
tRNA methyltransferase 11 homolog (S. cerevisiae)	<i>TRMT11</i>	218877_s_at	2.4	0.00074	0.5426
odz, odd Oz/ten-m homolog 2 (Drosophila)	<i>ODZ2</i>	231867_at	2.8	0.000927	0.646

plausible that SH3GLB1 has a role in the fission of membranes from the Golgi during autophagosome formation (24). The effects of *PAC SIN2* on autophagy is further supported by significant changes we observed in a second gene *C13orf18* (also known as *KIAA0226-like/PPP1R2P4*), which has recently been shown to be involved in autophagy (25). The *PAC SIN2* role in autophagy may also be involved in the mechanism of its effects on TPMT activity, as autophagy has been linked to degradation of the TPMT protein codified by variant *TPMT\*3A* and to a lesser extent wild-type *TPMT\*1* (26). Taken together, our results indicate that *PAC SIN2* may influence TPMT activity through an effect on TPMT mRNA levels and/or TPMT protein degradation. Future studies are warranted to fully elucidate the molecular mechanisms by which *PAC SIN2* alters TPMT activity.

The *PAC SIN2* SNP rs2413739 is located in an intronic region of the gene and, therefore, it is not clear how this SNP influences *PAC SIN2* mRNA and function. Interestingly, the UCSC Genome Browser (27) indicates that this SNP is located in an area enriched for a marker of acetylation of lysine 27 of the histone 3 protein (H3K27Ac histone mark) as determined by ChIP-Seq analyses (27,28) (Supplementary Material, Fig. S3). Therefore, the SNP rs2413739 may modulate transcription of the *PAC SIN2* gene by influencing the level of acetylation or the effects of the acetylation of this marker, that is thought to enhance transcription possibly by blocking the spread of the repressive histone mark H3K27Me3 (29).

In addition to showing a significant association of this *PAC SIN2* SNP on TPMT activity in cell lines and patients, we also show that patients with the *PAC SIN2* rs2413739 C genotype had higher TPMT activity and lower GI toxicity than patients with the T genotype. Furthermore, the

association of this *PAC SIN2* SNP with GI toxicity during consolidation therapy of ALL with mercaptopurine was identified in patients treated according to the St Jude Total 13B protocol and validated in an independent cohort of Italian patients. In the St Jude Total 13B protocol, patients were treated for a short period of time (2 weeks) with relatively high doses of mercaptopurine (75 mg/m<sup>2</sup>/day, regardless of TPMT genotype) and methotrexate (2 g/m<sup>2</sup> with folic acid rescue), which resulted in ~8% of patients exhibiting Grade 3–4 GI toxicity; in this context, *PAC SIN2* effects on GI toxicity were significant and independent from those of *TPMT* and *SLCO1B1*. In the validation cohort, the post-remission consolidation phase (protocol M) was quite different and involved lower mercaptopurine doses (25 mg/m<sup>2</sup>/day) and a longer treatment time (8 versus 2 weeks) and the overall incidence of severe GI toxicity was lower (~4% of patients versus ~8%); in this context the *PAC SIN2* SNP was significantly related to the incidence of GI toxicity, whereas no effects of *TPMT* and *SLCO1B1* could be detected. Patients with high TPMT activity may be protected from mercaptopurine toxicity (7,30) and in this study, the observation that *PAC SIN2* was associated with GI toxicity in both cohorts may indicate that its effects on patients with high TPMT activity are clinically relevant and evident across different protocols of consolidation therapy for pediatric ALL: indeed the most common TPMT coding SNPs do not identify patients with high TPMT activity (only low TPMT activity), whereas the *PAC SIN2* rs2413739 CC genotype does. Polymorphisms in the promoter region of TPMT have been previously associated with increased TPMT activity, although the clinical relevance of these observations is not clear (31). It is also plausible that *PAC SIN2* may influence mercaptopurine sensitivity independent of its effects on TPMT; indeed it has been shown recently

**Table 4.** Enrichment analysis of mRNA differentially expressed in human NALM6 leukemia cells, between PACSIN2 controls and knock-down

MetaCore GeneGo	Ranking	Pathway name	P-value	Ratio probe sets significant/in pathway	Significant mRNAs in pathway
GeneGo pathway maps	#1	wtCFTR and delta508 traffic/clathrin-coated vesicles formation (norm and CF)	1.36E-02	1/19	<i>SH3GLB1</i>
	#2	Development_Osteopontin signaling in osteoclasts and	2.15E-02	1/30	<i>SPP1</i>
	#2	Apoptosis and survival_Granzyme A signaling	2.15E-02	1/30	<i>GZMA</i>
GeneGoProcess networks	#3	Autophagy	2.29E-02	1/32	<i>SH3GLB1</i>
	#1	Proteolysis: proteolysis in cell cycle and apoptosis	1.01E-02	2/125	<i>GZMA, GZMK</i>
	#2	Cytoskeleton: actin filaments	1.95E-02	2/176	<i>PACSIN, PACSIN2</i>
GeneOntology Processes	#1	Cellular membrane organization	1.01E-04	5/653	<i>SH3GLB1, CCDC88A, ITSN1, PACSIN, PACSIN2</i>
	#2	Membrane organization	1.03E-04	5/656	<i>SH3GLB1, CCDC88A, ITSN1, PACSIN, PACSIN2</i>
	#3	Negative regulation of cellular component organization	1.61E-04	4/375	<i>MAP2, SPP1, PACSIN, PACSIN2</i>

to interact with Rac1 (32), which is implicated in the pharmacodynamics of thiopurines (33).

We have shown previously that during maintenance therapy of pediatric ALL according to Total 13B protocol, adjustment of mercaptopurine dose on the basis of TPMT phenotype or genotype mitigates the increased incidence of Grade 3–4 febrile neutropenia in patients with variant *TPMT* genotypes, allowing the effect of other genetic polymorphisms, such as those in inosine-triphosphate-pyrophosphatase to emerge (34). To explore whether the association of *PACSIN2* SNP with dose-dependent mercaptopurine toxicity is via its effects on TPMT activity, we evaluated whether the *PACSIN2* genotype was associated with the incidence of severe febrile neutropenia during Total 13B maintenance therapy and we could not find a significant association ( $P$ -value of a weighted logistic regression model considering time at risk = 0.41, Supplementary Material, Fig. S4). This suggests that the association of the *PACSIN2* genotype with mercaptopurine-related dose-dependent toxicity occurs through modulation of TPMT activity, and not through a direct effect of *PACSIN2* on cell sensitivity to mercaptopurine. However, this does not rule out tissue-specific effects of *PACSIN2* on mercaptopurine sensitivity occurring in GI tissue and not in white blood cells.

In summary, the current study has identified a new genetic polymorphism influencing TPMT activity and mercaptopurine toxicity in children being treated for ALL, providing new insights into the pharmacogenomics of ALL chemotherapy.

## MATERIALS AND METHODS

### Cell culture

Eighty-nine Epstein–Barr virus-transformed lymphoblastoid cell lines, forming the 30 CEPH family trios genotyped in the International HapMap project, were obtained from the Coriell Institute of Medicine (<http://www.ccr.coriell.org>). Cell lines were cultured in RPMI-1640 media with 15% fetal bovine serum. Two cell lines with poor growth were excluded from the study.

### TPMT activity in cell lines

Aliquots were obtained for each cell line while in the log phase and cytosolic cell lysates were prepared, as previously reported in detail (11). TPMT activity was measured adapting a previously described radiochemical assay (35). Briefly, the level of TPMT activity for each cell line was determined by measuring the incorporation of radioactively labeled *S*-adenosyl-L-methionine into mercaptopurine's inactive metabolite, methylmercaptopurine (11,35). One unit of enzyme activity represents the formation of 1 nmol of 6-methylmercaptopurine per hour of incubation. TPMT activity in cell lines was normalized per billion of cells evaluated.

### Genotypes and gene expression in cell lines

SNP genotypes for CEU cell lines were downloaded from release 22 on the International HapMap project Website (<http://www.HapMap.org>). Gene expression measured by the Affymetrix GeneChip Human Exon 1.0 ST Array was downloaded from the website:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7761>.

### Ethical

We studied children enrolled as patients in two single-institution (St Jude Total 13B and Total 15) and one multi-institution (AIEOP-BFM-ALL 2000) clinical protocol for the treatment of newly diagnosed ALL. All patients and/or their parents provided informed consent for the institutional review board-approved protocols.

### TPMT activity in patients

TPMT activity was measured in erythrocytes from pediatric patients with ALL enrolled at St Jude Children's Research Hospital in Total 13B or Total 15 protocols (36,37) using a previously described method (35); one unit of enzyme activity represents the formation of 1 nmol of 6-methylmercaptopurine per hour of incubation. TPMT activity in patients was normalized per milliliter of packed erythrocytes. TPMT activity was measured after patients were in complete remission from ALL

(i.e. at least 60 days from diagnosis) and at least 90 days from a red blood cell transfusion. There were a total of 323 measurements for 299 patients; for patients with multiple measurements, the median value was used.

### Toxicity in patients

Toxicity was evaluated during consolidation therapy for ALL according to St Jude Total 13B protocol and classified according to NCI criteria (8,36): patients developing relevant GI toxicity during consolidation therapy (Grade 3–4 mucositis and diarrhea) were defined as cases and they were compared with all other patients in the cohort who did not develop mucositis and or diarrhea and served as controls in the genetic analyses; other GI toxicity (hepatotoxicity, vomiting and pancreatic toxicity) were uncommon (1 Grade 3 hepatotoxicity, 1 Grade 3 vomiting and no pancreatic toxicity) and therefore were not considered in the present analysis. Consolidation therapy for Total 13B consisted of 2 weekly doses of high-dose methotrexate (2 g/m<sup>2</sup> over 2 h, followed by leucovorin rescue) and daily mercaptopurine (75 mg/m<sup>2</sup>/day for 2 weeks) (38). During consolidation therapy, the dosage of mercaptopurine therapy was the same for all patients, regardless of the TPMT genotype; mercaptopurine dosages were subsequently adjusted during continuation therapy based on the TPMT genotype (6,34). The entire cohort of Total 13B patients was considered for the genetic association discovery study: of 247 children with newly diagnosed ALL enrolled on the Total 13B study, 208 were evaluable herein. We excluded one patient who died shortly after diagnosis and did not have a remission DNA sample, five patients with Down syndrome, and one patient with cystic fibrosis because their underlying diseases can influence toxicity; we also excluded 32 patients who came off study for various reasons: induction failure ( $n = 1$ ), ALL relapse ( $n = 12$ ), second cancer ( $n = 3$ ), stem cell transplantation ( $n = 3$ ), non-compliance ( $n = 3$ ), non-GI toxicity ( $n = 3$ ), refused therapy ( $n = 2$ ), death unrelated to disease or therapy ( $n = 2$ ) or other reasons ( $n = 3$ ) (14). Validation cohort consisted of patients enrolled on the multi-institution AIEOP-BFM-ALL 2000 protocol. Post-remission consolidation therapy (protocol M) for the AIEOP-BFM-ALL 2000 protocol consisted of 8 weeks of treatment with four administrations of high-dose methotrexate (2 g/m<sup>2</sup> over 24 h followed by leucovorin rescue) and daily mercaptopurine (25 mg/m<sup>2</sup>/day for 8 weeks) (39). Sixty-seven patients without Down syndrome who developed Grade 3–4 mucositis and/or diarrhea during consolidation therapy served as cases and each of them was matched with two controls from the same protocol based on sex, age, ALL lineage and ALL risk classification. The toxicity was classified by the NCI criteria (39).

### Genotypes in patients

Genotyping of St Jude patients was performed for the major non-functional variant alleles of TPMT (*TPMT*\*2, *TPMT*\*3A and *TPMT*\*3C, defined by SNPs rs1142345, rs1800460 and rs1800462) using methods that we have previously described in detail (6). Genotyping of SNP rs11045879 in *SLCO1B1* in St Jude patients was obtained from Affymetrix 500K Array

Set and Affymetrix Genome-Wide Human SNP Array 6.0. SNPs rs2413739 of *PACSIN2* for St Jude patients was genotyped independently with two different methods, by DNAPrint Genomics (Sarasota, FL) and by iPLEX Sequenom assay done at the University of Chicago. Genotyping of the Italian validation cohort was performed at the University of Washington in St Louis, by Sequenom technology. Quality control of genotyping was verified by repeated analysis of the same samples, using different genotyping approaches; moreover, in the validation cohort, in addition to the SNPs in *TPMT*, *SLCO1B1* and *PACSIN2* genotyped in the St Jude cohort, two additional *PACSIN2* SNPs were genotyped: rs737782 and rs5996520, which were, respectively, in LD or not in LD with *PACSIN2* rs2413739.

### Knock-down of PACSIN2 in NALM6 heterologously expressing TPMT

The B lineage human leukemia cell line NALM6 was used to study effects of modulation of *PACSIN2* expression on TPMT activity.

The heterologous expression of *TPMT*\*1 and *TPMT*\*3A proteins was obtained by transfection using Nucleofector (Lonza) of native NALM6 with the appropriate plasmid (Origene), according to the manufacturer instructions. For selection of stably transfected cells, after transfection, culture medium supplemented with G418 was used.

*PACSIN2* knockdown was achieved in NALM6 heterologously expressing TPMT by RNA interference using a lentiviral vector-based shRNA approach from the MISSION<sup>TM</sup> TRC-Hs library (Sigma). Lentiviral particles corresponding to the MISSION shRNA SHVRS-NM\_007229 target set were used as well as the MISSION Non-Target shRNA control. The specificity and efficacy of the shRNA *PACSIN2* procedure were controlled by western blotting and real-time PCR, after transduction and puromycin selection.

TPMT activity was measured in these cell lines using the previously described radiochemical assay (11,35). *PACSIN2* and *TPMT* mRNA were quantified by real-time PCR using the commercial TaqMan Gene Expression Assay (Hs00200589\_m1 and Hs00909010\_g1, respectively) from Applied Biosystems.

### Microarray analysis

Global gene expression in NALM6 stably transfected with shRNA sequences for the knock-down of *PACSIN2* or scrambled sequences (control) were evaluated using microarray analysis. Total RNA of cells harvested during logarithmic growth was isolated using Trizol reagent. For quality control, RNA purity and integrity were evaluated by measuring the OD 260/280 ratio and analyzed on Agilent Bioanalyzer. Gene expression data was measured for 30 708 transcripts by the use of the Affymetrix Human Genome U133 Plus 2.0 Array, according to the manufacturer's instructions. Validation of a panel of differentially expressed genes was performed using gene expression real-time PCR assays from Applied Biosystem (Hs00899130\_m1 for *CSMD1*, Hs00228336\_m1 for *C13orf18/KIAA0266L/PPP1R2P4*, Hs00211220\_m1 for *SH3GLB1*).

## Statistical analysis

Analysis was done using the software R. To identify trans-genetic factors associated with TPMT activity in the HapMap cell lines, a linear mixed effects model adjusted for relatedness was used, with log<sub>10</sub> of TPMT activity as the dependent variable and gene expression and SNPs as the independent variables. Trans-genes were selected if they had SNPs associated with TPMT activity, using a cut-off of  $5 \times 10^{-4}$  for the *P*-value and then ranked according to the *P*-value for the association of gene expression with TPMT activity.

Effects of candidate SNPs on TPMT activity and GI toxicity in St Jude patients were evaluated by linear (for TPMT activity) or logistic regression (for toxicity) models, with formula phenotype–genotype; *P*-values are from the ANOVA test applied to these models, evaluating an additive effect of the genotype on the phenotype of interest. To check for the potential confounding effects of ancestry, analysis was performed even with models adjusted for ethnicity, determined as self-reported race [we have previously shown that in this population of patients, self-reported race was highly concordant with ethnicity determined using ancestry informative markers (14)]. CART analysis was used to evaluate interaction between genetic factors associated with toxicity in St Jude patients.

Validation of the effect of genotypes on GI toxicity in the Italian cohort was done using logistic regression models, with formula phenotype–genotype and *P*-values are from the ANOVA test applied to these models.

Comparison of measurements (e.g. TPMT activity, gene mRNA expression) in different genetically modified NALM6 cell lines was performed by Welch's *t*-test and *P*-values were adjusted for multiple testing by Holm's method (40).

Gene expression data from the Affymetrix chip was filtered by setting detection >500 and considering only probe sets called present by the MAS5 algorithm in at least 50% of samples. Selected gene signal values were log<sub>2</sub> transformed and normalized by the IQR method (so that inter-quartile ranges on all chips are set to their widest range), as implemented in the pre-process function of the LPE R package. The significance of the expression data was determined using the LPE test from the same R package and fold change, in which the null hypothesis was that no difference exists among the two groups (41). The FDR was controlled by adjusting *P*-values using the Benjamini–Hochberg algorithm (42). Pathway analysis of the genes differentially expressed was performed using MetaCore/GeneGo; enrichment analysis was performed evaluating GeneGo pathways, GeneOntology processes and GeneGo process networks.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

## ACKNOWLEDGEMENTS

We thank the patients and their parents for their participation in this study and our clinical staff for providing outstanding protocol-based patient care. We thank Natalya Lenchik, Yan

Wang, Siamac Salehy, Margaret Needham, May Chung and Yaqin Chu for their outstanding technical assistance. We thank Nancy Kornegay and Mark Wilkinson for their computer and database expertise, the staff from the St Jude Hartwell Center for Bioinformatics and Biotechnology and in particular Jay Morris, Geoff Neale and Jennifer Peters for assistance with microarray and *in vitro* experiments, and the staff of St Jude Biomedical Communications and in particular Julie Groff for the preparation of the figures.

**Conflict of Interest statement.** W.E.E. is an inventor on a patent awarded to SJCRH for the molecular diagnosis of TPMT deficiency based on the TPMT genotype and has received patent royalties. M.V.R. has also received such royalties. The other authors declared no conflict of interest.

## FUNDING

Funding sources include grants from the National Institutes of Health (R37 CA36401 to W.E.E., M.V.R., C.-H.P.; R01 CA78224 to W.E.E., M.V.R., C.-H.P.); NIH Pharmacogenomics Research Grant (U01 GM 92666 to M.V.R., W.E.E., C.C., W.Y., C.-H.P.), NCI Cancer Center Support Grant to St Jude (CA21765), F32 CA141752 to S.W.P., the American Lebanese Syrian Associated Charities (ALSAC) and the Associazione Genitori Malati Emopatici Neoplastici Friuli Venezia Giulia (AGMEN).

## REFERENCES

- Pui, C.H., Carroll, W.L., Meshinchi, S. and Arceci, R.J. (2012) Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *J. Clin. Oncol.*, **29**, 551–565.
- Pui, C.H. and Evans, W.E. (2006) Treatment of acute lymphoblastic leukemia. *N. Engl. J. Med.*, **354**, 166–178.
- Paugh, S.W., Stocco, G., McCorkle, J.R., Diouf, B., Crews, K.R. and Evans, W.E. (2011) Cancer pharmacogenomics. *Clin. Pharmacol. Ther.*, **90**, 461–466.
- Wang, L. and Weinshilboum, R. (2006) Thiopurine S-methyltransferase pharmacogenetics: insights, challenges and future directions. *Oncogene*, **25**, 1629–1638.
- Krynetski, E. and Evans, W.E. (2003) Drug methylation in cancer therapy: lessons from the TPMT polymorphism. *Oncogene*, **22**, 7403–7413.
- Relling, M.V., Gardner, E.E., Sandborn, W.J., Schmiegelow, K., Pui, C.H., Yee, S.W., Stein, C.M., Carrillo, M., Evans, W.E. and Klein, T.E. (2011) Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clin. Pharmacol. Ther.*, **89**, 387–391.
- Relling, M.V., Hancock, M.L., Rivera, G.K., Sandlund, J.T., Ribeiro, R.C., Krynetski, E.Y., Pui, C.H. and Evans, W.E. (1999) Mercaptopurine therapy intolerance and heterozygosity at the thiopurine S-methyltransferase gene locus. *J. Natl. Cancer Inst.*, **91**, 2001–2008.
- Trevino, L.R., Shimasaki, N., Yang, W., Panetta, J.C., Cheng, C., Pei, D., Chan, D., Sparreboom, A., Giacomini, K.M., Pui, C.H. *et al.* (2009) Germline genetic variation in an organic anion transporter polypeptide associated with methotrexate pharmacokinetics and clinical effects. *J. Clin. Oncol.*, **27**, 5972–5978.
- Peters, E.J., Kraja, A.T., Lin, S.J., Yen-Revollo, J.L., Marsh, S., Province, M.A. and McLeod, H.L. (2009) Association of thymidylate synthase variants with 5-fluorouracil cytotoxicity. *Pharmacogenet. Genomics*, **19**, 399–401.
- Wheeler, H.E. and Dolan, M.E. (2012) Lymphoblastoid cell lines in pharmacogenomic discovery and clinical translation. *Pharmacogenomics*, **13**, 55–70.
- Jones, T.S., Yang, W., Evans, W.E. and Relling, M.V. (2007) Using HapMap tools in pharmacogenomic discovery: the thiopurine methyltransferase polymorphism. *Clin. Pharmacol. Ther.*, **81**, 729–734.

12. Gamazon, E.R., Huang, R.S., Cox, N.J. and Dolan, M.E. (2010) Chemotherapeutic drug susceptibility associated SNPs are enriched in expression quantitative trait loci. *Proc. Natl. Acad. Sci. USA*, **107**, 9287–9292.
13. Nicolae, D.L., Gamazon, E., Zhang, W., Duan, S., Dolan, M.E. and Cox, N.J. (2010) Trait-associated SNPs are more likely to be eQTLs: annotation to enhance discovery from GWAS. *PLoS Genet.*, **6**, e1000888.
14. Kishi, S., Cheng, C., French, D., Pei, D., Das, S., Cook, E.H., Hijiya, N., Rizzari, C., Rosner, G.L., Frudakis, T. *et al.* (2007) Ancestry and pharmacogenetics of antileukemic drug toxicity. *Blood*, **109**, 4151–4157.
15. Tai, H.L., Fessing, M.Y., Bonten, E.J., Yanishevsky, Y., d'Azzo, A., Krynetski, E.Y. and Evans, W.E. (1999) Enhanced proteasomal degradation of mutant human thiopurine S-methyltransferase (TPMT) in mammalian cells: mechanism for TPMT protein deficiency inherited by TPMT\*2, TPMT\*3A, TPMT\*3B or TPMT\*3C. *Pharmacogenetics*, **9**, 641–650.
16. Stanulla, M., Schaeffeler, E., Flohr, T., Cario, G., Schrauder, A., Zimmermann, M., Welte, K., Ludwig, W.D., Bartram, C.R., Zanger, U.M. *et al.* (2005) Thiopurine methyltransferase (TPMT) genotype and early treatment response to mercaptopurine in childhood acute lymphoblastic leukemia. *J.A.M.A.*, **293**, 1485–1489.
17. Kessels, M.M. and Qualmann, B. (2004) The syndapin protein family: linking membrane trafficking with the cytoskeleton. *J. Cell Sci.*, **117**, 3077–3086.
18. Modregger, J., Ritter, B., Witter, B., Paulsson, M. and Plomann, M. (2000) All three PACSIN isoforms bind to endocytic proteins and inhibit endocytosis. *J. Cell Sci.*, **113**(Pt 24), 4511–4521.
19. Meng, H., Tian, L., Zhou, J., Li, Z., Jiao, X., Li, W.W., Plomann, M., Xu, Z., Lisanti, M.P., Wang, C. *et al.* (2011) PACSIN 2 represses cellular migration through direct association with cyclin D1 but not its alternate splice form cyclin D1b. *Cell Cycle*, **10**, 73–81.
20. Sznianowski, P., Corcelle-Termeau, E., Farkas, T., Hoyer-Hansen, M., Nylandsted, J., Kallunki, T. and Jaattela, M. (2011) A comprehensive siRNA screen for kinases that suppress macroautophagy in optimal growth conditions. *Autophagy*, **7**, 892–903.
21. Qualmann, B. and Kelly, R.B. (2000) Syndapin isoforms participate in receptor-mediated endocytosis and actin organization. *J. Cell. Biol.*, **148**, 1047–1062.
22. Wang, Q., Navarro, M.V., Peng, G., Molinelli, E., Goh, S.L., Judson, B.L., Rajashankar, K.R. and Sondermann, H. (2009) Molecular mechanism of membrane constriction and tubulation mediated by the F-BAR protein Pacsin/Syndapin. *Proc. Natl. Acad. Sci. U. S. A.*, **106**, 12700–12705.
23. Takahashi, Y., Coppola, D., Matsushita, N., Cualing, H.D., Sun, M., Sato, Y., Liang, C., Jung, J.U., Cheng, J.Q., Mule, J.J. *et al.* (2007) Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat. Cell. Biol.*, **9**, 1142–1151.
24. Takahashi, Y., Meyerkord, C.L., Hori, T., Runkle, K., Fox, T.E., Kester, M., Loughran, T.P. and Wang, H.G. (2011) Bif-1 regulates Atg9 trafficking by mediating the fission of Golgi membranes during autophagy. *Autophagy*, **7**, 61–73.
25. Behrends, C., Sowa, M.E., Gygi, S.P. and Harper, J.W. (2010) Network organization of the human autophagy system. *Nature*, **466**, 68–76.
26. Li, F., Wang, L., Burgess, R.J. and Weinshilboum, R.M. (2008) Thiopurine S-methyltransferase pharmacogenetics: autophagy as a mechanism for variant allozyme degradation. *Pharmacogenet. Genomics*, **18**, 1083–1094.
27. Raney, B.J., Cline, M.S., Rosenbloom, K.R., Dreszer, T.R., Learned, K., Barber, G.P., Meyer, L.R., Sloan, C.A., Malladi, V.S., Roskin, K.M. *et al.* (2011) ENCODE whole-genome data in the UCSC genome browser (2011 update). *Nucleic Acids Res.*, **39**, D871–D875.
28. Rosenbloom, K.R., Dreszer, T.R., Pheasant, M., Barber, G.P., Meyer, L.R., Pohl, A., Raney, B.J., Wang, T., Hinrichs, A.S., Zweig, A.S. *et al.* (2010) ENCODE whole-genome data in the UCSC Genome Browser. *Nucleic Acids Res.*, **38**, D620–D625.
29. Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M. and Haussler, D. (2002) The human genome browser at UCSC. *Genome Res.*, **12**, 996–1006.
30. McLeod, H.L., Coulthard, S., Thomas, A.E., Pritchard, S.C., King, D.J., Richards, S.M., Eden, O.B., Hall, A.G. and Gibson, B.E. (1999) Analysis of thiopurine methyltransferase variant alleles in childhood acute lymphoblastic leukaemia. *Br. J. Haematol.*, **105**, 696–700.
31. Roberts, R.L., Gearty, R.B., Bland, M.V., Sies, C.W., George, P.M., Burt, M., Marinaki, A.M., Arenas, M., Barclay, M.L. and Kennedy, M.A. (2008) Trinucleotide repeat variants in the promoter of the thiopurine S-methyltransferase gene of patients exhibiting ultra-high enzyme activity. *Pharmacogenet. Genomics*, **18**, 434–438.
32. de Kreuk, B.J., Nethé, M., Fernandez-Borja, M., Anthony, E.C., Hensbergen, P.J., Deelder, A.M., Plomann, M. and Hordijk, P.L. (2011) The F-BAR domain protein PACSIN2 associates with Rac1 and regulates cell spreading and migration. *J. Cell Sci.*, **124**, 2375–2388.
33. Tiede, I., Fritz, G., Strand, S., Poppe, D., Dvorsky, R., Strand, D., Lehr, H.A., Wirtz, S., Becker, C., Atreya, R. *et al.* (2003) CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *J. Clin. Invest.*, **111**, 1133–1145.
34. Stocco, G., Cheok, M.H., Crews, K.R., Dervieux, T., French, D., Pei, D., Yang, W., Cheng, C., Pui, C.H., Relling, M.V. *et al.* (2009) Genetic polymorphism of inosine triphosphate pyrophosphatase is a determinant of mercaptopurine metabolism and toxicity during treatment for acute lymphoblastic leukemia. *Clin. Pharmacol. Ther.*, **85**, 164–172.
35. McLeod, H.L., Lin, J.S., Scott, E.P., Pui, C.H. and Evans, W.E. (1994) Thiopurine methyltransferase activity in American white subjects and black subjects. *Clin. Pharmacol. Ther.*, **55**, 15–20.
36. Pui, C.H., Pei, D., Sandlund, J.T., Ribeiro, R.C., Rubnitz, J.E., Raimondi, S.C., Onciu, M., Campana, D., Kun, L.E., Jeha, S. *et al.* (2010) Long-term results of St Jude Total Therapy Studies 11, 12, 13A, 13B, and 14 for childhood acute lymphoblastic leukemia. *Leukemia*, **24**, 371–382.
37. Pui, C.H., Campana, D., Pei, D., Bowman, W.P., Sandlund, J.T., Kaste, S.C., Ribeiro, R.C., Rubnitz, J.E., Raimondi, S.C., Onciu, M. *et al.* (2009) Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N. Engl. J. Med.*, **360**, 2730–2741.
38. Pui, C.H., Sandlund, J.T., Pei, D., Campana, D., Rivera, G.K., Ribeiro, R.C., Rubnitz, J.E., Razzouk, B.I., Howard, S.C., Hudson, M.M. *et al.* (2004) Improved outcome for children with acute lymphoblastic leukemia: results of Total Therapy Study XIII B at St Jude Children's Research Hospital. *Blood*, **104**, 2690–2696.
39. Schrappe, M., Valsecchi, M.G., Bartram, C.R., Schrauder, A., Panzer-Grumayer, R., Moricke, A., Parasole, R., Zimmermann, M., Dworzak, M., Buldini, B. *et al.* (2011) Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. *Blood*, **118**, 2077–2084.
40. Holm, S. (1979) A simple sequentially rejective multiple test procedure. *Scand. J. Stat.*, **6**, 65–70.
41. Jain, N., Thatte, J., Braciale, T., Ley, K., O'Connell, M. and Lee, J.K. (2003) Local-pooled-error test for identifying differentially expressed genes with a small number of replicated microarrays. *Bioinformatics*, **19**, 1945–1951.
42. Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B*, **57**, 289–300.



# Multilocus genotypes of relevance for drug metabolizing enzymes and therapy with thiopurines in patients with acute lymphoblastic leukemia

Gabriele Stocco<sup>1,2\*</sup>, Raffaella Franca<sup>3</sup>, Federico Verzeznassi<sup>3</sup>, Margherita Londero<sup>4,5</sup>, Marco Rabusin<sup>3</sup> and Giuliana Decorti<sup>2</sup>

<sup>1</sup> Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, TN, USA

<sup>2</sup> Department of Life Sciences, University of Trieste, Trieste, Italy

<sup>3</sup> Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy

<sup>4</sup> Scuola di Dottorato di Ricerca in Scienze della Riproduzione, University of Trieste, Trieste, Italy

<sup>5</sup> Ospedale di San Daniele, Azienda per i Servizi Sanitari 4, Udine, Italy

## Edited by:

Kathrin Klein, Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Germany

## Reviewed by:

Branka Zukic, University of Belgrade, Serbia

Mirko Manchia, University of Cagliari, Italy

## \*Correspondence:

Gabriele Stocco, Department of Life Sciences, University of Trieste, Via Fleming 22, Trieste 34127, Italy.  
e-mail: gabriele.stocco@stjude.org; stocceg@units.it

Multilocus genotypes have been shown to be of relevance for using pharmacogenomic principles to individualize drug therapy. As it relates to thiopurine therapy, genetic polymorphisms of *TPMT* are strongly associated with the pharmacokinetics and clinical effects of thiopurines (mercaptopurine and azathioprine), influencing their toxicity and efficacy. We have recently demonstrated that *TPMT* and *ITPA* genotypes constitute a multilocus genotype of pharmacogenetic relevance for children with acute lymphoblastic leukemia (ALL) receiving thiopurine therapy. The use of high-throughput genomic analysis allows identification of additional candidate genetic factors associated with pharmacogenetic phenotypes, such as *TPMT* enzymatic activity: *PACSIN2* polymorphisms have been identified by a genome-wide analysis, combining evaluation of polymorphisms and gene expression, as a significant determinant of *TPMT* activity in the HapMap CEU cell lines and the effects of *PACSIN2* on *TPMT* activity and mercaptopurine induced adverse effects were confirmed in children with ALL. Combination of genetic factors of relevance for thiopurine metabolizing enzyme activity, based on the growing understanding of their association with drug metabolism and efficacy, is particularly promising for patients with pediatric ALL. The knowledge basis and clinical applications for multilocus genotypes of importance for therapy with mercaptopurine in pediatric ALL is discussed in the present review.

**Keywords:** acute lymphoblastic leukemia, mercaptopurine, pharmacogenetics, multilocus genotypes, *TPMT*, *ITPA*, *PACSIN2*

## INTRODUCTION

The principle of personalized therapy is the identification and application of features associated with treatment response, to select adequate medications and their doses, in order to offer to patients the most effective treatment, with the lower incidence of adverse events (Cheok and Evans, 2006). Among the several features that can be used to personalize therapy, demographic, clinical, and pharmacological ones have been considered. The application of therapy targeted according to these features, related to different treatment outcomes, has greatly improved the effectiveness and safety of therapy, in particular for patients with pediatric cancer, such as acute lymphoblastic leukemia (ALL).

## PERSONALIZED THERAPY IMPROVES TREATMENT EFFECTS: THE PARADIGM OF CHILDHOOD ALL

Optimal use of existing antileukemic agents and improved supportive care in contemporary clinical trials have improved the 5-year survival rate of childhood ALL above 85% in developed countries, a disease that was universally fatal in the sixties; moreover, molecular characteristics of leukemia cells have been shown

to influence treatment response (Pui and Evans, 2006; Pui et al., 2012).

Pharmacological therapy for childhood ALL consists in protocols in which specific treatment approaches may differ but consistently comprise three major treatment phases: remission induction therapy followed by consolidation/intensification therapy and then continuation/maintenance treatment to eliminate residual leukemic cells (Pui and Evans, 2006). Several medications are used in these treatment phases, comprising various lympholytic and cytotoxic drugs such glucocorticoids (i.e., prednisone, dexamethasone), asparaginase and vincristine, which are particularly important for the induction of disease remission. The purine analog mercaptopurine is a key medication for the successful treatment of childhood ALL, in particular for the consolidation and continuation therapies and is used in combination with the folate analog methotrexate: for the success of ALL treatment, the 18–24 months of adequate maintenance therapy comprising mercaptopurine and methotrexate have a key role and are necessary to prolong and consolidate the remission obtained during the initial treatment phases (Pui and Evans, 2006; Paugh et al., 2010; Stocco et al., 2010).

## GENETIC FEATURES MAY INFLUENCE RESPONSE TO THERAPY

Genetic polymorphisms for genes involved in drug metabolism, transport and molecular mechanism of action can alter the concentration of active metabolites and the molecular function of drugs' targets and therefore the efficacy and safety of pharmacological therapies (Paugh et al., 2011; Pinto et al., 2012). These genetic polymorphisms could therefore function as biomarkers for toxicity and efficacy, allowing the identification of patients with modified sensitivity, because of their genetic characteristics involving drug pharmacokinetics and pharmacodynamics. While many associations between single genetic polymorphisms and drug effects have been clearly demonstrated, showing that inherited genomic variation causes substantial interindividual differences in drug effects, the clinical implementation of these associations is still limited (Relling and Klein, 2011). This is due mainly to the lack of freely available, peer-reviewed, updatable, and detailed gene/drug clinical practice guidelines and even to the very high standards many clinicians and regulators hold for pharmacogenetic evidence (Relling et al., 2010). One of the main efforts to provide these guidelines, which could base the clinical implementation of pharmacogenomics, is that of the Clinical Pharmacogenetics Implementation Consortium (CPIC; Relling and Klein, 2011). CPIC was established in 2009 and consists of members of the Pharmacogenomics Research Network, the main US-based research network in this field, supported by the PharmGKB staff, one of the most important resources for curated pharmacogenomics knowledge (McDonagh et al., 2011), and other affiliated experts in pharmacogenetics, pharmacogenomics and laboratory medicine (Relling and Klein, 2011).

Currently, the CPIC has provided guidelines for pharmacogenetic implementation for 7 medications: abacavir, allopurinol, clopidogrel, codeine, simvastatin, thiopurines, and warfarin (<https://www.pharmgkb.org/page/cpic>). The process of guidelines definition and preparation is still ongoing and other potential guidelines may be of interest, such as inosine triphosphate pyrophosphatase (ITPA) genetic polymorphism and ribavirin (Fellay et al., 2010), for which a good amount of evidence and replication has been made (Ochi et al., 2010; Thompson et al., 2010; D'Avolio et al., 2012). Moreover, besides CPIC, other research groups have been putting together similar guidelines, such as the European Dutch and German translational pharmacogenomics research teams (Swen et al., 2008, 2011; Schwab and Brauch, 2012).

For the pharmacological therapy of pediatric ALL, several examples have been reported of genetic polymorphisms influencing drug response and toxicity, such as for prednisone polymorphisms of *SMARCB1* (Pottier et al., 2007, 2008) and *GST-M1* (Marino et al., 2009), for methotrexate solute carrier organic anion transporter family member 1B1 (*SLCO1B1*) (Trevino et al., 2009; Ramsey et al., 2012), for vincristine *ABCB1* and *CYP3A4/CYP3A5* (Paugh et al., 2010); however, the only drug – gene pair with a validated guideline published by CPIC that is fully relevant for therapy of pediatric ALL is that of mercaptopurine and thiopurine-S-methyltransferase (TPMT). Indeed, for mercaptopurine, genetic polymorphisms of *TPMT* have been demonstrated to influence drug metabolism and its effects, constituting one of the most studied and significant example of associations between drug clinical effects and a genetic polymorphism (Paugh et al., 2011).

In lymphoid tissues, mercaptopurine is converted to its active metabolites, the thioguanine nucleotides (TGNs) and is inactivated primarily to methylmercaptopurine by *TPMT* (Stocco et al., 2010; Zaza et al., 2010). *TPMT* is encoded by a gene that has non-synonymous single-nucleotide polymorphisms, leading to reduced *TPMT* activity. In the majority of world populations studied to date, ~1 in 180 to 1 in 3,700 individuals (depending on ethnicity) inherit two non-functional variants of the *TPMT* gene, 3–14% are heterozygous, and the rest are homozygous wild-type. With chronic conventional doses of mercaptopurine, patients who inherit two inactive *TPMT* alleles universally experience severe myelosuppression, because of accumulation of high levels of cellular TGNs; a high proportion (30–60%) of patients heterozygous for a *TPMT* variant allele does not tolerate full doses of mercaptopurine, again because of excessive TGNs. Three *TPMT* single-nucleotide polymorphisms account for more than 90% of inactivating alleles and therefore genotyping tests have a high likelihood of being informative. Characterization of *TPMT* deficiency by genotyping for the most common inactivating single-nucleotide polymorphisms can prospectively identify patients at higher risk of mercaptopurine hematopoietic toxicity; such genotyping is recommended in US Food and Drug Administration-approved labeling (Paugh et al., 2010, 2011).

The diagnosis of *TPMT* deficiency allows the rational reduction of mercaptopurine dosages while other concurrent cytotoxic agents remain at their usual unadjusted doses, thereby avoiding toxicity without compromising efficacy. For patients with ALL taking mercaptopurine, the CPIC guidelines indicate that these subjects with the homozygous variant should start with drastically reduced dose (i.e., reduce daily dose 10-fold and reduce frequency to thrice weekly instead of daily) and in case of myelosuppression, emphasis should be on reducing mercaptopurine over other agents; patients heterozygous for *TPMT* variant alleles (intermediate activity) should start at 30–70% of full dose and again, in case of myelosuppression, emphasis should be on reducing mercaptopurine over other agents. Patients with normal *TPMT* should begin therapy with normal starting dose and adjust doses of mercaptopurine (and of any other myelosuppressive therapy) without any special emphasis on mercaptopurine compared to other agents. For all genotypes, in case of dose adjustment, the guidelines specify to allow 2–6 weeks to reach steady state after each dose adjustment, with longer time needed for patients with inactive allele(s) as compared to patients with functional ones (Relling et al., 2011; **Table 1**). Indeed, it has been shown that, in an ALL protocol using mercaptopurine, prospective adjustment of mercaptopurine based on *TPMT* status allowed successful treatment of patients with variant *TPMT* at a reduced dose, with toxicity and efficacy comparable to those in patients with wild-type *TPMT* (Relling et al., 2006; Stocco et al., 2009).

## MULTILOCUS GENOTYPES

Most of the associations evaluated in the literature and those ready for clinical implementation, i.e. with published curated guidelines, are considering single gene influencing response to a particular drug. However, one of the most important, in terms of relevance and diffusion of drug treatment and potential improvement of guideline application to influence drug use in the clinical setting

**Table 1 | Recommended dosing of thiopurines by thiopurine methyltransferase phenotype.**

TPMT status	Mercaptopurine		Thioguanine	
	Effects on mercaptopurine metabolism	Dosing recommendations for mercaptopurine	Effects on thioguanine metabolism	Dosing recommendations for thioguanine
Homozygous wild-type or normal, high activity	Lower concentrations of TGNs metabolites, higher methylTIMP; this is the "normal" pattern	Start with normal starting dose (e.g., 75 mg/m <sup>2</sup> /day) and adjust doses of mercaptopurine (and of any other myelosuppressive therapy) without any special emphasis on mercaptopurine compared to other agents	Lower concentrations of TGNs metabolites, but note that TGNs after thioguanine are 5–10× higher than TGNs after mercaptopurine	Start with normal starting dose. Adjust doses of thioguanine and of other myelosuppressive therapy without any special emphasis on thioguanine.
Heterozygote or intermediate activity	Moderate to high concentrations of TGNs metabolites; low concentrations of methylTIMP	Start with reduced doses (start at 30–70% of full dose: e.g., at 50 mg/m <sup>2</sup> /day or 0.75 mg/kg/day) and adjust doses of mercaptopurine based on degree of myelosuppression and disease-specific guidelines. In those who require a dosage reduction based on myelosuppression, the median dose may be ~40% lower (44 mg/m <sup>2</sup> /day) than that tolerated in wild-type patients (75 mg/m <sup>2</sup> /day). In setting of myelosuppression, and depending on other therapy, emphasis should be on reducing mercaptopurine over other agents	Moderate to high concentrations of TGNs metabolites; but note that TGNs after thioguanine are 5–10× higher than TGNs after mercaptopurine	Start with reduced doses (reduce by 30–50%) and adjust doses of thioguanine based on degree of myelosuppression and disease-specific guidelines. In setting of myelosuppression, and depending on other therapy, emphasis should be on reducing thioguanine over other agents
Homozygous variant, mutant, low, or deficient activity	Extremely high concentrations of TGNs metabolites; fatal toxicity possible without dose decrease; no methylTIMP metabolites	Start with drastically reduced doses (reduce daily dose by 10-fold and reduce frequency to thrice weekly instead of daily, e.g., 10 mg/m <sup>2</sup> /day given just 3 days/week) and adjust doses of mercaptopurine based on degree of myelosuppression and disease-specific guidelines. In setting of myelosuppression, emphasis should be on reducing mercaptopurine over other agents	Extremely high concentrations of TGNs metabolites; fatal toxicity possible without dose decrease	Start with drastically reduced doses (reduce daily dose by 10-fold and dose thrice weekly instead of daily) and adjust doses of thioguanine based on degree of myelosuppression and disease-specific guidelines. In setting of myelosuppression, emphasis should be on reducing thioguanine over other agents

TGNs, thioguanine nucleotide; TIMP, thioinosine monophosphate (secondary metabolite of mercaptopurine); TPMT, thiopurine-S-methyl transferase (Relling et al., 2011).

are the guidelines for warfarin, the most commonly used oral anticoagulant worldwide (Johnson et al., 2011); indeed, these guidelines consider genetic variability at two loci: one for the hepatic drug metabolizing enzyme *CYP2C9* and one for the target enzyme of warfarin, that is vitamin K-epoxide reductase (*VKORC1*). *CYP2C9* and *VKORC1* genetic polymorphisms account for up to 18 and 30%, respectively, of the variance in stable warfarin dose among patients of European ancestry: these common polymorphisms in both genes affect warfarin pharmacokinetics (*CYP2C9*) and pharmacodynamics (*VKORC1*) and modulate the therapeutic dose necessary to maintain the optimal level of drug effect (i.e., anticoagulation), preventing the risk of adverse events due to low efficacy or excessive anticoagulation (i.e., respectively thrombosis or bleeding). Combination of *CYP2C9* and *VKORC1* genetic polymorphisms is important to select the most appropriate dose to start therapy with warfarin: patients are classified by the multilocus genotype in a  $2 \times 2$  table, according to the combined effects of the most relevant polymorphisms in each gene (two SNPs for *CYP2C9* and one SNP for *VKORC1*) in three levels of warfarin starting dose; this table is currently inserted in the US Food and Drug's Administration approved warfarin label. More complex algorithms, comprising even relevant demographic and clinical patient's characteristics affecting warfarin efficacy, such as age, smoking status and interacting drugs have been developed; some of these algorithms consider even additional genetic information besides *CYP2C9/VKORC1* multilocus genotype, such as polymorphisms of the *CYP4F2* and *GGCX* genes. It has been shown that warfarin dosing criteria considering genetics outperform non-genetic clinical algorithms and are particularly beneficial for patients requiring relatively low or high doses of the medication (i.e.,  $<21$  mg/week or  $>49$  mg/week), that however are  $\sim 40\%$  of all patients: thanks to genetic based dose selection, these patients reach their optimal dose level more quickly and therefore with a lower risk of developing adverse events. Moreover, these criteria are particularly important for patients starting warfarin therapy, while are less useful for already established treatments. The development of these important therapeutic guidelines, considering a multilocus genotype affecting warfarin dose requirements illustrates how genetic information in more than one gene can be of clinical relevance to guide therapy for a single medication. Other similar guidelines considering multiple loci are in development, such as *CYP2D6/CYP2C9* multilocus genotype for tricyclic antidepressants (Consortium, 2012).

## MULTILOCUS GENOTYPES OF RELEVANCE FOR THERAPY PERSONALIZATION OF PEDIATRIC ALL

### TPMT AND ITPA AND MAINTENANCE THERAPY FOR PEDIATRIC ALL

In addition to *TPMT*, other genetic factors may alter the effects of mercaptopurine, although their clinical importance has not been as well characterized. It has been shown that once mercaptopurine treatment for childhood ALL is individualized for *TPMT*, the effect of genetic polymorphisms in inosine triphosphate pyrophosphatase (*ITPA*) emerges (Stocco et al., 2010). *ITPA* is an enzyme that catalyzes the hydrolysis of inosine triphosphate (ITP) to inosine monophosphate (IMP). IMP is a central intermediate in purine metabolism and can be converted to ITP and to ATP via AMP or to GTP via GMP. The putative role of *ITPA*

is to protect cells from the accumulation of potentially harmful nucleotides, such as ITP or deoxy-ITP, which may be incorporated into nucleic acids; indeed, it has been demonstrated by knock-down experiments performed in HeLa cells, that *ITPA* has a significant role in preventing base analog induced apoptosis, DNA damage, and mutagenesis in human cells (Menezes et al., 2012). In humans, *ITPA* displays a genetically determined polymorphic activity (Marsh and Van Booven, 2009; Stocco et al., 2010). Characterization of *ITPA* haplotype structure has shown that the SNP rs1127354 is the most relevant polymorphism in determining *ITPA* low enzymatic activity (von Ahnen et al., 2008; Stocco et al., 2010). Our recent study assessed the influence of non-functional variant alleles of *TPMT* and *ITPA* on mercaptopurine metabolism and toxicity in patients with ALL whose mercaptopurine doses were adjusted based on *TPMT* genotype (Stocco et al., 2009). This study revealed that the cumulative incidence of severe adverse effects (grade 3–4 febrile neutropenia) in patients receiving maintenance therapy that includes mercaptopurine individualized for *TPMT* is significantly greater among patients who have inherited an *ITPA* variant allele; this association remained significant when the analysis was limited to only life threatening events (i.e., grade 4 fever and neutropenia). Our recent study has documented that inheritance of a non-functional variant allele for either *TPMT* or *ITPA* is associated with significant modification in the metabolism of mercaptopurine during treatment of ALL. Although the importance of the *TPMT* genetic polymorphism is very well known and characterized, this was the first report showing a significant effect of the *ITPA* genetic polymorphism in the context of mercaptopurine therapy that has been individualized based on *TPMT* genotype. We documented significantly higher concentrations of the methylated nucleotide metabolites of mercaptopurine in leukemia cells and erythrocytes of patients who have inherited a non-functional *ITPA* allele. In contrast, the inheritance of a variant *ITPA* allele was not associated with differences in TGN concentrations in either leukemia cells or erythrocytes. Although *ITPA* is known to be involved in mercaptopurine metabolism, the mechanism by which *ITPA* variant alleles influence the accumulation of methylated thionucleotides has not been fully elucidated (Stocco et al., 2009, 2010).

A recent study has replicated the observation of the effects of the combined *TPMT* and *ITPA* genotype on the mercaptopurine pharmacokinetics and in particular on the concentration of methylated-mercaptopurine-nucleotides: among 66 children with ALL, treated according to EORTC 58951 protocol, comprising mercaptopurine at a dose of  $50$  mg/m<sup>2</sup>/day and methotrexate at a dose of  $20$  mg/m<sup>2</sup>/week, methylated-mercaptopurine-nucleotides concentrations were low in patients with *TPMT* variant/*ITPA* wild-type multilocus genotype, intermediate in wild-type/wild-type patients and high in patients with wild-type *TPMT*/*ITPA* variant (Adam de Beaumais et al., 2010).

It is known that ethnic differences for genotype frequencies may influence treatment efficacy in ALL: for example, it has been reported that the component of genomic variation that co-segregated with Native-American ancestry was associated with risk of relapse, even after adjusting for known prognostic factors (Yang et al., 2011). The allele frequencies of *TPMT* and *ITPA* polymorphisms show significant inter-ethnic variability: in

particular for rs1127354 of *ITPA* allele frequency of the variant is known to be ~20% in Asian populations, ~6% in Caucasians, and ~2% in Hispanics, while for *TPMT*, the most common variants (rs1142345, rs1800460 and rs1800462) have a frequency of ~1% in Asians, ~5% in Caucasians, and ~10% in Hispanics. Therefore, it is interesting that for *TPMT* and *ITPA*, frequencies of the variant alleles associated with different metabolism of mercaptopurine, seem to be almost reversal in the two populations (Marsh and Van Booven, 2009) and *ITPA* variants seem to be predominant in the Asian population. Indeed several recent studies of patients of Asian ethnicity seem to underline significant effects of *ITPA* polymorphisms on thiopurines' efficacy and toxicity in patients with ALL, but even when these medications are used as immunosuppressants in other pathologies (Okada et al., 2009; Yamamoto et al., 2010). For children with ALL, a recent study in 90 Indian patients, on maintenance therapy according to the MCP-841 protocol (Advani et al., 1999) with mercaptopurine at a dose of 75 mg/m<sup>2</sup> for 12 weeks, showed an independent role for both *TPMT* and *ITPA* in terms of association with the incidence of hematological toxicity; moreover, the multilocus genotype *TPMT/ITPA* was associated with a gene-dosage effect: percentage of reduction in total leukocyte count (i.e., the average leukocyte count on days 43, 71, and 99 of maintenance therapy) resulted in ~40% for a patient with a wild-type genotype at both the *TPMT* and *ITPA* loci and increased proportionally to the number of risk alleles (i.e., variant inactive alleles for *TPMT* or *ITPA*) up to almost 70% in patients with three or more risk alleles at the *TPMT* and *ITPA* loci (Dorababu et al., 2012a). Analysis of epistasis by multifactor dimensionality reduction (Hahn et al., 2003) confirmed synergistic interactions between *TPMT* and *ITPA* variant alleles, in terms of their association with hematological toxicity during ALL maintenance therapy for this cohort of Indian children. Another recent study considered 100 Korean patients with pediatric ALL and evaluated in these patients 18 loci in 16 candidate genes of pharmacogenetic interest, including *TPMT* and *ITPA*, and their association with survival rate. Even if this study did not seem to confirm a strong difference for *TPMT* and *ITPA* gene variants between a western population of reference and the Korean patients, there was a significant effect of *ITPA* genotype, but not of *TPMT*, on the event free survival rate, which was lower in *ITPA* variants. *TPMT* genotype was however associated with the tolerance of mercaptopurine and methotrexate, evaluated as the dose of the medications used during the last cycle of maintenance therapy: indeed, as expected, patients with variant *TPMT* were selected to be treated with lower doses of mercaptopurine; unfortunately, data about the effect of *ITPA* genotype on the doses of antimetabolites was not reported (Kim et al., 2012).

Tanaka et al. (2012) have measured the activity of *ITPA* in 65 Japanese children with pediatric ALL, showing that patients with lower activity of this enzyme tolerated lower doses of mercaptopurine during maintenance therapy and presented increased probability of hepatotoxicity.

In Asian populations, therefore, polymorphisms of *ITPA* seem to be of particular relevance for the effects of mercaptopurine in children with ALL, given the low incidence of patients with variant *TPMT*, compared to patients of Caucasian ethnicity (Marsh and Van Booven, 2009). However, it is known that other

genetic polymorphisms may be of particular importance for Asian patients, such as SNP rs3765534 in the transporter *MRP4*, that is polymorphic only in patients of Asian ethnicity and that has been shown to modulate thiopurines intracellular levels by regulating the efflux of the thionucleotides (Krishnamurthy et al., 2008; Stocco et al., 2010).

On these bases, to understand the pharmacogenetics and improve treatment with thiopurines in the Asian populations, larger prospective studies are needed, considering even multilocus genotypes at loci of known relevance, such as *TPMT*, *ITPA*, and *MRP4*.

#### **MULTILOCUS GENOTYPE *TPMT* – *SLC01B1* – *PACSIN2* AND EFFECTS ON SEVERE MUCOSITIS DURING CONSOLIDATION THERAPY FOR PEDIATRIC ALL**

During consolidation therapy for pediatric ALL, patients are treated with weekly 24 h infusions of high dose methotrexate, up to 5 g/m<sup>2</sup> and daily oral mercaptopurine with doses that range from 25 to 50 mg/m<sup>2</sup>. Therapy with this association of antimetabolites has a very important role in preventing the relapse of the disease, after remission induction; however consolidation therapy is associated with the development of adverse effects, in particular gastrointestinal toxicity, such as stomatitis and mucositis, which cause major discomfort for the patient and can be severe, preventing the children from normal food intake and requiring parenteral nutrition. To avoid adverse events related to consolidation therapy, one of the most common approaches used in therapeutic protocols for ALL worldwide is the administration of leucovorin, a source of folic acid, that contrasts the cytotoxic effects of methotrexate and its association with mercaptopurine. Most protocols for ALL worldwide measure the concentration of methotrexate in patients' blood at the end of each infusion and administer leucovorin if methotrexate is then still present at significant concentrations: for example, in the Italian AIEOP-BFM ALL 2000 protocol, leucovorin was administered every 6 h at a dose of 7.5 mg/m<sup>2</sup>, if methotrexate concentration resulted higher than 0.5 μmol/l at 48 h from the beginning of the infusion and until methotrexate concentration dropped below 0.25 μmol/l (Conter et al., 2010; Schrappe et al., 2011). Consolidation therapy lasts from 2 weeks up to 3 months depending on the treatment protocol and therefore the length of the therapy is too short to implement therapeutic monitoring of mercaptopurine metabolites concentration, which are useful when the drug is taken for at least 2 months (Lennard and Lilleyman, 1989). Advanced protocols for treatment of ALL, developed at St. Jude Children's Research Hospital in Memphis, evaluate the clearance of methotrexate during the infusion and either adjust the speed of infusion of the drug to a target concentration in the subsequent course (Total XV protocol) or in the same course (Total XVI protocol). This procedure requires a quick and efficient turnaround of the samples for the measurement of methotrexate concentration, which need to be analyzed in a few hours timeframe, so that the clearance of methotrexate can be estimated during the infusion, and the medication's administration speed can be adapted to reach the desired concentration threshold (i.e., 33 μM for low risk patients, 65 μM for standard-high risk patients; Pui et al., 2009). Therapeutic monitoring of methotrexate during consolidation therapy has significantly improved patients'

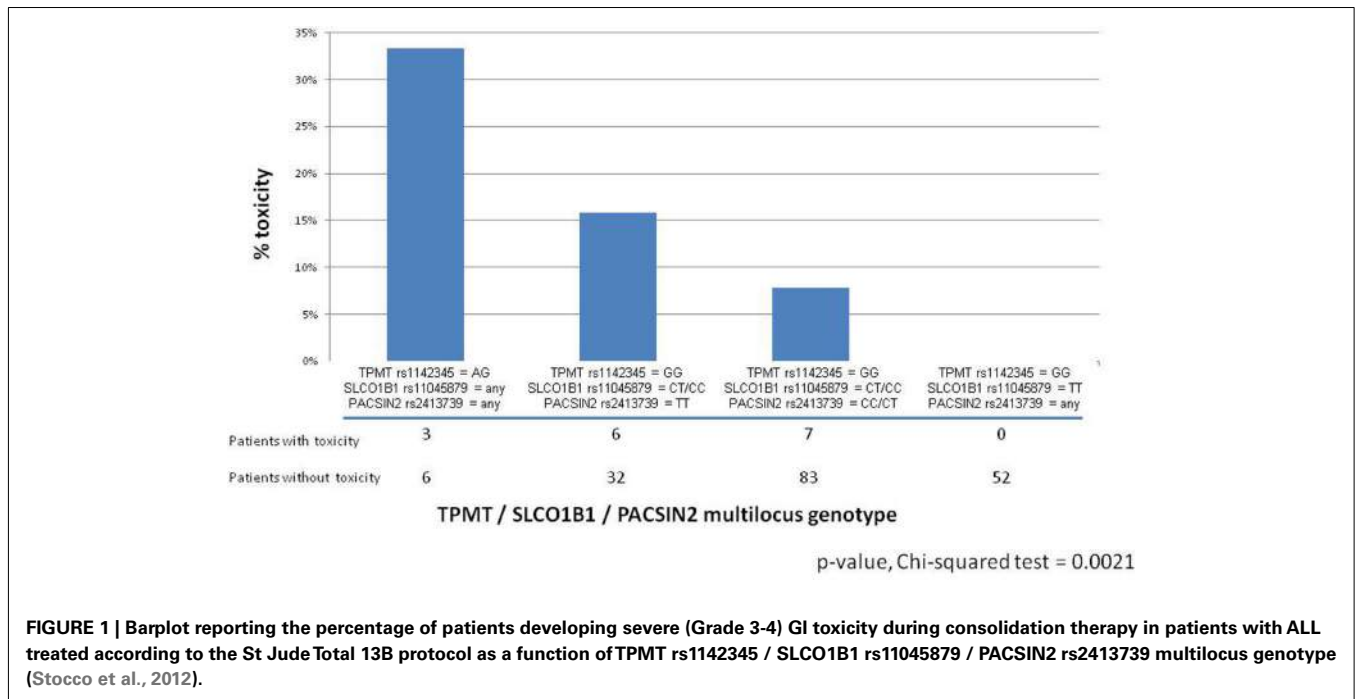
tolerance to this association treatment with antimetabolites; however, about 5% of pediatric patients still develop severe stomatitis/mucositis, with consequences that can be life threatening. The study of pharmacogenetic determinants of severe gastrointestinal (GI) toxicity during consolidation therapy has led to major breakthroughs in recent years, which hopefully will lead to even better treatment of patients with ALL, completely preventing the occurrence of this major adverse event. In particular, a recent genome-wide study analyzed 500,568 germline single-nucleotide polymorphisms to identify how inheritance affects methotrexate plasma disposition among 434 children with ALL who received 3,014 courses of methotrexate at 2–5 g/m<sup>2</sup> (Trevino et al., 2009). This study led to the identification of polymorphisms in *SLCO1B1*, as the most significant associations ( $p$ -value < 10<sup>-9</sup>) with methotrexate clearance, even after adjusting for age, race, sex, and methotrexate regimen. In particular, the most significant polymorphism was the intronic rs11045879, which is in linkage disequilibrium with the functional SNP rs4149056; these same polymorphisms were associated even with severe GI toxicity during consolidation therapy, mostly severe stomatitis and mucositis. This observation was confirmed by subsequent studies (Lopez-Lopez et al., 2011; Ramsey et al., 2012). Therefore *SLCO1B1* polymorphisms are significant determinants for the occurrence of severe GI toxicity and in particular stomatitis/mucositis, during consolidation therapy for pediatric ALL, by an effect on methotrexate disposition: indeed the gene product of *SLCO1B1* is a transporter which mediates the sodium-independent uptake of organic anions such as methotrexate and may play an important role in the clearance of bile acids and organic anions.

Another relevant study has considered the role of genetic determinants of mercaptopurine toxicity during consolidation therapy, together with *SLCO1B1*: this study on adverse effects considered 189 children with ALL and evaluated the association between genetic determinants of *TPMT* activity in patients and the incidence of severe GI toxicity. The frequency of GI toxicity (grade 3–4 mucositis) in this population was 8.5%; among these patients, deficiency in *TPMT* activity predisposed to an increased incidence of severe GI toxicity during consolidation therapy which included methotrexate (2 g/m<sup>2</sup>/week) and mercaptopurine (75 mg/m<sup>2</sup>/day, in all patients during consolidation therapy, regardless of *TPMT* genotype). Indeed, among nine patients with a variant *TPMT* allele, the frequency of GI toxicity was 33%, compared with 7.2% in patients with wild-type *TPMT*. As previously reported (Trevino et al., 2009), the *SLCO1B1* SNP rs11045879 was also associated with the incidence of GI toxicity: indeed none of the patients with the *SLCO1B1* CC or CT genotype had GI toxicity, whereas 11.8% of the patients with the wild-type *SLCO1B1* TT genotype had this side effect. Moreover, this study identified, through the HapMap model system, an additional determinant of *TPMT* activity, the *PACSIN2* gene which resulted as the highest correlated gene to *TPMT* activity, in an analysis combining polymorphisms and expression, all measured in the 30 HapMap CEU trios (Stocco et al., 2012). The most significant *PACSIN2* SNP in the HapMap analysis for *TPMT* activity, rs2413739, was also significantly associated with *TPMT* activity in patients with ALL, independently from *TPMT* genotype: the CC genotype for

the rs2413739 SNP displayed a higher *TPMT* activity in comparison to the TT genotype. Moreover, *PACSIN2* SNP rs2413739 also had a significant association with GI toxicity during consolidation therapy: the frequency of toxicity was 2.1, 9.1, and 13.2%, respectively, for the CC, CT, and TT genotype (Stocco et al., 2012). The effects of *PACSIN2* polymorphism on the incidence of severe mucositis during consolidation therapy for pediatric ALL were confirmed in another cohort of patients, considering 67 cases developing the adverse event during therapy according to the protocol AIEOP-BFM ALL 2000, which involves four weekly infusion of methotrexate at the dose of 2 g/m<sup>2</sup> and concomitant daily treatment with mercaptopurine at the dose of 25 mg/kg. Analysis in the validation cohort was done by a case-control design and each case was matched to two controls from the same protocol based on sex, age, ALL lineage and ALL risk classification, confirming a significant effect of *PACSIN2* SNP rs2413739 on the incidence of severe mucositis during the consolidation therapy of pediatric ALL (Stocco et al., 2012). Interestingly, in the discovery cohort, the effects of *TPMT*, *SLCO1B1* and *PACSIN2* polymorphisms were independent from each other, both in a multivariate logistic regression model and in a classification and regression tree analysis and could be combined in a multilocus genotype of potential importance to predict the incidence of severe mucositis in children with ALL treated with consolidation therapy comprising the combination of methotrexate and mercaptopurine (Figure 1).

#### GENOME-WIDE ANALYSIS OF SNPs ASSOCIATED TO CLINICAL RESPONSE IN PEDIATRIC ALL: IMPLICATIONS FOR THE PHARMACOGENETICS OF MERCAPTOPYRINE

Genome-wide analysis, if adequately powered, has great potential in elucidating and understanding the genomic component associated with interindividual differences in phenotypes, even of pharmacogenetic interest. This has been shown to be true in model systems like the HapMap cell lines, in which statistical power is obtained mainly by combining genomic information at the level of gene expression and genetic polymorphisms, with the advantage that the phenotypes can be characterized with great accuracy and consistency (Wheeler and Dolan, 2012). This has led for example to the identification of *PACSIN2* as a significant determinant of *TPMT* activity in the cell lines, with effects reproducible in patients with ALL mentioned above (Stocco et al., 2012). However, the greatest potential of the genome-wide approach resides really in the analysis of patients' samples: if the study is adequately designed and powered and the phenotypes are well collected, this approach can provide unpredictable insights on the phenotype of interest, potentially leading to major breakthroughs in the understanding of the genomic basis of inter-patient variability, even of pharmacogenetic traits. Several such examples exist in the literature: besides the already mentioned role of *SLCO1B1* in the disposition of methotrexate in children with ALL (Trevino et al., 2009), this same transporter was shown to be involved in statins' induced myopathy (Link et al., 2008); another example of genome-wide studies is the discovery of a role for *ITPA* in anemia induced by the anti-viral agent ribavirin (Fellay et al., 2010).



While in pediatric ALL genome-wide interrogation is complicated by the relative small number of patients available, St. Jude Children's Hospital was able to publish some genome-wide studies on leukemia pharmacogenetics, thanks to access to uniformly treated and well characterized patients and phenotypes (Trevino et al., 2009; Yang et al., 2009, 2012; Kawedia et al., 2011). Among these genome-wide studies of pharmacogenetic interest, some have generated data that could be of particular interest for the identification of multilocus genotypes of relevance for the treatment of ALL with thiopurines. In particular, these studies considered genetic polymorphisms associated with outcome to therapy evaluated as minimal residual disease (MRD) (Yang et al., 2009) or disease relapse (Yang et al., 2012); while these very important clinical phenotypes of patients with ALL are not related directly only to mercaptopurine, the genetic features identified are related even to disposition of antileukemic drugs and may be of relevance for mercaptopurine effects too and should be therefore considered.

The study on MRD considered two independent cohorts of children with newly diagnosed ALL: 318 patients in St Jude Total Therapy protocols XIII B and XV and 169 patients in Children's Oncology Group trial P9906. This study identified 102 SNPs associated with MRD in both cohorts, including five SNP in interleukin 15 (*IL15*). Twenty one of these SNPs were also associated with drug disposition (evaluated as methotrexate clearance, etoposide clearance, or methotrexate polyglutamates concentration), generally linking greater drug exposure with MRD eradication. While concentration of mercaptopurine metabolites was not evaluated in this study, the effects on the disposition of methotrexate, that is associated with mercaptopurine both during consolidation and maintenance therapy, suggest that these SNPs may be of interest to build multilocus genotypes useful for therapy personalization of pediatric ALL also with mercaptopurine.

#### FURTHER DEVELOPMENT OF MULTILOCUS GENOTYPES: EPISTASIS AND GENE-ENVIRONMENT INTERACTIONS

Phenotypes of pharmacogenetic interest are complex, particularly those describing patients' response to a medication, both in terms of efficacy and incidence of adverse events: it is likely that different genetic features, together with environmental factors, contribute to the interindividual variability of these phenotypes. Indeed, it is known that the effect of genetic polymorphisms is stronger when it refers to a pharmacokinetic phenotype and strength of the association reduces with the increasing complexity of the phenotype: for example, the effect of *TPMT* genotype is extremely strong on *TPMT* activity and the strength of the association is reduced, while still significant, considering more complex phenotypes such as the concentration of mercaptopurine metabolites and, even more, considering parameters of clinical response to the medication: the thinning of the association strength is due to the increasing complexity of the phenotype and the augmented potential role of environmental and additional genetic factors (Relling et al., 2011). Moreover, for complex phenotypes such as the response to a medication, the effects of a genetic factor may depend on other genetic variations and environmental factors, a phenomenon that is defined respectively as gene-gene interaction/epistasis or as gene-environment interaction (Moore and Williams, 2009). Methods have been developed to study consistently and efficiently the role of multiple genetic and environmental factors on complex phenotypes defined by discrete traits, such as those of pharmacogenetic interest (e.g., clinical response to a medication or occurrence of adverse events; Hahn et al., 2003; Gilbert-Diamond and Moore, 2011). This method is called multifactor dimensionality reduction (MDR) and allows collapsing multi-dimensional genetic information into a single dimension, thus permitting the detection of epistasis: MDR is a non-parametric method and

interactions are detected by a constructive induction approach, in particular by classifying multiple loci as high risk or low risk, depending on whether they are more common in affected or in unaffected subjects; this pooling allows reducing the dimensionality of the multilocus data to one dimension (Hahn et al., 2003). The new multilocus genotype variable is then evaluated for its ability to classify and predict the phenotype of interest (i.e., drug response): different approaches have been used to perform these computations; originally, however, it was done by cross-validation and permutation testing and recently extensions and variations of the method have been developed, which allow the calculation of odds ratios and application of Fisher's test to increase model robustness (Moore and Williams, 2009). Interestingly, it has been reported that MDR allows the identification of significant gene-gene interaction in the absence of a statistically significant main effect by a single genotype; moreover, it was mathematically proved that MDR is the best method to discriminate multilocus genotypes for clinical endpoints. MDR has been successfully applied to detecting gene-gene and gene-environment interactions for a wide variety of different complex phenotypes, such as incidence of human diseases and other clinical endpoints (Gilbert-Diamond and Moore, 2011). Recently this method has been applied even for studies of pharmacogenomics for thiopurines and methotrexate (Dervieux et al., 2012; Dorababu et al., 2012a,b; Kim et al., 2012), even if its application to this field is still limited and there is great

potential for discovery, in particular to detect and elucidate multilocus genotypes associated with genome-wide studies of complex pharmacogenetic phenotypes.

## CONCLUSION

Consideration of genetic biomarkers can improve therapy of pediatric ALL: the role of *TPMT* genetic polymorphism on mercaptopurine induced toxicity in children with ALL has been clearly defined and clinical guidelines have been developed to tailor treatment with this medication on the basis of *TPMT* status. Multilocus genotypes have been shown to be able to increase the amount of interindividual variability in a phenotype of clinical relevance explained: for example, the incidence of severe GI toxicity during consolidation therapy has been shown recently to be independently related to *TPMT*, *SLCO1B1*, and *PACSIN2* genetic polymorphisms. Identification, proper testing, and validation of multilocus genotypes hold great potential in further refining the clinical utility of pharmacogenetics to improve treatment of children with ALL by reducing treatment-related adverse events.

## ACKNOWLEDGMENTS

Dr. Stocco was a post-doctoral research fellow at St. Jude Children's Research Hospital when this manuscript was developed. We thank Dr. William E. Evans, CEO of SJCRH, for his helpful comments during the preparation of this manuscript.

## REFERENCES

- Adam de Beaumais, T., Dervieux, T., Fakhoury, M., Medard, Y., Azougagh, S., Zhang, D., et al. (2010). The impact of high-dose methotrexate on intracellular 6-mercaptopurine disposition during interval therapy of childhood acute lymphoblastic leukemia. *Cancer Chemother. Pharmacol.* 66, 653–658.
- Advani, S., Pai, S., Venzon, D., Adde, M., Kurkure, P. K., Nair, C. N., et al. (1999). Acute lymphoblastic leukemia in India: an analysis of prognostic factors using a single treatment regimen. *Ann. Oncol.* 10, 167–176.
- Cheok, M. H., and Evans, W. E. (2006). Acute lymphoblastic leukaemia: a model for the pharmacogenomics of cancer therapy. *Nat. Rev. Cancer* 6, 117–129.
- Consortium, C. P. I. (2012). *Clinical Pharmacogenetics Implementation Consortium (CPIC) Gene-drug pairs [Online]*. Available at: <http://www.pharmgkb.org/page/cpicGeneDrugPairs> [accessed Oct 22, 2012].
- Conter, V., Bartram, C. R., Valsecchi, M. G., Schrauder, A., Panzer-Grumayer, R., Moricke, A., et al. (2010). Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. *Blood* 115, 3206–3214.
- D'Avolio, A., Ciancio, A., Siccardi, M., Smedile, A., Baietto, L., Simiele, M., et al. (2012). Inosine triphosphatase polymorphisms and ribavirin pharmacokinetics as determinants of ribavirin-associate anemia in patients receiving standard anti-HCV treatment. *Ther. Drug. Monit.* 34, 165–170.
- Dervieux, T., Wessels, J. A., Kremer, J. M., Padyukov, L., Seddighzadeh, M., Saevardottir, S., et al. (2012). Patterns of interaction between genetic and non-genetic attributes and methotrexate efficacy in rheumatoid arthritis. *Pharmacogenet. Genomics* 22, 1–9.
- Dorababu, P., Nagesh, N., Linga, V. G., Gundeti, S., Kutala, V. K., Reddanna, P., et al. (2012a). Epistatic interactions between thiopurine methyltransferase (*TPMT*) and inosine triphosphate pyrophosphatase (*ITPA*) variations determine 6-mercaptopurine toxicity in Indian children with acute lymphoblastic leukemia. *Eur. J. Clin. Pharmacol.* 68, 379–387.
- Dorababu, P., Naushad, S. M., Linga, V. G., Gundeti, S., Nagesh, N., Kutala, V. K., et al. (2012b). Genetic variants of thiopurine and folate metabolic pathways determine 6-MP-mediated hematological toxicity in childhood ALL. *Pharmacogenomics* 13, 1001–1008.
- Fellay, J., Thompson, A. J., Ge, D., Gumbs, C. E., Urban, T. J., Shianna, K. V., et al. (2010). *ITPA* gene variants protect against anaemia in patients treated for chronic hepatitis C. *Nature* 464, 405–408.
- Gilbert-Diamond, D., and Moore, J. H. (2011). Analysis of gene-gene interactions. *Curr. Protoc. Hum. Genet.* Chap. 1, Unit 14.
- Hahn, L. W., Ritchie, M. D., and Moore, J. H. (2003). Multifactor dimensionality reduction software for detecting gene-gene and gene-environment interactions. *Bioinformatics* 19, 376–382.
- Johnson, J. A., Gong, L., Whirl-Carrillo, M., Gage, B. F., Scott, S. A., Stein, C. M., et al. (2011). Clinical pharmacogenetics implementation consortium guidelines for *CYP2C9* and *VKORC1* genotypes and warfarin dosing. *Clin. Pharmacol. Ther.* 90, 625–629.
- Kawedia, J. D., Kaste, S. C., Pei, D., Panetta, J. C., Cai, X., Cheng, C., et al. (2011). Pharmacokinetic, pharmacodynamic, and pharmacogenetic determinants of osteonecrosis in children with acute lymphoblastic leukemia. *Blood* 117, 2340–2347, quiz 2556.
- Kim, H., Kang, H. J., Kim, H. J., Jang, M. K., Kim, N. H., Oh, Y., et al. (2012). Pharmacogenetic analysis of pediatric patients with acute lymphoblastic leukemia: a possible association between survival rate and *ITPA* polymorphism. *PLoS ONE* 7:e45558. doi:10.1371/journal.pone.0045558
- Krishnamurthy, P., Schwab, M., Takenaka, K., Nachagari, D., Morgan, J., Leslie, M., et al. (2008). Transporter-mediated protection against thiopurine-induced hematopoietic toxicity. *Cancer Res.* 68, 4983–4989.
- Lennard, L., and Lilleyman, J. S. (1989). Variable mercaptopurine metabolism and treatment outcome in childhood lymphoblastic leukemia. *J. Clin. Oncol.* 7, 1816–1823.
- Link, E., Parish, S., Armitage, J., Bowman, L., Heath, S., Matsuda, F., et al. (2008). *SLCO1B1* variants and statin-induced myopathy – a genome wide study. *N. Engl. J. Med.* 359, 789–799.
- Lopez-Lopez, E., Martin-Guerrero, I., Ballesteros, J., Pinan, M. A., Garcia-Miguel, P., Navajas, A., et al. (2011). Polymorphisms of the *SLCO1B1* gene predict methotrexate-related toxicity in childhood acute lymphoblastic leukemia. *Pediatr. Blood Cancer* 57, 612–619.

- Marino, S., Verzeznassi, F., Tamaro, P., Stocco, G., Bartoli, F., Decorti, G., et al. (2009). Response to glucocorticoids and toxicity in childhood acute lymphoblastic leukemia: role of polymorphisms of genes involved in glucocorticoid response. *Pediatr. Blood Cancer* 53, 984–991.
- Marsh, S., and Van Booven, D. J. (2009). The increasing complexity of mercaptopurine pharmacogenomics. *Clin. Pharmacol. Ther.* 85, 139–141.
- McDonagh, E. M., Whirl-Carrillo, M., Garten, Y., Altman, R. B., and Klein, T. E. (2011). From pharmacogenomic knowledge acquisition to clinical applications: the PharmGKB as a clinical pharmacogenomic biomarker resource. *Biomark. Med.* 5, 795–806.
- Menezes, M. R., Waisertreiger, I. S., Lopez-Bertoni, H., Luo, X., and Pavlov, Y. I. (2012). Pivotal role of inosine triphosphate pyrophosphatase in maintaining genome stability and the prevention of apoptosis in human cells. *PLoS ONE* 7:e32313. doi:10.1371/journal.pone.0032313
- Moore, J. H., and Williams, S. M. (2009). Epistasis and its implications for personal genetics. *Am. J. Hum. Genet.* 85, 309–320.
- Ochi, H., Maekawa, T., Abe, H., Hayashida, Y., Nakano, R., Kubo, M., et al. (2010). ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy – a genome-wide study of Japanese HCV virus patients. *Gastroenterology* 139, 1190–1197.
- Okada, Y., Nakamura, K., Hiromura, K., Nojima, Y., Horiuchi, R., and Yamamoto, K. (2009). Pro32Thr polymorphism of inosine triphosphate pyrophosphatase gene predicts efficacy of low-dose azathioprine for patients with systemic lupus erythematosus. *Clin. Pharmacol. Ther.* 85, 527–530.
- Paugh, S. W., Stocco, G., and Evans, W. E. (2010). Pharmacogenomics in pediatric leukemia. *Curr. Opin. Pediatr.* 22, 703–710.
- Paugh, S. W., Stocco, G., McCorkle, J. R., Diouf, B., Crews, K. R., and Evans, W. E. (2011). Cancer pharmacogenomics. *Clin. Pharmacol. Ther.* 90, 461–466.
- Pinto, N., Cohn, S. L., and Dolan, M. E. (2012). Using germline genomics to individualize pediatric cancer treatments. *Clin. Cancer Res.* 18, 2791–2800.
- Pottier, N., Cheok, M. H., Yang, W., Assem, M., Tracey, L., Obenaus, J. C., et al. (2007). Expression of SMARCB1 modulates steroid sensitivity in human lymphoblastoid cells: identification of a promoter SNP that alters PARP1 binding and SMARCB1 expression. *Hum. Mol. Genet.* 16, 2261–2271.
- Pottier, N., Yang, W., Assem, M., Panetta, J. C., Pei, D., Paugh, S. W., et al. (2008). The SWI/SNF chromatin-remodeling complex and glucocorticoid resistance in acute lymphoblastic leukemia. *J. Natl. Cancer Inst.* 100, 1792–1803.
- Pui, C. H., Campana, D., Pei, D., Bowman, W. P., Sandlund, J. T., Kaste, S. C., et al. (2009). Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N. Engl. J. Med.* 360, 2730–2741.
- Pui, C. H., and Evans, W. E. (2006). Treatment of acute lymphoblastic leukemia. *N. Engl. J. Med.* 354, 166–178.
- Pui, C. H., Mullighan, C. G., Evans, W. E., and Relling, M. V. (2012). Pediatric acute lymphoblastic leukemia: where are we going and how do we get there? *Blood* 120, 1165–1174.
- Ramsey, L. B., Bruun, G. H., Yang, W., Trevino, L. R., Vattathil, S., Scheet, P., et al. (2012). Rare versus common variants in pharmacogenetics: SLCO1B1 variation and methotrexate disposition. *Genome Res.* 22, 1–8.
- Relling, M. V., Altman, R. B., Goetz, M. P., and Evans, W. E. (2010). Clinical implementation of pharmacogenomics: overcoming genetic exceptionalism. *Lancet Oncol.* 11, 507–509.
- Relling, M. V., Gardner, E. E., Sandborn, W. J., Schmiegelow, K., Pui, C. H., Yee, S. W., et al. (2011). Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clin. Pharmacol. Ther.* 89, 387–391.
- Relling, M. V., and Klein, T. E. (2011). CPIC: Clinical Pharmacogenetics Implementation Consortium of the Pharmacogenomics Research Network. *Clin. Pharmacol. Ther.* 89, 464–467.
- Relling, M. V., Pui, C. H., Cheng, C., and Evans, W. E. (2006). Thiopurine methyltransferase in acute lymphoblastic leukemia. *Blood* 107, 843–844.
- Schrapppe, M., Valsecchi, M. G., Bartram, C. R., Schrauder, A., Panzer-Grumayer, R., Moricke, A., et al. (2011). Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. *Blood* 118, 2077–2084.
- Schwab, M., and Brauch, H. (2012). *Fighting Drug Failure* [Online]. Available at: <http://www.fightingdrugfailure.net/> [accessed Oct 22, 2012].
- Stocco, G., Cheok, M. H., Crews, K. R., Dervieux, T., French, D., Pei, D., et al. (2009). Genetic polymorphism of inosine triphosphate pyrophosphatase is a determinant of mercaptopurine metabolism and toxicity during treatment for acute lymphoblastic leukemia. *Clin. Pharmacol. Ther.* 85, 164–172.
- Stocco, G., Crews, K. R., and Evans, W. E. (2010). Genetic polymorphism of inosine triphosphate-pyrophosphatase influences mercaptopurine metabolism and toxicity during treatment of acute lymphoblastic leukemia individualized for thiopurine-S-methyl-transferase status. *Expert Opin. Drug Saf.* 9, 23–37.
- Stocco, G., Yang, W., Crews, K. R., Thierfelder, W. E., Decorti, G., Londero, M., et al. (2012). PACSIN2 polymorphism influences TPMT activity and mercaptopurine-related gastrointestinal toxicity. *Hum. Mol. Genet.* 21, 4793–4804.
- Swen, J. J., Nijenhuis, M., De Boer, A., Grandia, L., Maitland-Van Der Zee, A. H., Mulder, H., et al. (2011). Pharmacogenetics: from bench to byte – an update of guidelines. *Clin. Pharmacol. Ther.* 89, 662–673.
- Swen, J. J., Wilting, I., De Goede, A. L., Grandia, L., Mulder, H., Touw, D. J., et al. (2008). Pharmacogenetics: from bench to byte. *Clin. Pharmacol. Ther.* 83, 781–787.
- Tanaka, Y., Manabe, A., Nakadate, H., Kondoh, K., Nakamura, K., Koh, K., et al. (2012). The activity of the inosine triphosphate pyrophosphatase affects toxicity of 6-mercaptopurine during maintenance therapy for acute lymphoblastic leukemia in Japanese children. *Leuk. Res.* 36, 560–564.
- Thompson, A. J., Fellay, J., Patel, K., Tillmann, H. L., Naggie, S., Ge, D., et al. (2010). Variants in the ITPA gene protect against ribavirin-induced hemolytic anemia and decrease the need for ribavirin dose reduction. *Gastroenterology* 139, 1181–1189.
- Trevino, L. R., Shimasaki, N., Yang, W., Panetta, J. C., Cheng, C., Pei, D., et al. (2009). Germline genetic variation in an organic anion transporter polypeptide associated with methotrexate pharmacokinetics and clinical effects. *J. Clin. Oncol.* 27, 5972–5978.
- von Ahlsen, N., Oellerich, M., and Armstrong, V. W. (2008). Characterization of the inosine triphosphatase (ITPA) gene: haplotype structure, haplotype-phenotype correlation and promoter function. *Ther. Drug Monit.* 30, 16–22.
- Wheeler, H. E., and Dolan, M. E. (2012). Lymphoblastoid cell lines in pharmacogenomic discovery and clinical translation. *Pharmacogenomics* 13, 55–70.
- Yamamoto, K., Okada, Y., Nakamura, K., Hiromura, K., Nojima, Y., and Nakamura, T. (2010). Inosine triphosphate pyrophosphatase 94C>A polymorphism: clinical implications for patients with systemic lupus erythematosus treated with azathioprine. *Expert Opin. Drug Saf.* 9, 447–457.
- Yang, J. J., Cheng, C., Devidas, M., Cao, X., Campana, D., Yang, W., et al. (2012). Genome-wide association study identifies germline polymorphisms associated with relapse of childhood acute lymphoblastic leukemia. *Blood* 120, 4197–4204.
- Yang, J. J., Cheng, C., Devidas, M., Cao, X., Fan, Y., Campana, D., et al. (2011). Ancestry and pharmacogenomics of relapse in acute lymphoblastic leukemia. *Nat. Genet.* 43, 237–241.
- Yang, J. J., Cheng, C., Yang, W., Pei, D., Cao, X., Fan, Y., et al. (2009). Genome-wide interrogation of germline genetic variation associated with treatment response in childhood acute lymphoblastic leukemia. *JAMA* 301, 393–403.
- Zaza, G., Cheok, M., Krynetskaia, N., Thorn, C., Stocco, G., Hebert, J. M., et al. (2010). Thiopurine pathway. *Pharmacogenet. Genomics* 20, 573–574.

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

Received: 22 October 2012; accepted: 12 December 2012; published online: 07 January 2013.

Citation: Stocco G, Franca R, Verzeznassi F, Londero M, Rabusin M and Decorti G (2013) Multilocus genotypes of relevance for drug metabolizing enzymes and therapy with thiopurines in patients with acute lymphoblastic leukemia. *Front. Gene.* 3:309. doi: 10.3389/fgene.2012.00309

This article was submitted to *Frontiers in Pharmacogenetics and Pharmacogenomics*, a specialty of *Frontiers in Genetics*.

Copyright © 2013 Stocco, Franca, Verzeznassi, Londero, Rabusin and Decorti. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

# Research Highlights

Highlights from the latest articles in acute lymphoblastic leukemia pharmacogenomics



## Systematic identification of host genomic variation related to treatment outcome of childhood acute lymphoblastic leukemia

Gabriele Stocco<sup>\*1</sup>,  
Raffaella Franca<sup>2</sup>,  
Margherita Londero<sup>3,4</sup>  
& Giuliana Decorti<sup>1</sup>

<sup>1</sup>Department of Life Sciences, University of Trieste, Trieste, Italy

<sup>2</sup>Institute for Maternal & Child Health IRCCS Burlo Garofolo, Trieste, Italy

<sup>3</sup>Scuola di Dottorato di Ricerca in Scienze della Riproduzione, University of Trieste, Trieste, Italy

<sup>4</sup>Ospedale di San Daniele, Azienda per i Servizi Sanitari 4, San Daniele del Friuli, Udine, Italy

\*Author for correspondence:

Tel.: +39 040 558 8634

Fax: +39 040 558 8634

stocceg@units.it

**Evaluation of:** Yang JJ, Cheng C, Devidas M *et al.* Genome-wide association study identifies germline polymorphisms associated with relapse of childhood acute lymphoblastic leukemia. *Blood* 120(20), 4197–4204 (2012).

The use of risk-directed chemotherapy for childhood acute lymphoblastic leukemia (ALL) has improved the success of therapy dramatically in the last 40 years; however, a substantial proportion of patients experience relapse and many of these have no known risk factors. Therefore, more accurate risk classification of newly diagnosed disease is needed to reduce relapses and improve the overall outcome. This study by Yang *et al.* aimed at agnostically testing, with a genome-wide approach, genotypes at 444,044 SNPs for their association with the risk of relapse in 2535 children with newly diagnosed ALL, the largest group of children with cancer ever studied for genetic determinants of outcome [1]. Germline DNA was collected at remission in children with ALL treated according to St Jude Children's Research Hospital (TN, USA) Total XIIIIB and XV protocols and the Children's Oncology Group (COG) P9906 or P9904/9905 studies [2]. Relapse was defined as disease recurrence in bone marrow and/or extramedullary sites. All genotype–relapse associations were stratified by the risk-adapted treatment arms of the various protocols considered and adjusted by patient characteristics at

diagnosis that are known to be risk factors (i.e., leukocyte count, age, molecular ALL subtypes, DNA index and minimal residual disease). Prognostic features of ALL are strongly dependent upon treatment; therefore, to maximize the identification of genotypes associated with relapse with an effect reproducible across diverse treatment regimens, an iterative two-step approach was used [3]: the cohort of patients was randomly split in a 1:1 ratio into a discovery and replication cohort, considering a congruent composition of each cohort in terms of genetic ancestry and treatment outcome. SNP significance was tested in both the discovery and replication group and the procedure was repeated 100-times, with each iteration generating a list of replicated SNPs: those replicated at least ten-times exceeded the frequency expected by chance and were designated as 'relapse SNPs'. With this approach, the authors identified 134 SNPs reproducibly associated with ALL relapse: these SNPs were all intronic except a synonymous coding variant in the *ATP8A2* gene (rs6491066). Of these 134 SNPs, 133 remained prognostic after adjusting for all known relapse risk factors and 111 were significant even among patients who achieved negative minimal residual disease after remission induction therapy. The C allele at rs7142143 in the *PYGL* gene was associated with 3.6-fold-higher risk of relapse than the T allele: this gene encodes glycogen phosphorylase, a target of AMP, which plays a critical role in response to antileukemic agents such as mercaptopurine and methotrexate [4].

### Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Future  
Medicine part of





All 134 relapse SNPs were selected for further analysis with additional prognostic phenotypes. In particular, the authors checked the effect of prognostic patients' characteristics at diagnosis and a majority of relapse SNPs (73 of 134) were related to one or more clinical presenting features, although all the SNPs remained prognostic after adjusting for those features. Moreover, the authors examined the association of the relapse SNPs with four pharmacokinetic and pharmacodynamic phenotypes: methotrexate plasma clearance, methotrexate polyglutamate concentrations, dexamethasone plasma

clearance and asparaginase antibody levels. Fourteen of the 134 SNPs, including variants in the *ABCB1* and *PDE4B* genes, were also associated with anti-leukemic drug pharmacokinetics and/or pharmacodynamics.

In conclusion, this study identified host genetic variations related to treatment outcome of childhood ALL, most of which were prognostic independent of known risk factors for relapse and some also influenced outcome by affecting host disposition of anti-leukemic drugs. These important insights into the molecular mechanism of poor outcome for anti-leukemic therapy could be the basis for further investigations needed to fully clarify the contribution of these genetic features on the phenotypes investigated.

## References

- 1 Yang JJ, Cheng C, Devidas M *et al.* Genome-wide association study identifies germline polymorphisms associated with relapse of childhood acute lymphoblastic leukemia. *Blood* 120(20), 4197–4204 (2012).
- 2 Borowitz MJ, Devidas M, Hunger SP *et al.* Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. *Blood* 111(12), 5477–5485 (2008).
- 3 Freidlin B, Jiang W, Simon R. The cross-validated adaptive signature design. *Clin. Cancer Res.* 16(2), 691–698 (2010).
- 4 Holleman A, Cheek MH, den Boer ML *et al.* Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *N. Engl. J. Med.* 351(6), 533–542 (2004).

# Processes for incorporation of pharmacogenetic tests and interpretations in medical records for clinical practice

**Evaluation of:** Hicks JK, Crews KR, Hoffman JM *et al.* A clinician-driven automated system for integration of pharmacogenetic interpretations into an electronic medical record. *Clin. Pharmacol. Ther.* 92(5), 563–566 (2012).

For several high-risk drugs, such as codeine or mercaptopurine, gene-based dosing guidelines have been developed, which translate laboratory test results into actionable prescribing decisions for these medications [1,2]. These guidelines are based on consolidated evidence connecting specific variants of genes involved in the pharmacokinetics and pharmacodynamics of a medication with clinical outcome, in terms of drug efficacy or toxicity; this therefore identifies the genetic status of the individual, represented by a diplotype, at these loci of pharmacogenetic relevance, which can be translated into a predicted clinical phenotype of drug response [2].

Clinical recommendations for the use of concerned drugs on the basis of patients' genotypes can therefore be provided either passively, as pharmacogenetic consultations,

or actively, as automated alerts that reach the clinician at the point of care. As pharmacogenetic knowledge advances, the number of clinically important genetic variants is continuously increasing, hence the correct communication and interpretation of these test results in the clinics becomes of critical importance. Computational tools that integrate directly into the electronic medical record (EMR) are therefore needed to translate the huge amount of genetic information into useful data that should facilitate gene-based drug prescribing [2].

Hicks *et al.* have recently developed a modular and modifiable system for construction of pharmacogenetic consultation templates, coupled with a system for point-of-care alerts, ready for implementation in patients' EMR [3]. The authors share their preliminary experience from the St Jude Children's Research Hospital (TN, USA) PG4KDS protocol, aimed at pre-emptively migrating array-based genotypes for clinically relevant genes into each patient's EMR. According to the study protocol, patients' DNA is genotyped using a commercial platform, the Affymetrix® DMET™ Plus array (Affymetrix, CA, USA), which probes for 1936 genomic

variants in 225 genes, supplemented with a *CYP2D6* copy-number assay. To organize the results of the genotyping, for each patient, 225 individual files containing results for all genes analyzed are generated and collected in a database. Data from two important pharmacogenes, *CYP2D6* and *TPMT*, have been chosen in this pilot study for selective migration into the EMR. Genomic variants assayed in the study have been translated into 29 *CYP2D6* alleles and nine *TPMT* alleles. Translation tables for *CYP2D6* and *TPMT* have been created, which relate each diplotype to a phenotype describing the activity status of the gene product, on the basis of the allele functionality, as reported in the literature [1,2,4]. Clinical priority status has also been organized and categorized as routine (normal) or high priority (high risk or abnormal). High-risk diplotypes are associated with a problem list entry and with rules for alerting clinicians of a priority pharmacogenetic test result at the point of care, when a high-risk drug is about to be prescribed. On this basis, a total of 187 diplotype-specific *CYP2D6* pharmacogenetic consultation templates and 31 diplotype-specific *TPMT* templates have been created. The *CYP2D6*



templates are built from the combination of five modular sections; that is, phenotype, diplotype interpretation, dosing recommendations, activity score and an educational link, and each section has multiple versions. The *TPMT* templates consist of four modular sections: phenotype, diplotype interpretation, dosing recommendations and an educational link. Diploidy results and pharmacogenetic consultations, after approval by a trained clinician, are made accessible via a customized 'pharmacogenetics' tab directly present in the EMR.

The pilot study enrolled 200 patients for which 37 test results were of high-priority status and were therefore highlighted to differentiate from routine results; in addition, these abnormal phenotypes were placed into the problem list of the EMR.

The authors conclude that this modular approach for creating pharmacogenetic interpretations has several advantages: as new data emerge, modifications need to be made only once in the database to alter all affected templates and the system can easily be expanded to include additional genes and drugs [3]. This automated system for integration of pharmacogenetic data into the EMR should have a positive effect in clinical practice, facilitating gene-based drug prescribing.

### References

- 1 Crews KR, Gaedigk A, Dunnenberger HM *et al.* Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines for codeine therapy in the context of cytochrome P450 2D6 (*CYP2D6*) genotype. *Clin. Pharmacol. Ther.* 91(2), 321–326 (2012).
- 2 Relling MV, Gardner EE, Sandborn WJ *et al.* Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clin. Pharmacol. Ther.* 89(3), 387–391 (2011).
- 3 Hicks JK, Crews KR, Hoffman JM *et al.* A clinician-driven automated system for integration of pharmacogenetic interpretations into an electronic medical record. *Clin. Pharmacol. Ther.* 92(5), 563–566 (2012).
- 4 Gaedigk A, Simon SD, Pearce RE *et al.* The *CYP2D6* activity score: translating genotype information into a qualitative measure of phenotype. *Clin. Pharmacol. Ther.* 83(2), 234–242 (2008).

## *ITPA* genetic polymorphism is possibly associated with survival rate in Korean children with acute lymphoblastic leukemia

**Evaluation of:** Kim H, Kang HJ, Kim HJ *et al.* Pharmacogenetic analysis of pediatric patients with acute lymphoblastic leukemia: a possible association between survival rate and *ITPA* polymorphism. *PLoS ONE* 7(9), e45558 (2012).

The study by Kim *et al.* is the first pharmacogenetic study of Korean children affected by acute lymphoblastic leukemia (ALL) [1]. According to institutional experience at the Seoul National University Hospital (Seoul, Korea), many patients could not tolerate full dosages of drugs used in western ALL protocols, showing in particular a reduced clinical tolerance to 6-mercaptopurine (6-MP). This study analyzed 100 patients (median age: 5.2 years; range: 1.4–16 years) treated with protocols adapted from the Children's Cancer Group (CCG-1881, CCG-1891 or CCG-1952 [2–4]) with 6-MP given at 50 instead of 75 mg/m<sup>2</sup>/day during

maintenance. Germline DNA was genotyped for 18 loci considered predictive of ALL therapy drug response and/or toxicity in previous studies on other ethnic populations: genetic variants analyzed were the 313A>G SNP in *GST-P1*, *CYP3A4\*1B*, *CYP3A5\*3*, 2677G>T/A (exon 21) and 3435C>T (exon 26) in *MDR1*, *FokI* (start-site) T>C and intron 8 G>A in *VDR*, 1088A>G in *NR3C1*, SNPs 238G>C, 460G>A and 719A>G in *TPMT*, 94C>A in *ITPA*, 80G>A in *RFC1*, 677C>T, 1298A>C in *MTHFR* and the enhancer repeats in *TYMS*. Genotype frequencies of ten and three loci were statistically different from those in western Caucasians and in Japanese, respectively. The *ITPA* 94AC/AA variant genotypes were the only independent risk factor for lower event-free survival in multivariate analysis (hazard ratio: 4.96; 95% CI: 1.1–22.7; p = 0.039), with events defined as any relapse, death or secondary malignancies. *TPMT* was not significantly associated with outcome.

The authors proposed that the influence of *ITPA* rather than *TPMT* on survival could be explained by the ethnic-specific genotype distribution [5]: Asians who have a low frequency of *TPMT* variant alleles may be more susceptible to the influence of *ITPA* variant alleles, while *TPMT* may have a predominant effect in Caucasians. However, in the study population, there was only a trend for *ITPA* for increased frequency in comparison to Caucasians.

The authors also evaluated the 6-MP and methotrexate dose percentage in the last maintenance chemotherapy cycle as a measure of the maximum tolerated dose: only 26 and 35% of patients could receive more than 75% of the planned doses, respectively. Median dose percentages of both drugs did not significantly differ by genotype in the 18 loci considered; however, as expected, carriers of *TPMT* variants (*TPMT*-deficient subjects) showed lower dose percentage than wild-type individuals (30.5 vs 50%, respectively); this is probably because they form higher concentrations of



the thioguanine nucleotides and are more susceptible to acute hematological toxicity at standard doses [6,7]. Of note, the dose of the single *TPMT* variant homozygote patient was not the lowest. No correlation was found between polymorphisms and toxicity (grade IV sepsis during total treatment, and grade III–IV hepatotoxicity and febrile neutropenia during maintenance). However, the statistical analysis did not consider the effect of the length of treatment, which is particularly prolonged in ALL therapy and could be related to cumulative adverse effects.

## References

- 1 Kim H, Kang HJ, Kim HJ *et al.* Pharmacogenetic analysis of pediatric patients with acute lymphoblastic leukemia: a possible association between survival rate and *ITPA* polymorphism. *PLoS ONE* 7(9), e45558 (2012).
- 2 Hutchinson RJ, Gaynon PS, Sather H *et al.* Intensification of therapy for children with lower-risk acute lymphoblastic leukemia: long-term follow-up of patients treated on Children's Cancer Group Trial 1881. *J. Clin. Oncol.* 21(9), 1790–1797 (2003).
- 3 Lange BJ, Bostrom BC, Cherlow JM *et al.* Double-delayed intensification improves event-free survival for children with intermediate-risk acute lymphoblastic leukemia: a report from the Children's Cancer Group. *Blood* 99(3), 825–833 (2002).
- 4 Broxson EH, Dole M, Wong R *et al.* Portal hypertension develops in a subset of children with standard risk acute lymphoblastic leukemia treated with oral 6-thioguanine during maintenance therapy. *Pediatr. Blood Cancer* 44(3), 226–231 (2005).
- 5 Marsh S, van Booven DJ. The increasing complexity of mercaptopurine pharmacogenomics. *Clin. Pharmacol. Ther.* 85(2), 139–141 (2009).
- 6 Cheok MH, Evans WE. Acute lymphoblastic leukaemia: a model for the pharmacogenomics of cancer therapy. *Nat. Rev. Cancer* 6(2), 117–129 (2006).
- 7 Adam de Beaumais T, Jacqz-Aigrain E. Pharmacogenetic determinants of mercaptopurine disposition in children with acute lymphoblastic leukemia. *Eur. J. Clin. Pharmacol.* 68(9), 1233–1242 (2012).

# DMET™ Plus array delivers results in good concordance with those of several lower-throughput genotyping methods in patient samples

**Evaluation of:** Fernandez CA, Smith C, Yang W *et al.* Concordance of DMET Plus genotyping results with those of orthogonal genotyping methods. *Clin. Pharmacol. Ther.* 92(3), 360–365 (2012).

Pharmacogenetics has the potential to improve medication use and drive the individualization of therapy through the pre-emptive detection of clinically significant genetic variants. The development of high-throughput array-based genotyping methods allows for simultaneous interrogation of a large number of variants in genes influencing the absorption, distribution, metabolism and elimination of drugs, such as those coding for metabolizing enzymes and drug transporters. Fernandez *et al.* [1] made a comparison between the novel DMET™ Plus array (Affymetrix, CA, USA) [2,3] and six other orthogonal genotyping methods in 220 pediatric patients enrolled on the PGEN5 protocol at St Jude Children's Research Hospital (TN, USA). The DMET Plus array genotypes

comprise both rare and common variants, biallelic and triallelic SNPs, copy-number variations and insertion/deletions, for a total of 1931 variants in 225 absorption, distribution, metabolism and elimination genes. Methodologically, it uses molecular inversion probe technology to amplify the sequence-specific targets at each polymorphism, then PCR products undergo enzymatic fragmentation and end-labeling followed by the hybridization to an array containing allele-specific oligonucleotides. The six orthogonal methods used for comparison were the Affymetrix Human Mapping 500K Array Set, the Affymetrix Genome-Wide Human SNP Array 6.0, the custom-designed Illumina® GoldenGate assay (Illumina, CA, USA), the iPLEX Gold assay on the MassARRAY® platform from Sequenom® (CA, USA)- and the Beckman Coulter GenomeLab™ SNPstream® (Beckman Coulter, CA, USA); major nonfunctional alleles of *TPMT* (\*2, \*3A and \*3C) were determined by a clinical reference laboratory (Prometheus Labs, CA, USA) using a TaqMan® (Invitrogen, CA, USA) probe.

In this study, the accuracy of DMET Plus array performance was determined for 1692 out of 1931 variants that passed the quality control criteria (call rates >98 and Hardy–Weinberg equilibrium at a p-value >0.001). Among these, the minor allele frequency of SNPs from patients of European ancestry was highly correlated with that in HapMap CEPH (Utah residents with ancestry from northern and western Europe) samples ( $R^2 = 0.9711$ ), giving further evidence of accuracy. Of the selected 1692 variants, 259 SNPs in genes coding for CYP450 and non-CYP450 metabolizing enzymes and drug transporters were genotyped by at least one independent method, and a total of 19,942 SNP–patient sample pairs were evaluated. The concordance rate was 99.9%, with only 28 genotype discordances observed. The DMET Plus array includes 164 SNPs in the eight genes deemed to be clinically relevant (*TPMT*, *CYP2D6*, *CYP2C19*, *CYP2C9*, *VKORC1*, *DPYD*, *UGT1A1* and *SLCO1B1*): 148 SNPs (90%) passed quality control and 46 of those were genotyped using at least one orthogonal method. A total



of 3799 SNP–patient sample pairs were evaluated within these eight genes, and there was an overall concordance rate of 99.96%

Authors conclude that the DMET Plus array performs well with primary patient samples, with results in good concordance with those of several lower-throughput genotyping methods.

---

## References

- 1 Fernandez CA, Smith C, Yang W *et al.* Concordance of DMET plus genotyping results with those of orthogonal genotyping methods. *Clin. Pharmacol. Ther.* 92(3), 360–365 (2012).
- 2 Deeken J. The Affymetrix DMET platform and pharmacogenetics in drug development. *Curr. Opin. Mol. Ther.* 11(3), 260–268 (2009).
- 3 Sissung TM, English BC, Venzon D, Figg WD, Deeken JF. Clinical pharmacology and pharmacogenetics in a genomics era: the DMET platform. *Pharmacogenomics* 11(1), 89–103 (2010).

# Pharmacogenomic Approaches for Tailored Anti-Leukemic Therapy in Children

G. Stocco<sup>\*1</sup>, R. Franca<sup>2</sup>, F. Verzegnassi<sup>2</sup>, M. Londero<sup>3,4</sup>, M. Rabusin<sup>2</sup> and G. Decorti<sup>1</sup>

<sup>1</sup>Department of Life Sciences, University of Trieste, Trieste, Italy; <sup>2</sup>Institute for Maternal and Child Health - IRCCS "Burlo Garofolo", Trieste, Italy; <sup>3</sup>"Scuola di Dottorato di Ricerca in Scienze della Riproduzione", University of Trieste, Trieste, Italy; <sup>4</sup>Pediatric Care Unit, "Sant'Antonio" General Hospital, San Daniele del Friuli, Udine, Italy

**Abstract:** Several lympholytic and cytotoxic agents are used in acute lymphoblastic leukemia (ALL) polychemotherapy. Genetic variants for cellular components involved in the pharmacokinetics and pharmacodynamics of these drugs can influence the pharmacological response, and molecular characterization of these genetic variants could be helpful for the comprehension of the mechanisms of resistance or increased sensitivity. The purpose of this review is to carry out an update of recent publications on genes that might influence ALL treatment in terms of outcome and/or toxicity and to underlie the role of genetic variants, particularly single nucleotide polymorphisms (SNP), in predicting clinical response, with particular reference to the current protocol for ALL therapy used in Italy, AIEOP-BFM ALL 2009.

**Keywords:** Acute lymphoblastic leukemia, chemotherapy, pharmacogenetics.

## INTRODUCTION

Acute lymphoblastic leukemia (ALL) polychemotherapy comprises glucocorticoids (GCs), antimetabolites, asparaginase (ASNase), alkylating agents, antimitotic drugs and antibiotics combined together at different dosages and timing according to the patient's class of risk. Any of the cellular components directly or indirectly involved in the pharmacokinetics and pharmacodynamics of these agents could potentially be a carrier of a genetic variant affecting the pharmacological response. The molecular characterization of these genetic variants could be helpful for the comprehension of the mechanism of resistance or increased sensitivity in ALL patients.

In Italy, childhood ALL is treated according to official guidelines provided by the Associazione Italiana di Ematologia ed Oncologia Pediatrica (AIEOP) and the protocol currently in use is the AIEOP-Berlin-Frankfurt-Munster (BFM) ALL 2009 (ClinicalTrials.gov identifier NCT01117441, <http://clinicaltrials.gov>). The chemotherapy is organized in phases: a remission/induction, followed by a consolidation and then by a continuation/maintenance treatment to eliminate residual leukemic cells [1]. A schematic representation of the treatment phases is presented in (Figs. 1 and 2).

The purpose of this review is to carry out an update of recent publications on genes that might influence ALL treatment in terms of outcome and/or toxicity and to underlie the role of genetic variants, particularly single nucleotide polymorphisms (SNPs), in predicting ALL clinical response [2].

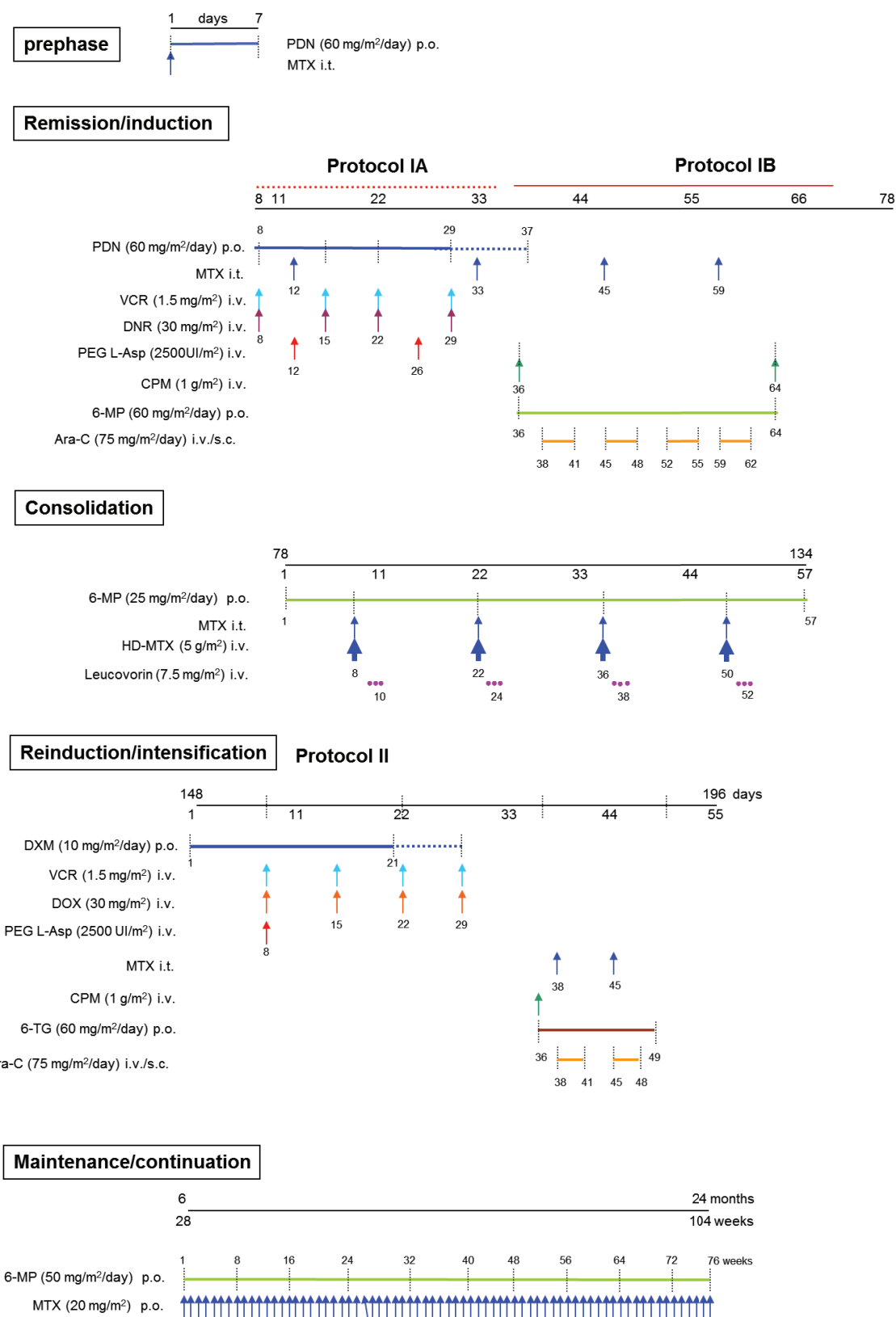
## THIOPURINES

The thiopurines, 6-mercaptopurine (6MP) and 6-thioguanine (6TG), are the backbone of pediatric ALL treatment worldwide. In the AIEOP-BFM ALL 2009 protocol, 6MP is administered daily for about 8 weeks during consolidation therapy at a dose of 25 mg/m<sup>2</sup>/day, associated with biweekly high-dose infusions of methotrexate (MTX, 5 g/m<sup>2</sup>), while during maintenance therapy 6MP it is used for about two years at a dose of 50 mg/m<sup>2</sup>/day combined with relatively low doses of oral MTX (20 mg/m<sup>2</sup>/week) (Fig. 1). 6MP (60 mg/m<sup>2</sup>/day for 4 weeks) is also used in the induction phase, while 6TG (60 mg/m<sup>2</sup>/day for 2 weeks) is preferred in the reinduction protocols.

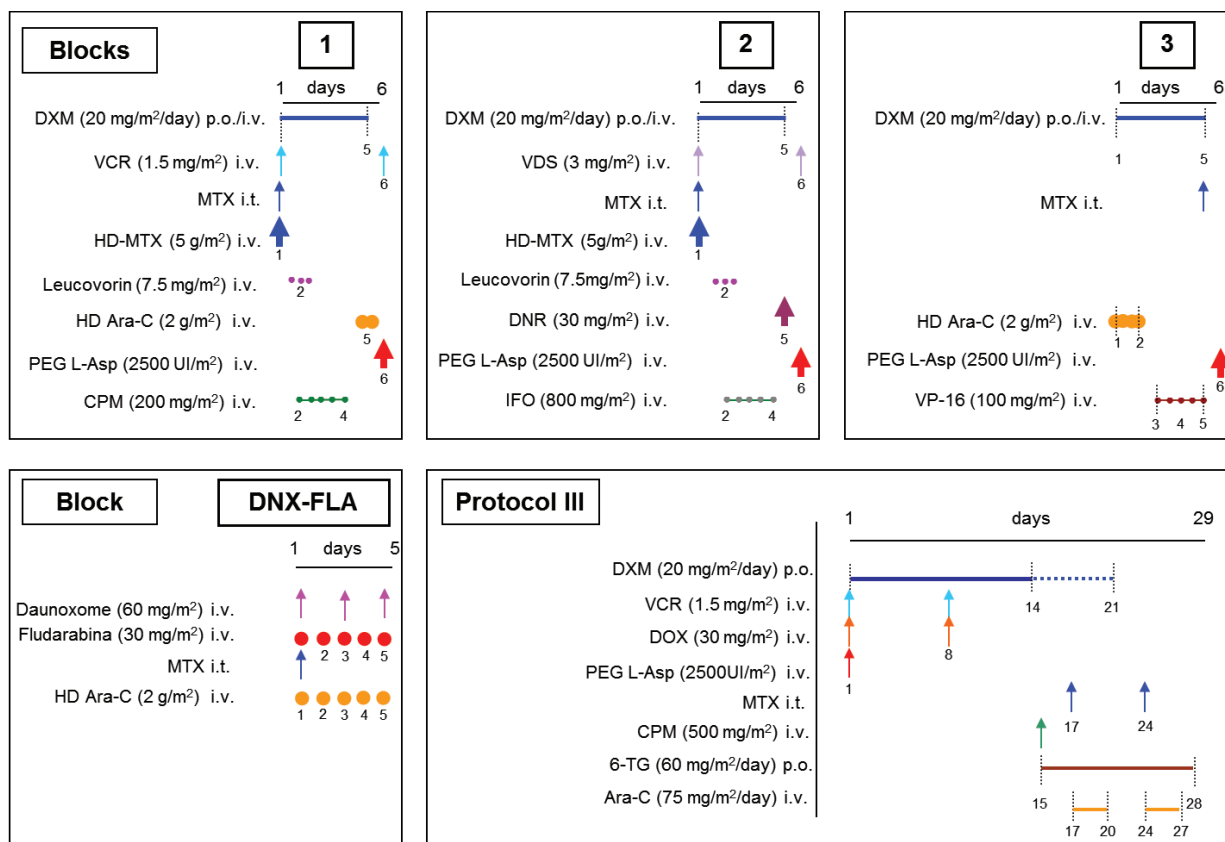
To exert the antileukemic effects both 6MP and 6TG have to be converted to the active thioguanine nucleotides (TGN) by enzymes of the purine salvage pathway, starting with hypoxanthine phosphoribosyl transferase (HPRT). In white blood cells thiopurines are inactivated by the enzyme thiopurine-S-methyl transferase (TPMT), which catalyzes the methylation of the thiol moiety, preventing their activation by HPRT [3]. TPMT activity displays inter-individual differences due to common genetic polymorphisms. Patients that inherit variant inactive alleles show increased production of active TGN metabolites and, consequently, an increased risk of adverse events during treatment with conventional doses of thiopurines. Recently pharmacogenetic-based guidelines have been developed to provide dosing recommendations for 6MP and 6TG according to TPMT status in patients with ALL (Table 1) [4].

As mentioned above, during consolidation therapy 6MP is administered in the AIEOP-BFM ALL 2009 at a daily dose of 25 mg/m<sup>2</sup>; however, in a recent study, performed in 38 children with ALL during post remission treatment with

\*Address correspondence to this author at the Department of Life Sciences, University of Trieste, Via Fleming 22, Trieste, Italy, 34127; Tel/Fax: 39 040 5588634; E-mail: stoccog@units.it



**Fig. (1).** Scheme of treatment phases for current standard AIEOP-BFM ALL 2009. Details for the treatment phases are provided for each medication in the text of the manuscript and are available online (ClinicalTrials.gov identifier NCT01117441, <http://clinicaltrials.gov>). 6-MP = mercaptopurine, 6-TG = 6-thioguanine, Ara-C = cytarabine, CPM = cyclophosphamide, DNR = daunorubicin, DOX = doxorubicin, DXM = dexamethasone, HD-MTX = high dose methotrexate, MTX = methotrexate, PDN = prednisone, PEG-L-ASP = PEG-asparaginase, VCR = vincristine.



**Fig. (2).** Scheme of blocks and reinduction phases for high-risk AIEOP-BFM ALL 2009. Prephases and Protocol IA-IB are identical to the current standard protocol (Fig. 1), with the exception of randomized additional weekly administration of PEG-L-ASP in phase IB. In the high-risk patients, Blocks I-II-III replace the Consolidation therapy and Protocol III is repeated three times intercalated by 29 days of ad interim maintenance therapy with daily 6-MP 50 mg/m<sup>2</sup> and weekly MTX 20 mg/m<sup>2</sup>. 6-TG = 6-thioguanine, Ara-C = cytarabine, CPM = cyclophosphamide, DNR = daunorubicin, DOX = doxorubicin, DXM = dexamethasone, HD Ara-C = high dose cytarabine, HD-MTX = high dose methotrexate, IFO = ifosfamide, MTX = methotrexate, PEG-L-ASP = PEG-asparaginase, VCR = vincristine, VDS = vindesine, VP16 = etoposide.

high-dose MTX, according to the Nordic Society of Paediatric Haematology and Oncology ALL 2008 protocol, 6MP dose could be increased from 25 mg/m<sup>2</sup> to 75 mg/m<sup>2</sup>, on the basis of the clinical response, with no increase in toxicity in 81% of patients [5]. This pilot study indicates that in current protocols using standard 6MP consolidation treatment of 25 mg/m<sup>2</sup>/day, only one-third of patients are treated with the maximum tolerated dose. Since the cumulative 6MP dose has been shown to be an important determinant of survival in pediatric ALL [6], a titrated approach for increased doses of 6MP during consolidation therapy could be considered even for the Italian protocol AIEOP-BFM ALL 2009.

Various clinical trials have shown that the efficacy of 6MP and 6TG are comparable in maintaining remission of pediatric ALL [7, 8]; however, prolonged treatment with 6TG is associated with the development of a severe idiosyncratic adverse event: a hepatic veno-occlusive disease (VOD), also known as sinusoidal obstructive syndrome or nodular regenerative hyperplasia [7, 8]. For this reason, 6TG is not commonly used for prolonged treatment, such as consolidation or maintenance therapy. However, current clinical protocols use 6TG for short period of time at the end of reinduction therapy (60 mg/m<sup>2</sup>/day for two weeks). A mouse model to study VOD has been recently developed [9]: it con-

sists of C57Bl/6 mice with specific genes altered to elucidate the molecular mechanisms responsible for VOD. This model confirmed that only 6TG and not 6MP or its methylated metabolites caused VOD, in dose related manner. VOD did not occur in HPRT-deficient mice, demonstrating that this complication requires conversion to TGNs. Mice deficient in P- and E-selectins on the surface of vascular endothelial cells showed markedly reduced incidence of VOD, demonstrating a major role for blood leukocytes. Splitting the dose of 6TG in two daily administrations instead of the standard single one markedly attenuated the incidence of VOD without compromising immunosuppressive activity. The authors suggested that VOD related to 6TG administration may be avoided by either inhibition of endothelial activation or by simple changes in 6TG dosing regimens [9].

TPMT genetic polymorphism has been generally associated with dose dependent bone marrow toxicity due to 6MP administration [10]; however, it has been reported that patients with reduced TPMT may show increased probability of VOD during treatment with 6TG. Indeed, Lennard *et al.* have shown that, among 1492 children with ALL randomized to be treated with either 6TG or 6MP, 11% of those treated with 6TG and none of those treated with 6MP, presented VOD, with no improvement in event-free survival

**Table 1. Recommended Dosing of Thiopurines by Thiopurine Methyltransferase Phenotype. (See Reference 4)**

TPMT Status	6MP		6TG	
	Effects on 6MP Metabolism	Dosing Recommendations for 6MP	Effects on 6TG Metabolism	Dosing Recommendations for 6TG
Homozygous wild-type or normal, high activity	Lower concentrations of 6TGN metabolites, higher methyl-TIMP, this is the “normal” pattern	Start with normal starting dose (e.g., 75 mg/m <sup>2</sup> /day) and adjust doses of 6MP (and of any other myelosuppressive therapy) without any special emphasis on 6MP compared to other agents.	Lower concentrations of 6TGN metabolites, but note that 6TGN after 6TG are 5–10 higher than 6TGN after 6MP or azathioprine	Start with normal starting dose. Adjust doses of 6TG and of other myelosuppressive therapy without any special emphasis on 6TG. Allow 2 weeks to reach steady state after each dose adjustment.
Heterozygote or intermediate activity	Moderate to high concentrations of 6TGN metabolites; low concentrations of methyl-TIMP	Start with reduced doses (start at 30–70% of full dose: e.g., at 50 mg/m <sup>2</sup> /day or 0.75 mg/kg/day) and adjust doses of 6MP based on degree of myelosuppression and disease-specific guidelines. In those who require a dosage reduction based on myelosuppression, the median dose may be ~40% lower (44 mg/m <sup>2</sup> /day) than that tolerated in wild-type patients (75 mg/m <sup>2</sup> /day). In setting of myelosuppression, and depending on other therapy, emphasis should be on reducing 6MP over other agents.	Moderate to high concentrations of 6TGN metabolites; but note that 6TGN after 6TG are 5–10 higher than 6TGN after 6MP or azathioprine	Start with reduced doses (reduce by 30–50%) and adjust doses of 6TG based on degree of myelosuppression and disease-specific guidelines. In setting of myelosuppression, and depending on other therapy, emphasis should be on reducing 6TG over other agents.
Homozygous variant, mutant, low, or deficient activity	Extremely high concentrations of 6TGN metabolites; fatal toxicity possible without dose decrease; no methyl-TIMP metabolites	Start with drastically reduced doses (reduce daily dose by 10-fold and reduce frequency to thrice weekly instead of daily, e.g., 10 mg/m <sup>2</sup> /day given just 3 days/week) and adjust doses of 6MP based on degree of myelosuppression and disease-specific guidelines. In setting of myelosuppression, emphasis should be on reducing 6MP over other agents.	Extremely high concentrations of 6TGN metabolites; fatal toxicity possible without dose decrease	Start with drastically reduced doses (reduce daily dose by 10-fold and dose thrice weekly instead of daily) and adjust doses of 6TG based on degree of myelosuppression and disease-specific guidelines. In setting of myelosuppression, emphasis should be on reducing 6TG over other agents.

6MP, mercaptopurine; 6TG, thioguanine; 6TGN, thioguanine nucleotide; TIMP, secondary metabolite of 6MP; TPMT, thiopurine-S-methyl transferase (see reference 4).

rate. TPMT activity was significantly lower in children which developed 6TG-related VOD, potentially providing a mean of identifying at-risk patients [8].

Interestingly, some patients with normal TPMT still present thiopurine-related adverse events: other factors therefore, such as clinical, environmental and genetic ones, may have a role in the determination of these adverse events. Among the additional genetic factors, it has been shown that polymorphisms of inosine-triphosphate-pyrophosphatase (ITPA), an enzyme involved in 6MP metabolism [3] and in particular rs1127354, a SNP determining reduced ITPA ac-

tivity [11], are associated with the cumulative incidence of grade 3-4 febrile neutropenia in patients receiving maintenance therapy that includes 6MP doses individualized for TPMT. Moreover, it has been documented that patients who have inherited a non-functional ITPA allele show significantly higher concentrations of the methylated nucleotide metabolites of 6MP (MMPN) in leukemia cells and erythrocytes [12, 13]. A recent study has replicated the observation of the combined effects of TPMT and ITPA genotypes on 6MP pharmacokinetics: among 66 children with ALL, treated according to the EORTC 58951 protocol, comprising 50 mg/m<sup>2</sup>/day of 6MP and 20 mg/m<sup>2</sup>/week of MTX, concen-

trations of MMPN were low in patients with TPMT variant/ITPA wild-type multilocus genotype, intermediate in wild-type/wild type patients and high in patients with TPMT wild-type /ITPA variant [14].

The allele frequencies of TPMT and ITPA polymorphisms show significant inter-ethnic variability: in particular for ITPA rs1127354, allele frequency of the variant is known to be ~20% in Asian populations, ~6% in Caucasians and ~2% in Hispanics, while for TPMT, the most common variants (rs1142345, rs1800460 and rs1800462) have a frequency of ~1% in Asians, ~5% in Caucasians and ~10% in Hispanics. Therefore, it is interesting that for TPMT and ITPA, frequencies of the variant alleles seem to be almost reversal in the two populations [15] and ITPA variants seem to be predominant in Asians. Indeed several recent studies of ALL patients from this ethnic group underline significant effects of ITPA polymorphisms on 6MP efficacy and toxicity [16-19]; therefore consideration of ITPA genetic polymorphisms may further improve personalized treatment with 6MP in patients with pediatric ALL and this could be particularly relevant in Asian children [20].

A recent study on 6MP adverse effects considered 189 children with ALL treated according to St. Jude Total 13B protocol consolidation therapy, which included MTX (2 g/m<sup>2</sup>/week) and 6MP (75 mg/m<sup>2</sup>/day in all patients, regardless of TPMT genotype). This report evaluated the association between genetic determinants of TPMT activity in patients and the incidence of severe gastrointestinal toxicity. Among these patients, deficiency in TPMT activity predisposed to an increased incidence of severe gastrointestinal toxicity (grade 3-4 mucositis): the frequency of toxicity was 33% in TPMT variant patients in comparison to 7% in TPMT wild-type. A SNP in the transporter SLCO1B1, rs11045879, was also associated with the incidence of gastrointestinal toxicity in this population, by its effects on MTX clearance [21]. Moreover, this study identified, through the HapMap model system (CEU trios), an additional determinant of TPMT activity, the PACSIN2 gene, which resulted as the most highly correlated gene to TPMT activity, in an analysis combining polymorphisms and gene expression [22]. The most significant PACSIN2 SNP identified rs2413739, was also significantly associated with TPMT activity in patients with ALL, independently from TPMT genotype. Moreover, PACSIN2 SNP rs2413739 also had a significant association with gastrointestinal toxicity during consolidation therapy [22]. The effects of PACSIN2 polymorphism on the incidence of severe mucositis during consolidation therapy for pediatric ALL was confirmed in another cohort of patients, considering 67 cases developing the adverse event during therapy according to the protocol AIEOP-BFM ALL 2000, which involves 4 weekly infusions of MTX at the dose of 2 mg/m<sup>2</sup> and concomitant daily treatment with 6MP at the dose of 25 mg/m<sup>2</sup>. Interestingly, in the discovery cohort, the effects of TPMT, SLCO1B1 and PACSIN2 polymorphisms were independent from each other, both in a multivariate logistic regression model and in a classification and regression tree analysis and could be combined in a multilocus genotype of potential importance to predict the incidence of severe mucositis [20].

A recent comprehensive evaluation of genes involved in the thiopurines pathway in terms of their association with 6MP and 6TG cytotoxic activity in a human cell line model (Corriel Human Variation Panel) has identified an effect of ecto-5-nucleotidase, which combines with TPMT and MRP4. These interesting *in vitro* observations need validation in the clinical setting [23].

## METHOTREXATE

MTX is a folic acid analogue that in the AIEOP-BFM ALL 2009 protocol is used during consolidation therapy by infusion at high doses (5 g/m<sup>2</sup>) and during maintenance therapy, when MTX is administered orally at a weekly dose of 20 mg/m<sup>2</sup>; moreover, MTX is also given intrathecally at a dose of 8-12 mg at different time points throughout the protocol. MTX inhibits different target enzymes, mainly in the folate pathway, such as dihydrofolate reductase and thymidylate synthase. MTX is metabolized intracellularly by addition of up to five glutamic acid residues, originating the MTX polyglutamates, which have increased cytotoxic activity. The pharmacokinetics and pharmacodynamics of MTX show large interpatient variability regardless of the route of administration [24].

Several proteins involved in the transport and biotransformation of MTX or in molecular processes inhibited by the compound can influence its activity. Indeed, MTX pharmacogenetic is complex and several SNPs and other features (e.g., promoter methylation, aberrant splicing [25, 26]) have been related to MTX effects, sometimes with discordant results [2, 27, 28].

One of the most studied genetic polymorphisms associated with MTX efficacy and toxicity is that of methylenetetrahydrofolate reductase (MTHFR) which catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate, required for purine and thymidine synthesis, to 5-methyltetrahydrofolate, required for protein synthesis and nucleic acid methylation. Alterations in reduced folate pools, as a consequence of changes in MTHFR activity, may have a significant effect on the responsiveness of malignant and non-malignant cells to MTX and increase its cytotoxic effects.

Salazar *et al.* have shown that the MTHFR genotype can be useful to adjust MTX dosage during consolidation therapy in 141 patients with pediatric ALL treated according to ALL/SHOP-2005 protocol. Patients with a genotype corresponding to increased or normal MTHFR activity were treated with a dose of 5 g/m<sup>2</sup> MTX; these patients had better survival, with no increased toxicity, in comparison to patients with a variant MTHFR, corresponding to reduced enzymatic activity, treated with infusions of 3 g/m<sup>2</sup> MTX [29].

Several clinical studies have shown that MTHFR polymorphisms are associated with the incidence of adverse events during chemotherapy with MTX in children with ALL. However, a recent systematic meta-analysis performed on 238 studies, could not validate such association, possibly because of differences between treatment protocols. This indicates that MTHFR polymorphisms are not good markers of MTX-related toxicity in children with ALL [30]. Interestingly, another recent meta-analysis on the same topic did

find significant effects of MTHFR genotype on MTX related adverse events: this analysis considered 42 studies on patients with different pathologies (12 on ALL). The analysis highlighted a significant association of C677T (rs1801133) polymorphism with overall MTX toxicity, hepatotoxicity, hematological toxicity, and neurotoxicity [31].

One of the major recent breakthroughs in MTX pharmacogenetics has been the identification, in a genome-wide association study in children with ALL, treated according to St. Jude Total 13B and Total 15 protocols, of multiple common genetic polymorphisms of SLCO1B1 (rs11045879 and rs4149081) as major determinants of MTX clearance during high-doses infusions in consolidation therapy [21].

The effect of SLCO1B1 on MTX pharmacokinetics has been recently replicated by other investigators: Lopez-Lepez *et al.* have shown that in 115 children with ALL, MTX plasma concentrations were significantly related, among a pool of candidate genes, only to SLCO1B1 SNPs that could therefore be suitable as markers of toxicity [32].

A recent study has shown that even rare variants in SLCO1B1 can affect MTX clearance. Deep resequencing of exons gene was performed in 699 ALL pediatric patients, identifying 93 SNPs, 15 of which were nonsynonymous. Among these, three were common (minor allele frequency, MAF >5%), one had low frequency (MAF 1–5%) and 11 were rare (MAF <1%). Non-synonymous SNPs (common or rare) predicted to be functionally damaging were more likely to be found among patients with the lowest MTX clearance. Lower function was verified *in vitro* for four SLCO1B1 haplotypes that were associated with reduced MTX clearance. In a multivariate stepwise regression analysis, adjusting for other genetic and non-genetic covariates, SLCO1B1 variants accounted for 10.7% of the population variability in clearance. Common non synonymous variants accounted for the majority of that variability; interestingly, rare damaging non-synonymous variants (1.9% of total variation) had larger effect sizes than the common ones. These results confirm a significant role of genetic variants of SLCO1B1 on MTX clearance and show that rare variants could have an important effect on pharmacogenetic phenotypes [33].

Besides the pharmacogenetic approach, personalization of the therapy with high-dose MTX can be performed by assessing drug's clearance during the infusion and the infusion rate can be adapted to reach the desired concentration threshold (i.e., 33 M for low-risk patients, 65 M for standard and high-risk patients). Therapeutic monitoring of MTX during consolidation therapy has significantly improved patients' tolerance to MTX high-dose infusion [34, 35].

In pediatric ALL, MTX and 6MP are administered together both during consolidation and maintenance therapy: *in vitro* these medications show a synergistic interaction, since MTX, by inhibiting the earliest steps in purine synthesis, elevates the intracellular concentration of phosphoribosyl pyrophosphate, the cofactor required for 6MP activation by HPRT. This synergistic interaction is present during maintenance therapy for pediatric ALL, when MTX is used at lower doses (e.g., 20 mg/m<sup>2</sup>) than during consolidation therapy. However, a recent clinical study performed on 20 children with ALL taking high dose MTX infusions (5 g/m<sup>2</sup>) together

with MP at 25 mg/m<sup>2</sup>, has shown that high-doses infusion produced a rapid reduction in TGN 24 hours after the start of MTX, and this effect persisted at least until the third day [14]. Moreover, it has been recently shown that TPMT genetic polymorphisms are associated with severe mucositis during consolidation therapy with high-dose MTX (1 g/m<sup>2</sup>) and 6MP (75 mg/m<sup>2</sup>), further proving the clinical relevance of this interaction [3]. These data warrant additional studies elucidating the optimal MTX dose synergizing with 6MP, in order to provide the best consolidation treatment with these medications in children with ALL.

## GLUCOCORTICOIDS

Synthetic glucocorticoids (GCs) are steroid-based structures, whose pharmacokinetic and pharmacodynamic properties mimic those of endogenous cortisol. Being lipophilic in nature, they are rapidly absorbed and transported in the blood by corticosteroid-binding globulin and albumin. Free GCs are able to diffuse passively across cell plasma membranes and interact specifically with the cytosolic glucocorticoid receptor (GR, encoded by NR3C1 gene). According to the classical mechanism of steroid action, the GC-bound GR enters the nucleus and interacts with GC-responsive elements (GRE) on DNA [36], thus modulating the expression of downstream genes. In lymphoblasts, the transactivation and/or transrepression of downstream target genes ultimately result in the induction of apoptosis.

GCs commonly used in the treatment of childhood leukemia are prednisone (PDN) and dexamethasone (DXM). In the AIEOP-BFM ALL 2009 protocol, PDN doses of 60 mg/m<sup>2</sup>/day are used in the first week of therapy after diagnosis and a good response to this initial phase (day+8) is known to be strongly predictive of a successful long-term clinical outcome [37–39]. PDN is also used in combination with vincristine (VCR), PEG-ASNase, daunorubicin (DNR) and intrathecal MTX during the subsequent induction phase (Fig. 1), whereas DXM 10 mg/m<sup>2</sup> is given in the reinduction protocols.

A recent review reports on the role of pharmacogenetics in predicting GC clinical response in several diseases, investigating the GC molecular mechanism of action and on genes and variants that can thus affect drug response [40]. Although extensively studied, only few polymorphisms were found to be significantly associated to GC response.

Recently, a novel two-phase study design was applied to select, from the 1999 ALL patients treated according to the AIEOP-BFM ALL 2000 trial, a sub-sample of 614 children to be genotyped for the deletions of glutathione S-transferase (GST) genes. No overall effect was found, but the GST-T1 deletion was associated with a worse outcome in the standard-risk group and within day+8 prednisone good responders, whereas the GST-M1 deletion was associated with a lower risk of relapse within high risk/day+8 prednisone poor responder patients [41]. The GST-M1 non-null genotype was also found to be significantly associated with increased hematologic recurrence in ALL patients treated with the St. Jude Total 13B study protocol high-risk arm [42].

Many adverse effects have been related to GC therapy, among which osteonecrosis, hypertension, obesity and hy-

pertriglyceridemia. Osteonecrosis is one of the dose-limiting GC-induced toxicity phenotypes [43]. In a prospective study of St Jude Children's Hospital, 364 patients treated with Protocol Total 15 underwent magnetic resonance imaging of hips and knees during the first 6 to 8 months of continuation therapy, regardless of symptoms. Older age and increased intensity of treatment were identified as non genetic risk factors for osteonecrosis. Genome-wide analysis identified significant SNPs in a locus on chromosome 2 encoding for acid phosphatase 1 (ACP1) and SH3YL1. Patients with at least one A allele at ACP1 SNP rs12714403 had a higher risk of symptomatic osteonecrosis, lower albumin, and higher cholesterol levels, after adjustment for age and treatment arm [44]. ACP1 has a role in controlling serum cholesterol and triglyceride levels [45] and regulates osteoblast differentiation via Src kinase [46]. These findings suggest that ACP1 might act via multiple mechanisms to affect bone homeostasis and DXM-induced osteonecrosis.

A polymorphism in the plasminogen activator inhibitor-1 (PAI-1) gene (rs6092) was reported by the Children's Oncology Group (COG) to be also associated with the incidence of osteonecrosis: the presence of at least one minor allele was associated with approximately a 2.8-fold increased risk in an analysis adjusted for gender, age, and treatment arm in 361 patients older than 10 years of age treated as part of the Children's Cancer Group (CCG) 1882 study. The effect was independent from traditional risk factors for osteonecrosis development. It was suggested that increased PAI-1 levels associated with this polymorphism might lead to reduced fibrinolysis. The resulting increase in intraosseous venous pressure, reducing blood flow to the femoral head, may culminate in hypoxic bone death [47]. The importance of this polymorphism on the risk of osteonecrosis was not confirmed by the previously mentioned St. Jude genome-wide study [44] nor by a pharmacogenetic investigation on 100 children treated as part of the Medical Research Council UK ALL 2003 trial, whose protocol for high-risk cases is similar to the one of CCG 1882, with the exception that DXM (6 mg/m<sup>2</sup>) replaces PDN and all patients receive PEG-asparaginase [48].

The genetic risk factors for GC-related hypertension in ALL patients have been investigated in 602 children who were normotensive at diagnosis and were treated with PDN at 40 mg/m<sup>2</sup>/day during the 28-day period of remission induction according to the St. Jude Total Therapy 13A, 13B, 14 and 15 protocols. None of the presenting features (age, sex, race, white blood cell count, risk group, body mass index or serum creatinine) was associated with this adverse effect, whereas 12 SNPs in 8 candidate genes (CNTNAP2 rs2286128, LEPR rs1137101, CRHR1 rs242941, rs1876828 and rs1876829, NTAN1 rs1135999, rs1136001 and rs16966957, SLC12A3 rs11643718, ALPL rs3738099, BGLAP rs1800247 and APOB rs693) were found to be significant predictors of hypertension. Among these, the contactin-associated protein-like-2 (CNTNAP2, rs2286128) genotype was the most significant determinant, with a protective effect of the minor T allele [49].

## VINCRISTINE

VCR is a commonly used chemotherapeutic agent for treating various solid tumors, lymphomas and leukemias.

This vinca alkaloid acts as tubulin-binding agent, interfering with microtubule formation and therefore with cell cytoskeleton and mitotic spindle dynamics, disrupting cell structure and blocking mitotic cells in metaphase. In the AIEOP-BFM ALL 2009 protocol, VCR is used in combination chemotherapy at doses of 1.5 mg/m<sup>2</sup> i.v. (maximum 2 mg) during the remission induction and intensification phases (4 administrations each).

Despite the extensive experience with this drug, large intra-patient, inter-patient and inter-ethnic variability is observed in response and a frequent, unpredictable and clinically important dose-limiting neurotoxicity occurs. VCR is mainly metabolized at the hepatic level by cytochrome P450 (CYP) 3A4/5. CYP3A5 is up to 14 times more efficient in catalyzing the formation of the major secondary amine metabolite M1 compared to CYP3A4 [50], and clearance of VCR has been found to be 5-fold higher in patients with CYP3A5 high expression than low expression using a bank of human liver microsomes phenotyped for both isoenzymes [51]. *In vivo*, an extensive VCR metabolizer may be underdosed while a poor metabolizer may be at higher risk of toxicity with conventional dosing strategies. Among Caucasians, only ~20% of subjects express functionally relevant levels of CYP3A5 [52]. Indeed this gene is highly polymorphic in this population, with the CYP3A5\*3 allele (rs776746) being the most common genetic variant, coding for a splicing defect generating a modified mRNA and a non-functional protein [53, 54]. In the absence of at least one functioning wild-type CYP3A5\*1 allele, VCR metabolism becomes dependent on the less selective CYP3A4 protein. Although several low-frequency genetic variants of the CYP3A4 gene have been identified so far, there are conflicting results on their effect on gene transcription and no clear consensus on their significance for drug disposition [55]. A further confounding variable is represented by the induction of CYP3A4 by steroids, generally given concomitantly to VCR in the remission induction and intensification phases in ALL therapy. Indeed, Groningen *et al.* measured VCR clearance after a single dose in 70 newly diagnosed ALL children, finding that it was substantially lower than the clearance reported previously for children receiving VCR as part of combination chemotherapy with steroids [56].

Different studies have investigated the role of CYP3A polymorphisms in ALL. A recent study on precursor B cell ALL pediatric patients, considered 107 children treated according to the following protocols: CCG1961 and CCG1991 and COG studies AALL0331, AALL0232, AALL0434 and AALL01P1; all treatment regimens included a standard VCR dose of 1.5 mg/m<sup>2</sup>, with a maximum dose of 2 mg administered weekly during induction chemotherapy. Patients carrying one or two copies of the active CYP3A5\*1 allele experienced less VCR-related neurotoxicity and produced more M1 metabolite in comparison to CYP3A5 non-expressor [57].

In another recent study on 616 ALL patients, CYP3A5\*3 genotype was not associated with differences in event-free survival. However, T-ALL patients with low expression of CYP3A5 had a worse prognosis and eight-fold increased risk of experiencing an event when compared to those having at least one functional allele [58].

In a retrospective analysis on 533 patients (409 non relapsed and 124 relapsed) treated with the CCG-1891 pediatric ALL trial, no association between variants and relapse was found. Patients with the CYP3A4\*1B and CYP3A5\*3 genotypes showed a decreased risk of peripheral neuropathy in univariate analysis [59].

Recently PharmGKB published an updated pharmacogenetic summary for CYP3A5 [60] and other genes of relevance for VCR efficacy and/or toxicity, such as GST-T1 and ABCB1/MDR1, respectively involved in VCR metabolism and cellular efflux, however these summaries consider anti-neoplastic regimens for oncological diseases different from pediatric ALL [61, 62].

Apoptosis occurs as the ultimate result of VCR-induced cytotoxicity through molecular mechanisms that are not yet completely understood. A very recent report [63] demonstrates that, during the mitotic arrest triggered by antitubulin agents, the antiapoptotic protein myeloid cell leukemia sequence 1 (MCL1) is degraded through a post-translational mechanism, thus potentiating cell death. MCL1 decline is driven by the ubiquitin-proteasome system: phosphorylation of MCL1 directs its interaction with the tumor-suppressor protein FBW7, which mediates the polyubiquitination of MCL1 and targets it for proteasomal degradation. The degradation of MCL1 is blocked in patient-derived tumor cells that lack or have loss-of-function mutations in FBW7, conferring resistance to antitubulin agents. Additionally, genetic features resulting in FBW7 inactivation and elevated MCL1 levels are more frequent in primary tumor samples. In the NCBI SNP database (dbSNP, build 130), accessed in January 2013, there are 187 and 2825 SNPs in the human MCL1 and FBW7 genes, respectively. These genes and variants could play a role in determining patients' response to remission induction therapy for ALL and could hence represent novel candidates of pharmacogenetic interest for VCR resistance [64, 65]. Indeed, FBW7 mutations have been reported to be common in T-ALL and are believed to contribute to T-ALL pathogenesis by activating the NOTCH pathway [66-68]; moreover, in a recent study on a large group of 301 children with T-ALL treated with the AIEOP-BFM ALL 2000 protocol, patients with FBW7 mutations showed an excellent prednisone (day +8) and early minimal residual disease response (at day +33) [69].

## ASPARAGINASE

Current pediatric ALL treatment regimens include a widespread use of ASNase for remission induction and reinduction/intensification therapies. ASNases are bacterial-derived enzymes that catalyze the extracellular conversion of asparagine to aspartic acid and ammonia. In most human tissues, asparagine is intracellularly synthesized from L-aspartate under physiological conditions, in an ATP-dependent reaction catalyzed by asparagine synthetase (ASNS). Primary ALL cells exhibit a particularly low level of ASNS expression in comparison to normal cells [70-72], lack the ability to up-regulate the enzyme and hence take the required asparagine from plasma [73]. Therefore ALL blasts are not able to overcome starvation of this amino acid and ASNase selectively affects blasts without inducing toxic effects in normal cells. ASNase-resistant ALL cell lines ex-

hibit elevated expression of ASNS [74, 75] and display several adaptive changes in cellular transport and metabolism to support the asparagine biosynthesis [76]. *In vitro* studies on MOLT-4 human leukaemia cell line show that over-expression of exogenous ASNS protein results in an ASNase-resistant phenotype [75]. Also in patients, higher expression of ASNS is related to resistance to ASNase. On the contrary in TEL/AML1-positive leukemic cells, ASNS expression levels have not been correlated to ASNase resistance; however TEL/AML1 are more sensitive to ASNase, therefore a molecular mechanism independent from ASNS concentration has been suggested [77-79].

In the current AIEOP-BFM ALL 2009 protocol, a pegylated form of the native *E. coli* asparaginase (pegaspargase) has been introduced, that is given at a dose of 2500 U/m<sup>2</sup> (max 3750 U) twice during induction and once during intensification therapy (Fig. 1). Pegylation increases the drug half-life and decreases the probability of developing anti-ASNase antibodies, that cause clinical hypersensitivity in up to 40% of patients [80]. These antibodies also cause rapid drug inactivation in approximately 30% of patients, a phenomenon commonly referred to as 'silent hypersensitivity' or 'silent inactivation', that results in suboptimal asparagine depletion [81, 82]. A recent study has also demonstrated that higher DXM clearance was associated with increased anti-ASNase antibodies and reduced exposure to both medications, significantly affecting the risk of relapse [83]. Of note, in the AIEOP-BFM ALL 2009 protocol, ASNase and DXM are administered concomitantly during the reinduction phase.

A recent study combining genome-wide SNP and gene expression analyses on HapMap human lymphoblastoid cell lines, has identified variants and genes of the aspartate metabolism pathway (according to the KEGG database, [84, 85]) as the most significant for the ASNase *in vitro* sensitivity (measured as IC<sub>50</sub>, the drug concentration necessary to inhibit 50% of cell growth). The role of variants and genes belonging to the aspartate metabolism pathway has been investigated also in bone marrow samples from 54 patients with primary childhood ALL. The highest-ranked SNP (rs3102475) has been found in the adenylosuccinate synthetase (ADSS) gene. ADSS encodes an enzyme that plays an important role in purine biosynthesis by converting L-aspartate and inosine monophosphate to adenylosuccinate, in turn transformed into adenosine monophosphate (AMP) by adenylosuccinate lyase. Other SNPs significantly associated with ASNase *in vitro* sensitivity were located in adenylosuccinate synthetase like-1 (ADSSL1: rs2494731), argininosuccinate synthetase 1 (ASS1: rs553696, rs544701, rs4740158), aspartyl-tRNA synthetase (DARS: rs2068871, rs2759328, rs941988, rs2227589, rs16846526) and asparaginyl-tRNA synthetase 2 (NARS2: rs11237537) genes [86]. ASS1 is a crucial enzyme involved in the arginine biosynthetic pathway and, indirectly, in the de novo synthesis of asparagine; furthermore, up-regulation of ASS1 has been identified by microarray analysis in cell lines with ASNase resistance [87]. DARS and NARS2 are cytoplasmic enzymes involved in protein synthesis.

Rousseau *et al.* [88] report on 14 tag-SNPs in the regulatory and coding regions of ASNS, ASS1, ATF5 and their association with ALL clinical outcome. ATF5 (activating

transcription factor 5) is a stress related transcription factor that responds to amino acid limitation and upregulates ASNS transcription. ATF5 is differentially expressed in ASNS resistant or sensitive leukemic cells [87, 89, 90]. Rousseau *et al.* found that, in patients of two independent cohorts who received *E. coli* ASNSase, lower event-free survival was associated with the T allele of rs11554772 SNP, located in the 5'-UTR of ATF5; the presence of the T allele showed a higher promoter activity in gene-reporter assay [88].

Important ASNSase-related adverse effects include pancreatitis (4–18% of pediatric patients), abnormalities of hemostasis (2–4%), hyperglycemia and alterations of lipid metabolism [81, 82]. The already mentioned hypersensitivity was particularly frequent in patients treated with the native *E. coli* formulation and is nowadays reduced by the use of pegaspargase. Inter-individual differences in ASNSase hypersensitivity have been recently investigated by a genome-wide approach in newly diagnosed childhood ALL treated on St. Jude Total Therapy 15 protocol. Five intronic germline variations (rs10070447, rs6890057, rs4958676, rs6889909 and particularly rs4958381) in the GRIA1 gene were associated with allergic reactions in both discovery and validation cohorts. GRIA1 encodes for a subunit of the AMPA receptor, a tetrameric ligand-gated ion channel that transmits glutamatergic signals in the brain. Beside its role as neurotransmitter, it has been recently shown that glutamate has also a role as an immunomodulator [91, 92].

## ANTHRACYCLINES

Anthracyclines used in ALL treatment, daunorubicin (DNR) and doxorubicin (DOX), are antibiotics that exploit antileukemic activity by intercalating in DNA, inhibiting topoisomerase II and by generating free radicals and oxidative stress. In the AIEOP-BFM ALL 2009 protocol, DNR is used during the remission induction phase at the dose of 30 mg/m<sup>2</sup> i.v. weekly (4 administrations), whereas DOX is preferred in reinduction with the same dosing scheme (Fig. 1). The liposomal formulation of DNR (daunoxome) is used in combination with fludarabine, MTX and high doses of cytarabine for high risk patients who did not achieve remission and are eligible for transplantation.

Both DNR and DOX have similar pharmacokinetic properties and exhibit long terminal plasma half-lives with extensive tissue binding. DNR and DOX are both metabolized by the cytosolic aldo/keto reductases [93] to form their 13-hydroxylated metabolites, daunorubicinol and doxorubicinol, respectively. Daunorubicinol has about 10% of the cytotoxic activity of DNR in bone marrow stem cells [94], and doxorubicinol has approximately 5% of the antitumor activity of DOX [95], but may be a more potent cardiotoxin [96].

These agents cause an unusual and often irreversible cardiomyopathy, the occurrence of which is related to the cumulative dose of the drug. Other established risk factors for cardiomyopathy are concomitant cardiac irradiation as well as higher dose, shorter infusion time, younger age, longer time since treatment and female sex. However, these risk factors alone are insufficient to accurately stratify patients into groups at high and low risk for cardiomyopathy and pharmacogenetic studies may be of help. Recently, a clinical study has been conducted considering 2,977 SNPs in 220

key drug biotransformation genes in a discovery cohort of 156 anthracycline-treated children from British Columbia, with replication in a second cohort of 188 children across Canada and further replication of the top SNP in a third cohort of 96 patients from Amsterdam, the Netherlands. The study identified a highly significant protective association of a synonymous coding variant (rs7853758) within the SLC28A3 gene with anthracycline-induced cardiotoxicity [97]; this synonymous SNP may have functional consequences since carriers of the rs7853758 minor allele exhibited reduced SLC28A3 mRNA expression in monocytes [97, 98]. Additional associations with risk and protective variants in other genes including SLC28A1 and several adenosine triphosphate-binding cassette transporters (ABCB1, ABCB4, and ABCC1), were present. SLC28A3 and SLC28A1 can transport anthracyclines into cells, while ABC transporters extrude these agents, providing a potential mechanism by which these variants could affect the incidence of cardiomyopathy [99].

## CYTARABINE

Cytarabine (1- $\beta$ -arabinofuranosylcytosine, ara-C) is the mainstay of acute myeloid leukemia (AML) chemotherapy and part of ALL treatment in children (Fig. 1 and 2).

At plasma concentrations of 0.5-1  $\mu$ M (achieved with standard-low dose regimen, 100 mg/m<sup>2</sup>) ara-C enters leukemic cells via a transmembrane nucleoside carrier, the human equilibrative nucleoside transporter 1 (hENT1) [100]. Phosphorylation by deoxycytidine kinase (DCK, the rate-limiting enzyme catalyzing the first-step activation of nucleoside analogues) to ara-C monophosphate, and then by pyrimidine kinases, leads to production of the active metabolite ara-CTP (1- $\beta$ -D-arabinofuranosylcytosine-5'-triphosphate), which exerts antiproliferative activity by inhibition of DNA polymerase and, after incorporation into DNA, chain termination with block of DNA synthesis [101, 102]; importantly, a direct correlation exists between intracellular concentrations of ara-CTP and sensitivity to ara-C [103]. Opposite to DCK, dephosphorylation by 5' nucleotidases (NT5C2) and deamination by cytidine deaminase (CDA) induce ara-CTP depletion, while pyrimidine biosynthesis, catalyzed by cytidine-5'-triphosphate synthetase (CTPS) and ribonucleotide reductase (RRM1 and 2), provides competing substrates for DNA polymerase (dCTP), with feedback inhibition of DCK and allosteric activation of CDA [104].

Only few reports provide insights on the relation between germline SNPs and response to ara-C. hENT1, NT5C2, DCK, and CDA expression has been associated *in vivo* and *in vitro* with ara-C sensitivity and treatment outcome [105-110], however a correlation between polymorphisms and response to ara-C has been so far identified for only a few genes. In cell lines from the International HapMap Project panels (n = 90 each) with European (CEU) or African Yoruba (YRI) ancestry, sixty-four genetic polymorphisms, including three non-synonymous coding changes were identified and resulted associated with DCK activity. Lymphoblast cell lines from subjects heterozygous for the coding changes had significantly lower DCK activity compared with homozygous wild type subjects. In an exploratory analysis, the C allele of SNP rs4643786 was also associated with

lower blast ara-CTP levels in acute myeloid leukemia patients receiving ara-C as continuous infusion [111]. A common coding polymorphism in CDA gene, resulting in decreased enzyme activity, was determined in 457 children with AML treated on the CCG 2941 and 2961 protocols. Post-induction treatment-related mortality was significantly higher in children with the mutated genotype and this was most evident in children who received a polychemotherapeutic regimen which comprised ara-C [112]. Recently, genome-wide expression studies [113] and integrative analysis of gene expression and phenotype analysis in patients with AML identified genes involved in the apoptotic pathway and intracellular signaling which predicted outcome and sensitivity to ara-C [114], offering new opportunities for future research.

### CYCLOPHOSPHAMIDE

Cyclophosphamide (CPM) is the combination of chloroethane and phosphoric amide; it's an alkylating and cell cycle nonspecific agent clinically used to treat leukemia and other malignancies. In AIEOP-BFM ALL 2009, CPM is administered during induction and reinduction therapy (Fig. 1). Devoid of cytotoxic activity *in vitro*, CPM is a prodrug requiring enzymatic activation, catalyzed *in vivo* by different CYPs, to 4-hydroxyCPM, which is responsible for alkylation and toxicity in tumor cells [115]. Activation of CPM to 4-hydroxyCPM is catalyzed by the hepatic isozymes CYP2B6, 2C9 and 3A4 (with 2A6, 2C8 and 2C19 contributing to a lesser extent). Competing with C-4 hydroxylation of CPM is a minor (~10%) oxidative pathway that leads to N-dechloroethylation and to the formation of the neurotoxic chloroacetaldehyde. CYP3A4 is primarily responsible for this undesirable side-chain oxidation with a minor contribution from CYP2B6 [116, 117]. Several-fold differences in the extent of metabolite formation have been observed among patients and these inter-individual differences may be due to polymorphisms in CYP enzymes [116].

Moreover, CYP450 oxidoreductase (POR) is a flavoprotein that contains both flavin mononucleotide and flavin adenine dinucleotide as cofactors, and uses NADPH as the source of electrons; it supplies electrons to all microsomal CYPs for their catalytic activity [118]. As the only flavoprotein-transferring electrons from NADPH to CYP, POR was indicated to play an important role in CYP2B6 mediated CPM metabolism [119]. Studies have found that the gene encoding human POR is highly polymorphic. In particular one study demonstrated that cells transfected with POR A287P were much more tolerant to CPM [115]. POR and its genetic polymorphisms play an essential role in the hepatic metabolism of CPM and of other drugs and could hence be important in determining their efficacy and toxicity, allowing it to become a new target in the field of pharmacogenetics [115].

### INNOVATIVE AGENTS FOR PEDIATRIC ALL

#### Imatinib

Imatinib mesylate (IM, STI571, Glivec, Gleevec) is the first member of a class of "targeted" anticancer agents. It is a highly selective inhibitor of tyrosine kinase enzymes [120], such as the BCR-ABL fusion protein, the platelet derived

growth factor receptors (PDGFR), and the c-KIT receptors [121]. The drug is used in chronic myeloid leukemia (CML), however, in conjunction with intensive chemotherapy and allogeneic stem-cell transplantation is employed also for the treatment of children with Philadelphia-chromosome-positive ALL [122]. The AIEOP-BFM ALL 2009 protocol participates in the EsPhALL European protocol to treat patients with Philadelphia-chromosome-positive ALL: current indications are that starting on day +15 of therapy, children are treated with daily IM at a dose of 300 mg/m<sup>2</sup> until the completion of chemotherapy (i.e., for all the 104 weeks of therapy) [122].

IM is well absorbed after oral administration, with a bioavailability exceeding 90%. It is extensively metabolized by the hepatic cytochrome P450 enzyme system (particularly by the CYP3A4 and CYP3A5 members) to the active metabolite CGP 74588 [123]. In the blood, the drug is extensively bound to the plasma protein (free fraction 4%), mostly to the  $\alpha_1$ -acid glycoprotein [124].

The most common chromosomal translocation occurring in CML is the t(9;22)(q34;q11), in which a region of the Bcr gene on chromosome 22 recombines with the Abl gene on chromosome 9 to form the hybrid BCR-ABL gene with leukemogenic properties. This translocation, also known as Philadelphia chromosome, leads to a fusion protein with a constitutively active tyrosine kinase. More than a dozen mutations in the BCR-ABL fusion gene lead to IM resistance through a variety of mechanisms, including P-loop or activation loop conformational change and steric clashing in the binding site [125]. This is particularly troubling because targeted inhibition of IM may lead to clonal selection of BCR-ABL resistant mutants. However, if a significant number of mutations leading to resistance are due to the inability of BCR-ABL to achieve the closed conformation required for IM binding, molecules targeting the open conformation may be an effective alternative or concomitant therapeutic strategy. Inhibition of signaling pathways downstream of BCR-ABL could also overcome BCR-ABL kinase domain mutations resistance to IM. Prospective testing for IM-resistant BCR-ABL mutations in CML provides valuable clinical information because a small number of mutation account for the majority of resistant cases [125].

The decision as to whether to treat patients with IM is based on the presence of genetic biomarkers, including BCR-ABL, as well as c-KIT and PDGFR gene rearrangements. It was demonstrated that there is a good response to IM in presence of c-KIT mutations in exon 11, and a partial response in presence of c-KIT mutations in exon 9. Resistance is seen in presence of c-KIT mutations in exon 13 and 17, of PDGFR mutations (especially exon 18) and after acquisition of secondary mutations in the kinase domain [126].

A review on IM pharmacogenetics has been recently published by Dulucq *et al.* [127]. Pharmacokinetic genetic variants in cellular transporters or metabolizing enzymes have been investigated for their role on efficacy of IM treatment with controversial results. Candidate genes carrying potentially important SNPs were the solute carrier SLC22A1 (encoding for the human organic cation transporter 1, hOCT1) mediating cellular IM uptake, the efflux pumps belonging to the ATP-binding cassette family ABCB1 (MDR1) and

ABCG2 (BCRP), the CYP3A4 and CYP3A5, and the plasma alpha 1-acid glycoprotein AGP. Although some groups reported an *in vivo* functional impact for SLC22A1 rs34130495 and rs683369, for ABCB1 rs1045642, rs1128503 and rs2032582, for ABCG2 rs2231142 and rs2231137, for CYP3A5 rs776746, there was no clear consensus on the importance of such a genetic contribution. Discrepancies among studies could be explained by different response criteria, sample size, IM dosage and treatment protocols.

In a subset of 189 newly diagnosed, previously untreated CML patients receiving IM in the framework of the TOPS phase III trial, a combination of non-synonymous SNP in SLC22A1 or in different genes involved in IM uptake SLC22A1 / SLC22A4 (encoding respectively for the organic cation transporters hOCT1 and OCTN1) and SLCO1A2 (encoding for the organic anion transporter OATP1A2) was significantly associated with major molecular response (MMR, defined as 3-log reduction in Bcr-Abl transcript level from a standardized baseline value), regardless of ethnicity [128]. Analyses restricted to Caucasians highlighted also the significant association of the MDR1 CC genotype (rs1045642) with a complete molecular response (defined as at least 4-log reduction corresponding to undetectable Bcr-Abl transcript by real-time reverse transcription polymerase chain reaction). An interesting finding was the identification of the SLC22A4 rs1050152C allele (major allele) as a significant favorable determinant of MMR achievement in both the overall population and Caucasians [128].

Other tyrosine kinase inhibitors are being considered for the treatment of pediatric ALL: in particular the second generation tyrosine kinase inhibitor dasitinib is being tested in an international clinical trial that enrolls patients from Europe and the US (COG protocol).

### Fludarabine

Fludarabine is a prodrug that is converted to the free nucleoside 9-beta-D-arabinosyl-2 fluoroadenine (F-ara-A) which enters cells and accumulates mainly as the 5'-triphosphate, F-ara-ATP. The rate-limiting step in the formation of triphosphate is conversion of F-ara-A to its monophosphate, which is catalyzed by deoxycytidine kinase. F-ara-ATP has multiple mechanisms of action, which are mostly directed toward DNA, and include inhibition of ribonucleotide reductase, incorporation into DNA resulting in repression of further DNA polymerization and inhibition of DNA ligase and DNA primase. Collectively these actions affect DNA synthesis [129].

The primary mechanism underlying fludarabine resistance appears to be the insufficient level of F-ara-ATP, which may be caused by inefficient cellular uptake of fludarabine, due to low levels and/or activity of the transporter hENTI (SLC29A1), reduced levels of activating enzymes, primarily DCK, and increased cellular dCTP pools that are regulated by the enzyme ribonucleotide reductase. Genetic variations in these candidate genes could predict its antileukemic effect and the clinical outcome [104]. Recently, Rivero *et al.* [130] have reported on the relationship between genetic variants of DCK and the toxicity of a fludarabine-based regimen in 74 patients with follicular lymphoma. Using sequencing and high resolution melting curve analysis on

amplified genomic DNA corresponding to exons and the promoter region of DCK, they observed five single nucleotide polymorphisms with different frequencies. The 6 patients heterozygous for the synonymous rs11544786 situated in exon 3 had a lower risk of lymphopenia whereas they were more susceptible to neutropenia than the 58 wild type patients.

Combination of fludarabine with other DNA damaging agents to inhibit DNA repair processes has been highly effective against acute leukemia. The combination of fludarabine with ara-C, granulocyte colony-stimulating factor and anthracyclines, known as FLAG-IDA, appears to have a synergistic effect and has been administered successfully in children with refractory or relapsed ALL and AML [131], and is now included in the European protocols of treatment of relapsed ALL and AML in children.

### Clofarabine

Clofarabine is a second-generation purine nucleoside analog, rationally designed to incorporate the best attributes of its predecessors, fludarabine and cladribine. Clofarabine must first be phosphorylated into its monophosphate derivative by DCK and, following additional phosphorylation steps, is converted to the active triphosphate metabolite [132]. Reasonably, genetic variations in DCK should influence its expression and activity but no data have been published yet.

The drug is toxic to non-dividing cells as well as to cells which are proliferating and its anti-cancer activity is considered to be due to three mechanisms that are: inhibition of DNA synthesis and repair, inhibition of ribonucleotide reductase with reduction of deoxynucleotide triphosphate pools, and induction of apoptosis through direct and indirect action on mitochondria [133, 134].

The drug was proposed, as single agent, in two phase 2 trials in pediatric patients with relapsed or refractory ALL but, due to its different mechanism of anti-cancer activity, in combination with other drugs such as ara-C, CPM and etoposide. This therapeutic approach has provided interesting results in both ALL and AML relapsed after front line therapies [135] and, for this reason, the combination of clofarabine, etoposide and CPM (CLOVE) will be included in the next European protocol for the treatment of relapsed ALL.

Finally, *in vitro* experiments in lymphoid tumor cell lines showed that clofarabine, at a very low concentration, induces DNA hypomethylation and increases the expression of cancer testis antigen sperm protein 17 and SPAN-Xb [136]. The potential use of low-dose clofarabine appears to be an interesting field for future research.

### GENOME-WIDE APPROACHES TO IDENTIFY MARKERS FOR OUTCOME IN CHILDREN WITH ALL

Genome-wide analysis, if adequately powered, has great potential in elucidating and understanding the genomic component associated with inter-individual differences in phenotypes, even of pharmacogenetic interest. The greatest potential of the genome-wide approach resides in the analysis of

patients' samples: if the study is adequately designed and powered, and the phenotypes are well collected, this approach can provide unpredictable insights on the phenotype of interest, potentially leading to major breakthroughs in the understanding of the genomic basis of inter-patient variability [137]. Several such examples exist in the literature: besides the already mentioned role of *SLCO1B1* in the disposition of MTX in children with ALL [21], this same transporter was shown to be involved in statin's induced myopathy [138].

Considering studies focusing on pediatric ALL, genome-wide interrogation is complicated by the relative small number of cases, compared to other pathologies, and therefore by the need for large multicenter studies, which may render the collection of consistent phenotypes more difficult. St. Jude Children's research hospital is a pioneer in applying the genome-wide approach to clinical phenotypes of importance for therapy of pediatric ALL: some of the most important recent studies considered genetic polymorphisms associated with the outcome of therapy evaluated as MRD [139] or disease relapse [140].

The study on MRD considered two independent cohorts of children with newly diagnosed ALL: 318 patients in St. Jude Total Therapy protocols 13B, 15 and 169 patients in COG trial P9906. This study identified 102 SNPs associated with MRD in both cohorts, including 5 SNPs in interleukin 15 (*IL15*). Twenty-one of these SNPs were also associated with drug disposition (evaluated as MTX clearance, etoposide clearance or MTX polyglutamates concentration), generally linking greater drug exposure with MRD eradication [139].

The study on relapse considered the relationships between genotypes at 444,044 SNPs and the risk of relapse in 2,535 children with newly diagnosed ALL, after adjusting for genetic ancestry and treatment regimen. 134 SNPs were identified that were reproducibly associated with ALL relapse. Of these 134 relapse SNPs, 133 remained prognostic after adjusting for all known relapse risk factors, including MRD, and 111 were significant even among patients who were negative for MRD after remission induction therapy. According to the authors, one of the most interesting finding is that the C allele at rs7142143 in the *PYGL* gene was associated with a ~4-fold higher risk of relapse than the T allele. Fourteen of the 134 relapse SNPs, including variants *ABCB1*, were also associated with antileukemic drug pharmacokinetics and/or pharmacodynamics [140].

## CONCLUSION

Chemotherapy of pediatric ALL is one of the most successful treatments applied in the field of oncology. Indeed, it is truly remarkable that more than 80% of children are cured today of a disease that was universally fatal within weeks to months in the early 1960s [1, 34, 141]. Considering ALL a cancer that is effectively treated is tempting, but several significant improvements could be made to the therapy. In particular, one of the current aims of ALL research is to guarantee survival in 100% of patients, therefore identifying treatment strategies that allow response in patients with higher risk disease, e.g. identifying patients resistant or not compliant to current therapy, as the application of pharmacogenetic

based therapy personalization should allow [141]. Given the acute nature of the disease, it is likely that biological therapy will not be applicable to ALL; in addition due to the lack of clear univocal molecular drivers of ALL, specific useful inhibitors will likely not emerge. Therefore, one of the most important improvements necessary for chemotherapy of ALL is to reduce treatment's adverse events and toxicity, that if too intense may guarantee survival but at the same time may have significant negative impact on the long-term quality of life of survivor patients. Indeed ALL survivors have been reported to present more adverse events in general and mental health, functional impairment and activity limitations compared with sibling without ALL; rates of marriage, university graduation and employment were all lower in ALL patients [142]. In particular, ALL survivors who received irradiation have been reported to present, in comparison to the general population, a higher incidence of second neoplasms, a slight excess in mortality and increased unemployment rate [143]. With marked reduction in the use of cranial radiation and risk-directed therapy that stratifies low-risk patients to receive a less toxic therapy, we can also expect a significant improvement in the quality of life of survivors. To reduce the use of cranial irradiation, improvement of treatment personalization with strategies considering pharmacogenomics are required, as it has been demonstrated by studies performed at St. Jude Children Research Hospital [34].

In conclusion, application of pharmacogenomic approaches for tailored anti-leukemic therapy in children with ALL should significantly contribute in providing the best care for these children, in particular in order to reduce unnecessary toxic effect and guarantee a higher quality of life to ALL survivors.

## CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

## ACKNOWLEDGEMENTS

The financial support of the Associazione Genitori Malati Emopatici Neoplastici Friuli Venezia Giulia (AGMEN) is acknowledged. Dr. Raffaella Franca is supported by a fellowship of IRCCS Burio Garofolo, Trieste.

## REFERENCES

- [1] Pui, C.H.; Evans, W.E. Treatment of acute lymphoblastic leukemia. *N. Engl. J. Med.*, **2006**, *354*, 166-178.
- [2] Paugh, S.W.; Stocco, G.; Evans, W.E. Pharmacogenomics in pediatric leukemia. *Curr. Opin. Pediatr.*, **2010**, *22*, 703-710.
- [3] Zaza, G.; Cheok, M.; Krynetskaia, N.; Thom, C.; Stocco, G.; Hebert, J.M.; McLeod, H.; Weinshilboum, R.M.; Relling, M.V.; Evans, W.E.; Klein, T.E.; Altman, R.B. Thiopurine pathway. *Pharmacogenet. Genomics*, **2009**, *20*, 573-574.
- [4] Relling, M.V.; Gardner, E.E.; Sandborn, W.J.; Schmiegelow, K.; Pui, C.H.; Yee, S.W.; Stein, C.M.; Carrillo, M.; Evans, W.E.; Klein, T.E. Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clin. Pharmacol. Ther.*, **2011**, *89*, 387-391.
- [5] Frandsen, T.L.; Abrahamsson, J.; Lausen, B.; Vetterranta, K.; Heyman, M.; Behrentz, M.; Castor, A.; Wehner, P.S.; Frost, B.M.; Andersen, E.W.; Schmiegelow, K. Individualized toxicity-titrated 6-mercaptopurine increments during high-dose methotrexate consolidation treatment of lower risk childhood acute lympho

- blastic leukaemia. A Nordic Society of Paediatric Haematology and Oncology (NOPHO) pilot study. *Br. J. Haematol.*, **2011**, *155*, 244-247.
- [6] Relling, M.V.; Hancock, M.L.; Boyett, J.M.; Pui, C.H.; Evans, W.E. Prognostic importance of 6-mercaptopurine dose intensity in acute lymphoblastic leukemia. *Blood*, **1999**, *93*, 2817-2823.
- [7] Stork, L.C.; Matloub, Y.; Broxson, E.; La, M.; Yanofsky, R.; Sather, H.; Hutchinson, R.; Heerema, N.A.; Sorrell, A.D.; Master son, M.; Bleyer, A.; Gaynon, P.S. Oral 6-mercaptopurine versus oral 6-thioguanine and veno-occlusive disease in children with standard-risk acute lymphoblastic leukemia: report of the Children's Oncology Group CCG-1952 clinical trial. *Blood*, **2010**, *115*, 2740-2748.
- [8] Lennard, L.; Richards, S.; Cartwright, C.S.; Mitchell, C.; Lilleyman, J.S.; Vora, A. The thiopurine methyltransferase genetic polymorphism is associated with thioguanine-related veno-occlusive disease of the liver in children with acute lymphoblastic leukemia. *Clin. Pharmacol. Ther.*, **2006**, *80*, 375-383.
- [9] Oancea, I.; Png, C.W.; Das, I.; Lourie, R.; Winkler, I.G.; Eri, R.; Subramaniam, N.; Jinnah, H.A.; McWhinney, B.C.; Levesque, J.P.; McGuckin, M.A.; Duley, J.A.; Florin, T.H. A novel mouse model of veno-occlusive disease provides strategies to prevent thioguanine-induced hepatic toxicity. *Gut*, **2012**, *3*, 62, 594-605.
- [10] Relling, M.V.; Hancock, M.L.; Rivera, G.K.; Sandlund, J.T.; Ribeiro, R.C.; Krynetski, E.Y.; Pui, C.H.; Evans, W.E. Mercaptopurine therapy intolerance and heterozygosity at the thiopurine S-methyltransferase gene locus. *J. Natl. Cancer Inst.*, **1999**, *91*, 2001-2008.
- [11] Stocco, G.; Crews, K.R.; Evans, W.E. Genetic polymorphism of inosine-triphosphate-pyrophosphatase influences mercaptopurine metabolism and toxicity during treatment of acute lymphoblastic leukemia individualized for thiopurine-S-methyl-transferase status. *Expert Opin. Drug Saf.*, **2010**, *9*, 23-37.
- [12] Stocco, G.; Cheok, M.H.; Crews, K.R.; Dervieux, T.; French, D.; Pei, D.; Yang, W.; Cheng, C.; Pui, C.H.; Relling, M.V.; Evans, W.E. Genetic polymorphism of inosine triphosphate pyrophosphatase is a determinant of mercaptopurine metabolism and toxicity during treatment for acute lymphoblastic leukemia. *Clin. Pharmacol. Ther.*, **2009**, *85*, 164-172.
- [13] Paugh, S.W.; Stocco, G.; McCorkle, J.R.; Diouf, B.; Crews, K.R.; Evans, W.E. Cancer pharmacogenomics. *Clin. Pharmacol. Ther.*, **2011**, *90*, 461-466.
- [14] Adam de Beaumais, T.; Dervieux, T.; Fakhoury, M.; Medard, Y.; Azougagh, S.; Zhang, D.; Yakouben, K.; Jacqz-Aigrain, E. The impact of high-dose methotrexate on intracellular 6-mercaptopurine disposition during interval therapy of childhood acute lymphoblastic leukemia. *Cancer Chemother. Pharmacol.*, **2010**, *66*, 653-658.
- [15] Marsh, S.; Van Booven, D.J. The increasing complexity of mercaptopurine pharmacogenomics. *Clin. Pharmacol. Ther.*, **2009**, *85*, 139-141.
- [16] Dorababu, P.; Nagesh, N.; Linga, V.G.; Gundeti, S.; Kutala, V.K.; Reddanna, P.; Digumarti, R. Epistatic interactions between thiopurine methyltransferase (TPMT) and inosine triphosphate pyrophosphatase (ITPA) variations determine 6-mercaptopurine toxicity in Indian children with acute lymphoblastic leukemia. *Eur. J. Clin. Pharmacol.*, **2012**, *68*, 379-387.
- [17] Dorababu, P.; Naushad, S.M.; Linga, V.G.; Gundeti, S.; Nagesh, N.; Kutala, V.K.; Reddanna, P.; Digumarti, R. Genetic variants of thiopurine and folate metabolic pathways determine 6-MP-mediated hematological toxicity in childhood ALL. *Pharmacogenomics*, **2012**, *13*, 1001-1008.
- [18] Kim, H.; Kang, H.J.; Kim, H.J.; Jang, M.K.; Kim, N.H.; Oh, Y.; Han, B.D.; Choi, J.Y.; Kim, C.W.; Lee, J.W.; Park, K.D.; Shin, H.Y.; Ahn, H.S. Pharmacogenetic Analysis of Pediatric Patients with Acute Lymphoblastic Leukemia: A Possible Association between Survival Rate and ITPA Polymorphism. *PLoS One*, **2012**, *7*, e45558.
- [19] Tanaka, Y.; Manabe, A.; Nakadate, H.; Kondoh, K.; Nakamura, K.; Koh, K.; Utano, T.; Kikuchi, A.; Komiyama, T. The activity of the inosine triphosphate pyrophosphatase affects toxicity of 6-mercaptopurine during maintenance therapy for acute lymphoblastic leukemia in Japanese children. *Leuk. Res.*, **2012**, *36*, 560-564.
- [20] Stocco, G.; Franca, R.; Verzegnassi, F.; Londero, M.; Rabusin, M.; Decorti, G. Multilocus genotypes of relevance for drug metabolizing enzymes and therapy with thiopurines in patients with acute lymphoblastic leukemia. *Front. Genet.*, **2013**, *2*, 3, 309.
- [21] Trevino, L.R.; Shimasaki, N.; Yang, W.; Panetta, J.C.; Cheng, C.; Pei, D.; Chan, D.; Sparreboom, A.; Giacomini, K.M.; Pui, C.H.; Evans, W.E.; Relling, M.V. Germline genetic variation in an organic anion transporter polypeptide associated with methotrexate pharmacokinetics and clinical effects. *J. Clin. Oncol.*, **2009**, *27*, 5972-5978.
- [22] Stocco, G.; Yang, W.; Crews, K.R.; Thierfelder, W.E.; Decorti, G.; Londero, M.; Franca, R.; Rabusin, M.; Valsecchi, M.G.; Pei, D.; Cheng, C.; Paugh, S.W.; Ramsey, L.B.; Diouf, B.; McCorkle, J.R.; Jones, T.S.; Pui, C.H.; Relling, M.V.; Evans, W.E. PACSIN2 polymorphism influences TPMT activity and mercaptopurine-related gastrointestinal toxicity. *Hum. Mol. Genet.*, **2012**, *21*, 4793-4804.
- [23] Li, F.; Fridley, B.L.; Matimba, A.; Kalari, K.R.; Pellemounter, L.; Moon, I.; Ji, Y.; Jenkins, G.D.; Batzler, A.; Wang, L.; Weinschl boum, R.M. Ecto-5'-nucleotidase and thiopurine cellular circulation: association with cytotoxicity. *Drug Metab. Dispos.*, **2010**, *38*, 2329-2338.
- [24] Mikkelsen, T.S.; Thorn, C.F.; Yang, J.J.; Ulrich, C.M.; French, D.; Zaza, G.; Dunnenberger, H.M.; Marsh, S.; McLeod, H.L.; Giacomini, K.; Becker, M.L.; Gaedigk, R.; Leeder, J.S.; Kager, L.; Relling, M.V.; Evans, W.; Klein, T.E.; Altman, R.B. PharmGKB summary: methotrexate pathway. *Pharmacogenet. Genomics*, **2011**, *21*, 679-686.
- [25] Stark, M.; Wichman, C.; Avivi, I.; Assaraf, Y.G. Aberrant splicing of folylpolyglutamate synthetase as a novel mechanism of antifolate resistance in leukemia. *Blood*, **2009**, *113*, 4362-4369.
- [26] Cheng, Q.; Cheng, C.; Crews, K.R.; Ribeiro, R.C.; Pui, C.H.; Relling, M.V.; Evans, W.E. Epigenetic regulation of human gamma-glutamyl hydrolase activity in acute lymphoblastic leukemia cells. *Am. J. Hum. Genet.*, **2006**, *79*, 264-274.
- [27] Schmiegelow, K. Advances in individual prediction of methotrexate toxicity: a review. *Br. J. Haematol.*, **2009**, *146*, 489-503.
- [28] de Beaumais, T.A.; Jacqz-Aigrain, E. Intracellular disposition of methotrexate in acute lymphoblastic leukemia in children. *Curr. Drug. Metab.*, **2012**, *13*, 822-834.
- [29] Salazar, J.; Altes, A.; del Rio, E.; Estella, J.; Rives, S.; Tasso, M.; Navajas, A.; Molina, J.; Villa, M.; Vivanco, J.L.; Torrent, M.; Baiget, M.; Badell, I. Methotrexate consolidation treatment according to pharmacogenetics of MTHFR ameliorates event-free survival in childhood acute lymphoblastic leukaemia. *Pharmacogenomics J.*, **2012**, *12*, 379-385.
- [30] 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. *Pharmacogenomics J.*, **2012**, doi: 10.1038/tpj.2012.44. [Epub ahead of print].
- [31] Spyridopoulou, K.P.; Dimou, N.L.; Hamodrakas, S.J.; Bagos, P.G. Methylene tetrahydrofolate reductase gene polymorphisms and their association with methotrexate toxicity: a meta-analysis. *Pharmacogenet. Genomics*, **2012**, *22*, 117-133.
- [32] Lopez-Lopez, E.; Martin-Guerrero, I.; Ballesteros, J.; Pinan, M.A.; Garcia-Miguel, P.; Navajas, A.; Garcia-Orad, A. Polymorphisms of the SLC01B1 gene predict methotrexate-related toxicity in childhood acute lymphoblastic leukemia. *Pediatr. Blood Cancer*, **2011**, *57*, 612-619.
- [33] Ramsey, L.B.; Bruun, G.H.; Yang, W.; Trevino, L.R.; Vattathil, S.; Scheet, P.; Cheng, C.; Rosner, G.L.; Giacomini, K.M.; Fan, Y.; Sparreboom, A.; Mikkelsen, T.S.; Corydon, T.J.; Pui, C.H.; Evans, W.E.; Relling, M.V. Rare versus common variants in pharmacogenetics: SLC01B1 variation and methotrexate disposition. *Genome Res.*, **2012**, *22*, 1-8.
- [34] Pui, C.H.; Campana, D.; Pei, D.; Bowman, W.P.; Sandlund, J.T.; Kaste, S.C.; Ribeiro, R.C.; Rubnitz, J.E.; Raimondi, S.C.; Onciu, M.; Coustan-Smith, E.; Kun, L.E.; Jeha, S.; Cheng, C.; Howard, S.C.; Simmons, V.; Bayles, A.; Metzger, M.L.; Boyett, J.M.; Leung, W.; Handgretinger, R.; Downing, J.R.; Evans, W.E.; Relling, M.V. Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N. Engl. J. Med.*, **2009**, *360*, 2730-2741.
- [35] Mikkelsen, T.S.; Sparreboom, A.; Cheng, C.; Zhou, Y.; Boyett, J.M.; Raimondi, S.C.; Panetta, J.C.; Bowman, W.P.; Sandlund, J.T.; Pui, C.H.; Relling, M.V.; Evans, W.E. Shortening infusion time for high-dose methotrexate alters antileukemic effects: a

- randomized prospective clinical trial. *J. Clin. Oncol.*, **2011**, *29*, 1771-1778.
- [36] Nordeen, S.K.; Suh, B.J.; Kuhnel, B.; Hutchison, C.A., 3rd. Structural determinants of a glucocorticoid receptor recognition element. *Mol. Endocrinol.*, **1990**, *4*, 1866-1873.
- [37] Kaspers, G.J.; Pieters, R.; Van Zantwijk, C.H.; Van Wering, E.R.; Van Der Does-Van Den Berg, A.; Veerman, A.J. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood*, **1998**, *92*, 259-266.
- [38] Hongo, T.; Yajima, S.; Sakurai, M.; Horikoshi, Y.; Hanada, R. *In vitro* drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia. *Blood*, **1997**, *89*, 2959-2965.
- [39] Den Boer, M.L.; Harms, D.O.; Pieters, R.; Kazemier, K.M.; Gobel, U.; Korholz, D.; Graubner, U.; Haas, R.J.; Jorch, N.; Spaar, H.J.; Kaspers, G.J.; Kamps, W.A.; Van der Does-Van den Berg, A.; Van Wering, E.R.; Veerman, A.J.; Janka-Schaub, G.E. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *J. Clin. Oncol.*, **2003**, *21*, 3262-3268.
- [40] De Iudicibus, S.; Franca, R.; Martelossi, S.; Ventura, A.; Decorti, G. Molecular mechanism of glucocorticoid resistance in inflammatory bowel disease. *World J. Gastroenterol.*, **2011**, *17*, 1095-1108.
- [41] Franca, R.; Rebori, P.; Basso, G.; Biondi, A.; Cazzaniga, G.; Crovella, S.; Decorti, G.; Fagioli, F.; Giarin, E.; Locatelli, F.; Poggi, V.; Valsecchi, M.G.; Rabusin, M. Glutathione S-transferase homozygous deletions and relapse in childhood acute lymphoblastic leukemia: a novel study design in a large Italian AIEOP cohort. *Pharmacogenomics*, **2012**, *16*, 1905-1916.
- [42] Rocha, J.C.; Cheng, C.; Liu, W.; Kishi, S.; Das, S.; Cook, E.H.; Sandlund, J.T.; Rubnitz, J.; Ribeiro, R.; Campana, D.; Pui, C.H.; Evans, W.E.; Relling, M.V. Pharmacogenetics of outcome in children with acute lymphoblastic leukemia. *Blood*, **2005**, *105*, 4752-4758.
- [43] Relling, M.V.; Yang, W.; Das, S.; Cook, E.H.; Rosner, G.L.; Neel, M.; Howard, S.; Ribeiro, R.; Sandlund, J.T.; Pui, C.H.; Kaste, S.C. Pharmacogenetic risk factors for osteonecrosis of the hip among children with leukemia. *J. Clin. Oncol.*, **2004**, *22*, 3930-3936.
- [44] Kawedia, J.D.; Kaste, S.C.; Pei, D.; Panetta, J.C.; Cai, X.; Cheng, C.; Neale, G.; Howard, S.C.; Evans, W.E.; Pui, C.H.; Relling, M.V. Pharmacokinetic, pharmacodynamic, and pharmacogenetic determinants of osteonecrosis in children with acute lymphoblastic leukemia. *Blood*, **2011**, *117*, 2340-2347.
- [45] Bottini, N.; MacMurray, J.; Peters, W.; Rostamkhani, M.; Comings, D.E. Association of the acid phosphatase (ACP1) gene with triglyceride levels in obese women. *Mol. Genet. Metab.*, **2002**, *77*, 226-229.
- [46] Zambuzzi, W.F.; Granjeiro, J.M.; Parikh, K.; Yuvaraj, S.; Peppelenbosch, M.P.; Ferreira, C.V. Modulation of Src activity by low molecular weight protein tyrosine phosphatase during osteoblast differentiation. *Cell Physiol. Biochem.*, **2008**, *22*, 497-506.
- [47] French, D.; Hamilton, L.H.; Mattano, L.A., Jr.; Sather, H.N.; Devidas, M.; Nachman, J.B.; Relling, M.V. A PAI-1 (SERPINE1) polymorphism predicts osteonecrosis in children with acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Blood*, **2008**, *111*, 4496-4499.
- [48] Bond, J.; Adams, S.; Richards, S.; Vora, A.; Mitchell, C.; Goulden, N. Polymorphism in the PAI-1 (SERPINE1) gene and the risk of osteonecrosis in children with acute lymphoblastic leukemia. *Blood*, **2011**, *118*, 2632-2633.
- [49] Kamdem, L.K.; Hamilton, L.; Cheng, C.; Liu, W.; Yang, W.; Johnson, J.A.; Pui, C.H.; Relling, M.V. Genetic predictors of glucocorticoid-induced hypertension in children with acute lymphoblastic leukemia. *Pharmacogenet. Genomics*, **2008**, *18*, 507-514.
- [50] Dennison, J.B.; Kulanthaivel, P.; Barbuch, R.J.; Renbarger, J.L.; Ehlhardt, W.J.; Hall, S.D. Selective metabolism of vincristine *in vitro* by CYP3A5. *Drug. Metab. Dispos.*, **2006**, *34*, 1317-1327.
- [51] Dennison, J.B.; Jones, D.R.; Renbarger, J.L.; Hall, S.D. Effect of CYP3A5 expression on vincristine metabolism with human liver microsomes. *J. Pharmacol. Exp. Ther.*, **2007**, *321*, 553-563.
- [52] Xie, H.G.; Wood, A.J.; Kim, R.B.; Stein, C.M.; Wilkinson, G.R. Genetic variability in CYP3A5 and its possible consequences. *Pharmacogenomics*, **2004**, *5*, 243-272.
- [53] Hustert, E.; Haberl, M.; Burk, O.; Wolbold, R.; He, Y.Q.; Klein, K.; Nuessler, A.C.; Neuhaus, P.; Klattig, J.; Eiselt, R.; Koch, I.; Zibat, A.; Brockmoller, J.; Halpert, J.R.; Zanger, U.M.; Wojnowski, L. The genetic determinants of the CYP3A5 polymorphism. *Pharmacogenetics*, **2001**, *11*, 773-779.
- [54] Kuehl, P.; Zhang, J.; Lin, Y.; Lamba, J.; Assem, M.; Schuetz, J.; Watkins, P.B.; Daly, A.; Wrighton, S.A.; Hall, S.D.; Maurel, P.; Relling, M.; Brimer, C.; Yasuda, K.; Venkataramanan, R.; Strom, S.; Thummel, K.; Boguski, M.S.; Schuetz, E. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat. Genet.*, **2001**, *27*, 383-391.
- [55] Lamba, J.K.; Lin, Y.S.; Schuetz, E.G.; Thummel, K.E. Genetic contribution to variable human CYP3A-mediated metabolism. *Adv. Drug. Deliv. Rev.*, **2002**, *54*, 1271-1294.
- [56] Groninger, E.; Meeuwse-de Boar, T.; Koopmans, P.; Uges, D.; Sluiter, W.; Veerman, A.; Kamps, W.; de Graaf, S. Pharmacokinetics of vincristine monotherapy in childhood acute lymphoblastic leukemia. *Pediatr. Res.*, **2002**, *52*, 113-118.
- [57] Egbelakin, A.; Ferguson, M.J.; MacGill, E.A.; Lehmann, A.S.; Topletz, A.R.; Quinney, S.K.; Li, L.; McCammack, K.C.; Hall, S.D.; Renbarger, J.L. Increased risk of vincristine neurotoxicity associated with low CYP3A5 expression genotype in children with acute lymphoblastic leukemia. *Pediatr. Blood Cancer*, **2011**, *56*, 361-367.
- [58] Borst, L.; Wallerek, S.; Dalhoff, K.; Rasmussen, K.K.; Wesenberg, F.; Wehner, P.S.; Schmiegelow, K. The impact of CYP3A5\*3 on risk and prognosis in childhood acute lymphoblastic leukemia. *Eur. J. Haematol.*, **2011**, *86*, 477-483.
- [59] Aplenc, R.; Glatfelter, W.; Han, P.; Rappaport, E.; La, M.; Cnaan, A.; Blackwood, M.A.; Lange, B.; Rebbeck, T. CYP3A genotypes and treatment response in paediatric acute lymphoblastic leukaemia. *Br. J. Haematol.*, **2003**, *122*, 240-244.
- [60] Lamba, J.; Hebert, J.M.; Schuetz, E.G.; Klein, T.E.; Altman, R.B. PharmGKB summary: very important pharmacogene information for CYP3A5. *Pharmacogenet. Genomics*, **2012**, *22*, 555-558.
- [61] Thorn, C.F.; Ji, Y.; Weinshilboum, R.M.; Altman, R.B.; Klein, T.E. PharmGKB summary: very important pharmacogene information for GSTT1. *Pharmacogenet. Genomics*, **2012**, *22*, 646-651.
- [62] Hodges, L.M.; Markova, S.M.; Chinn, L.W.; Gow, J.M.; Kroetz, D.L.; Klein, T.E.; Altman, R.B. Very important pharmacogene summary: ABCB1 (MDR1, P-glycoprotein). *Pharmacogenet. Genomics*, **2011**, *21*, 152-161.
- [63] Wertz, I.E.; Kusam, S.; Lam, C.; Okamoto, T.; Sandoval, W.; Anderson, D.J.; Helgason, E.; Ernst, J.A.; Eby, M.; Liu, J.; Belmont, L.D.; Kaminker, J.S.; O'Rourke, K.M.; Pujara, K.; Kohli, P.B.; Johnson, A.R.; Chiu, M.L.; Lill, J.R.; Jackson, P.K.; Fairbrother, W.J.; Seshagiri, S.; Ludlam, M.J.; Leong, K.G.; Dueber, E.C.; Maecker, H.; Huang, D.C.; Dixit, V.M. Sensitivity to anti-tubulin chemotherapeutics is regulated by MCL1 and FBW7. *Nature*, **2011**, *471*, 110-114.
- [64] Wang, Z.; Fukushima, H.; Gao, D.; Inuzuka, H.; Wan, L.; Lau, A.W.; Liu, P.; Wei, W. The two faces of FBW7 in cancer drug resistance. *Bioessays*, **2011**, *33*, 851-859.
- [65] Inuzuka, H.; Shaik, S.; Onoyama, I.; Gao, D.; Tseng, A.; Maser, R.S.; Zhai, B.; Wan, L.; Gutierrez, A.; Lau, A.W.; Xiao, Y.; Christie, A.L.; Aster, J.; Settleman, J.; Gygi, S.P.; Kung, A.L.; Look, T.; Nakayama, K.I.; DePinho, R.A.; Wei, W. SCF(FBW7) regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. *Nature*, **2011**, *471*, 104-109.
- [66] Maser, R.S.; Choudhury, B.; Campbell, P.J.; Feng, B.; Wong, K.K.; Protopopov, A.; O'Neil, J.; Gutierrez, A.; Ivanova, E.; Perna, I.; Lin, E.; Mani, V.; Jiang, S.; McNamara, K.; Zaghlul, S.; Edkins, S.; Stevens, C.; Brennan, C.; Martin, E.S.; Wiedemeyer, R.; Kabbarah, O.; Nogueira, C.; Histen, G.; Aster, J.; Mansour, M.; Duke, V.; Feroni, L.; Fielding, A.K.; Goldstone, A.H.; Rowe, J.M.; Wang, Y.A.; Look, A.T.; Stratton, M.R.; Chin, L.; Futreal, P.A.; DePinho, R.A. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. *Nature*, **2007**, *447*, 966-971.
- [67] O'Neil, J.; Grim, J.; Strack, P.; Rao, S.; Tibbitts, D.; Winter, C.; Hardwick, J.; Welcker, M.; Meijerink, J.P.; Pieters, R.; Draetta, G.; Sears, R.; Clurman, B.E.; Look, A.T. FBW7 mutations in leukemic

- cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J. Exp. Med.*, **2007**, *204*, 1813-1824.
- [68] Thompson, B.J.; Buonamici, S.; Sulis, M.L.; Palomero, T.; Vilimas, T.; Basso, G.; Ferrando, A.; Aifantis, I. The SCFFB7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. *J. Exp. Med.*, **2007**, *204*, 1825-1835.
- [69] Cox, C.; Zimmermann, M.; Stanulla, M.; Leible, S.; Schrappe, M.; Ludwig, W.D.; Koehler, R.; Tolle, G.; Bandapalli, O.R.; Breit, S.; Muckenthaler, M.U.; Kulozik, A.E. The favorable effect of activating NOTCH1 receptor mutations on long-term outcome in T-ALL patients treated on the ALL-BFM 2000 protocol can be separated from FBXW7 loss of function. *Leukemia*, **2010**, *24*, 2005-2013.
- [70] Haskell, C.M.; Canellos, G.P. L-asparaginase resistance in human leukemia--asparagine synthetase. *Biochem. Pharmacol.*, **1969**, *18*, 2578-2580.
- [71] Horowitz, B.; Madras, B.K.; Meister, A.; Old, L.J.; Boyes, E.A.; Stockert, E. Asparagine synthetase activity of mouse leukemias. *Science*, **1968**, *160*, 533-535.
- [72] Kiriya, Y.; Kubota, M.; Takimoto, T.; Kitoh, T.; Tanizawa, A.; Akiyama, Y.; Mikawa, H. Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. *Leukemia*, **1989**, *3*, 294-297.
- [73] Hutson, R.G.; Kitoh, T.; Moraga Amador, D.A.; Cosic, S.; Schuster, S.M.; Kilberg, M.S. Amino acid control of asparagine synthetase: relation to asparaginase resistance in human leukemia cells. *Am. J. Physiol.*, **1997**, *272*, C1691-1699.
- [74] den Boer, M.L.; Pieters, R.; Kazemier, K.M.; Rottier, M.M.; Zwaan, C.M.; Kaspers, G.J.; Janka-Schaub, G.; Henze, G.; Creutzig, U.; Scheper, R.J.; Veerman, A.J. Relationship between major vault protein/lung resistance protein, multidrug resistance-associated protein, P-glycoprotein expression, and drug resistance in childhood leukemia. *Blood*, **1998**, *91*, 2092-2098.
- [75] Aslanian, A.M.; Fletcher, B.S.; Kilberg, M.S. Asparagine synthetase expression alone is sufficient to induce L-asparaginase resistance in MOLT-4 human leukaemia cells. *Biochem. J.*, **2001**, *357*, 321-328.
- [76] Aslanian, A.M.; Kilberg, M.S. Multiple adaptive mechanisms affect asparagine synthetase substrate availability in asparaginase-resistant MOLT-4 human leukaemia cells. *Biochem. J.*, **2001**, *358*, 59-67.
- [77] Krejci, O.; Starkova, J.; Otova, B.; Madzo, J.; Kalinova, M.; Hrusak, O.; Trka, J. Upregulation of asparagine synthetase fails to avert cell cycle arrest induced by L-asparaginase in TEL/AML1-positive leukaemic cells. *Leukemia*, **2004**, *18*, 434-441.
- [78] Stams, W.A.; den Boer, M.L.; Holleman, A.; Appel, I.M.; Beverloo, H.B.; van Wering, E.R.; Janka-Schaub, G.E.; Evans, W.E.; Pieters, R. Asparagine synthetase expression is linked with L-asparaginase resistance in TEL-AML1-negative but not TEL-AML1-positive pediatric acute lymphoblastic leukemia. *Blood*, **2005**, *105*, 4223-4225.
- [79] Stams, W.A.; den Boer, M.L.; Beverloo, H.B.; Meijerink, J.P.; Stigter, R.L.; van Wering, E.R.; Janka-Schaub, G.E.; Slater, R.; Pieters, R. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. *Blood*, **2003**, *101*, 2743-2747.
- [80] Panetta, J.C.; Gajjar, A.; Hijiyi, N.; Hak, L.J.; Cheng, C.; Liu, W.; Pui, C.H.; Relling, M.V. Comparison of native E. coli and PEG asparaginase pharmacokinetics and pharmacodynamics in pediatric acute lymphoblastic leukemia. *Clin. Pharmacol. Ther.*, **2009**, *86*, 651-658.
- [81] Kearney, S.L.; Dahlberg, S.E.; Levy, D.E.; Voss, S.D.; Sallan, S.E.; Silverman, L.B. Clinical course and outcome in children with acute lymphoblastic leukemia and asparaginase-associated pancreatitis. *Pediatr. Blood Cancer*, **2009**, *53*, 162-167.
- [82] Pieters, R.; Hunger, S.P.; Boos, J.; Rizzari, C.; Silverman, L.; Baruchel, A.; Goekbuget, N.; Schrappe, M.; Pui, C.H. L-asparaginase treatment in acute lymphoblastic leukemia: a focus on Erwinia asparaginase. *Cancer*, **2011**, *117*, 238-249.
- [83] Kawedia, J.D.; Liu, C.; Pei, D.; Cheng, C.; Fernandez, C.A.; Howard, S.C.; Campana, D.; Panetta, J.C.; Bowman, W.P.; Evans, W.E.; Pui, C.H.; Relling, M.V. Dexamethasone exposure and asparaginase antibodies affect relapse risk in acute lymphoblastic leukemia. *Blood*, **2012**, *119*, 1658-1664.
- [84] Kanehisa, M.; Goto, S.; Sato, Y.; Furumichi, M.; Tanabe, M. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.*, **2012**, *40*, D109-114.
- [85] Kanehisa Alanine, aspartate and glutamate metabolism - Reference pathway [http://www.genome.jp/kegg-bin/show\\_pathway?map00250](http://www.genome.jp/kegg-bin/show_pathway?map00250) (2013-01-03),
- [86] Chen, S.H.; Yang, W.; Fan, Y.; Stocco, G.; Crews, K.R.; Yang, J.J.; Paugh, S.W.; Pui, C.H.; Evans, W.E.; Relling, M.V. A genome-wide approach identifies that the aspartate metabolism pathway contributes to asparaginase sensitivity. *Leukemia*, **2011**, *25*, 66-74.
- [87] Estes, D.A.; Lovato, D.M.; Khawaja, H.M.; Winter, S.S.; Larson, R.S. Genetic alterations determine chemotherapy resistance in childhood T-ALL: modelling in stage-specific cell lines and correlation with diagnostic patient samples. *Br. J. Haematol.*, **2007**, *139*, 20-30.
- [88] Rousseau, J.; Gagne, V.; Labuda, M.; Beaubois, C.; Sinnett, D.; Laverdiere, C.; Moghrabi, A.; Sallan, S.E.; Silverman, L.B.; Neuberger, D.; Kutok, J.L.; Krajcinovic, M. ATF5 polymorphisms influence ATF function and response to treatment in children with childhood acute lymphoblastic leukemia. *Blood*, **2011**, *118*, 5883-5890.
- [89] Al Sarraj, J.; Vinson, C.; Thiel, G. Regulation of asparagine synthetase gene transcription by the basic region leucine zipper transcription factors ATF5 and CHOP. *Biol. Chem.*, **2005**, *386*, 873-879.
- [90] Richards, N.G.; Kilberg, M.S. Asparagine synthetase chemotherapy. *Annu. Rev. Biochem.*, **2006**, *75*, 629-654.
- [91] Franco, R.; Pacheco, R.; Lluís, C.; Ahern, G.P.; O'Connell, P.J. The emergence of neurotransmitters as immune modulators. *Trends Immunol.*, **2007**, *28*, 400-407.
- [92] Pacheco, R.; Gallart, T.; Lluís, C.; Franco, R. Role of glutamate on T-cell mediated immunity. *J. Neuroimmunol.*, **2007**, *185*, 9-19.
- [93] Huffman, D.H.; Benjamin, R.S.; Bachur, N.R. Daunorubicin metabolism in acute nonlymphocytic leukemia. *Clin. Pharmacol. Ther.*, **1972**, *13*, 895-905.
- [94] Dessypris, E.N.; Brenner, D.E.; Hande, K.R. Toxicity of doxorubicin metabolites to human marrow erythroid and myeloid progenitors *in vitro*. *Cancer Treat. Rep.*, **1986**, *70*, 487-490.
- [95] Schott, B.; Robert, J. Comparative activity of anthracycline 13-dihydrimetabolites against rat glioblastoma cells in culture. *Biochem. Pharmacol.*, **1989**, *38*, 4069-4074.
- [96] Olson, R.D.; Mushlin, P.S.; Brenner, D.E.; Fleischer, S.; Cusack, B.J.; Chang, B.K.; Boucek, R.J., Jr. Doxorubicin cardiotoxicity may be caused by its metabolite, doxorubicinol. *Proc. Natl. Acad. Sci. U. S. A.*, **1988**, *85*, 3585-3589.
- [97] Visscher, H.; Ross, C.J.; Rassekh, S.R.; Barhdadi, A.; Dube, M.P.; Al-Saloo, H.; Sandor, G.S.; Caron, H.N.; van Dalen, E.C.; Kremer, L.C.; van der Pal, H.J.; Brown, A.M.; Rogers, P.C.; Phillips, M.S.; Rieder, M.J.; Carleton, B.C.; Hayden, M.R. Pharmacogenomic prediction of anthracycline-induced cardiotoxicity in children. *J. Clin. Oncol.*, **2012**, *30*, 1422-1428.
- [98] Zeller, T.; Wild, P.; Szymczak, S.; Rotival, M.; Schillert, A.; Castagne, R.; Maouche, S.; Germain, M.; Lackner, K.; Rossmann, H.; Eleftheriadis, M.; Sinning, C.R.; Schnabel, R.B.; Lubos, E.; Menerich, D.; Rust, W.; Perret, C.; Proust, C.; Nicaud, V.; Loscalzo, J.; Hubner, N.; Tregouet, D.; Munzel, T.; Ziegler, A.; Tiret, L.; Blankenberg, S.; Cambien, F. Genetics and beyond--the transcriptome of human monocytes and disease susceptibility. *PLoS One*, **2010**, *5*, e10693.
- [99] Nagasawa, K.; Nagai, K.; Ohnishi, N.; Yokoyama, T.; Fujimoto, S. Contribution of specific transport systems to anthracycline transport in tumor and normal cells. *Curr. Drug Metab.*, **2001**, *2*, 355-366.
- [100] Wiley, J.S.; Jones, S.P.; Sawyer, W.H.; Paterson, A.R. Cytosine arabinoside influx and nucleoside transport sites in acute leukemia. *J. Clin. Invest.*, **1982**, *69*, 479-489.
- [101] Kessel, D.; Hall, T.C.; Wodinsky, I. Transport and phosphorylation as factors in the antitumor action of cytosine arabinoside. *Science*, **1967**, *156*, 1240-1241.
- [102] Kufe, D.W.; Major, P.P.; Egan, E.M.; Beardsley, G.P. Correlation of cytotoxicity with incorporation of ara-C into DNA. *J. Biol. Chem.*, **1980**, *255*, 8997-8900.
- [103] Kufe, D.W.; Munroe, D.; Herrick, D.; Egan, E.; Spriggs, D. Effects of 1-beta-D-arabinofuranosylcytosine incorporation on eukaryotic DNA template function. *Mol. Pharmacol.*, **1984**, *26*, 128-134.

- [104] Lamba, J.K. Genetic factors influencing cytarabine therapy. *Pharmacogenomics*, **2009**, *10*, 1657-1674.
- [105] Galmarini, C.M.; Thomas, X.; Calvo, F.; Rousselot, P.; Rabilloud, M.; El Jaffari, A.; Cros, E.; Dumontet, C. *In vivo* mechanisms of resistance to cytarabine in acute myeloid leukaemia. *Br. J. Haematol.*, **2002**, *117*, 860-868.
- [106] Stam, R.W.; den Boer, M.L.; Meijerink, J.P.; Ebus, M.E.; Peters, G.J.; Noordhuis, P.; Janka-Schaub, G.E.; Armstrong, S.A.; Korsmeyer, S.J.; Pieters, R. Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. *Blood*, **2003**, *101*, 1270-1276.
- [107] Galmarini, C.M.; Cros, E.; Thomas, X.; Jordheim, L.; Dumontet, C. The prognostic value of cN-II and cN-III enzymes in adult acute myeloid leukemia. *Haematologica*, **2005**, *90*, 1699-1701.
- [108] Galmarini, C.M.; Thomas, X.; Calvo, F.; Rousselot, P.; El Jafaari, A.; Cros, E.; Dumontet, C. Potential mechanisms of resistance to cytarabine in AML patients. *Leuk. Res.*, **2002**, *26*, 621-629.
- [109] Jahns-Streubel, G.; Reuter, C.; Auf der Landwehr, U.; Unterhalt, M.; Schleyer, E.; Wormann, B.; Buchner, T.; Hiddemann, W. Activity of thymidine kinase and of polymerase alpha as well as activity and gene expression of deoxycytidine deaminase in leukemic blasts are correlated with clinical response in the setting of granulocyte-macrophage colony-stimulating factor-based priming before and during TAD-9 induction therapy in acute myeloid leukemia. *Blood*, **1997**, *90*, 1968-1976.
- [110] Schroder, J.K.; Kirch, C.; Seeber, S.; Schutte, J. Structural and functional analysis of the cytidine deaminase gene in patients with acute myeloid leukaemia. *Br. J. Haematol.*, **1998**, *103*, 1096-1103.
- [111] Lamba, J.K.; Crews, K.; Pounds, S.; Schuetz, E.G.; Gresham, J.; Gandhi, V.; Plunkett, W.; Rubnitz, J.; Ribeiro, R. Pharmacogenetics of deoxycytidine kinase: identification and characterization of novel genetic variants. *J. Pharmacol. Exp. Ther.*, **2007**, *323*, 935-945.
- [112] Bhatla, D.; Gerbing, R.B.; Alonzo, T.A.; Conner, H.; Ross, J.A.; Meshinchi, S.; Zhai, X.; Zamzow, T.; Mehta, P.A.; Geiger, H.; Perentesis, J.; Davies, S.M. Cytidine deaminase genotype and toxicity of cytosine arabinoside therapy in children with acute myeloid leukemia. *Br. J. Haematol.*, **2009**, *144*, 388-394.
- [113] Hartford, C.M.; Duan, S.; Delaney, S.M.; Mi, S.; Kistner, E.O.; Lamba, J.K.; Huang, R.S.; Dolan, M.E. Population-specific genetic variants important in susceptibility to cytarabine arabinoside cytotoxicity. *Blood*, **2009**, *113*, 2145-2153.
- [114] Lamba, J.K.; Crews, K.R.; Pounds, S.B.; Cao, X.; Gandhi, V.; Plunkett, W.; Razzouk, B.I.; Lamba, V.; Baker, S.D.; Raimondi, S.C.; Campana, D.; Pui, C.H.; Downing, J.R.; Rubnitz, J.E.; Ribeiro, R.C. Identification of predictive markers of cytarabine response in AML by integrative analysis of gene-expression profiles with multiple phenotypes. *Pharmacogenomics*, **2011**, *12*, 327-339.
- [115] Hu, L.; Zhuo, W.; He, Y.J.; Zhou, H.H.; Fan, L. Pharmacogenetics of P450 oxidoreductase: implications in drug metabolism and therapy. *Pharmacogenet. Genomics*, **2012**, *22*, 812-819.
- [116] Whirl-Carrillo, M.; McDonagh, E.M.; Hebert, J.M.; Gong, L.; Sangkuhl, K.; Thorn, C.F.; Altman, R.B.; Klein, T.E. Pharmacogenomics knowledge for personalized medicine. *Clin. Pharmacol. Ther.*, **2012**, *92*, 414-417.
- [117] Thorn, C.F.; Lamba, J.K.; Lamba, V.; Klein, T.E.; Altman, R.B. PharmGKB summary: very important pharmacogene information for CYP2B6. *Pharmacogenet. Genomics*, **2010**, *20*, 520-523.
- [118] Porter, T.D.; Coon, M.J. Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J. Biol. Chem.*, **1991**, *266*, 13469-13472.
- [119] Chen, L.; Yu, L.J.; Waxman, D.J. Potentiation of cytochrome P450/cyclophosphamide-based cancer gene therapy by coexpression of the P450 reductase gene. *Cancer Res.*, **1997**, *57*, 4830-4837.
- [120] Klein, T.E.; Chang, J.T.; Cho, M.K.; Easton, K.L.; Fergerson, R.; Hewett, M.; Lin, Z.; Liu, Y.; Liu, S.; Oliver, D.E.; Rubin, D.L.; Shafa, F.; Stuart, J.M.; Altman, R.B. Integrating genotype and phenotype information: an overview of the PharmGKB project. Pharmacogenetics Research Network and Knowledge Base. *Pharmacogenomics J.*, **2001**, *1*, 167-170.
- [121] Buchdunger, E.; Zimmermann, J.; Mett, H.; Meyer, T.; Muller, M.; Druker, B.J.; Lydon, N.B. Inhibition of the Abl protein-tyrosine kinase *in vitro* and *in vivo* by a 2-phenylaminopyrimidine derivative. *Cancer Res.*, **1996**, *56*, 100-104.
- [122] Biondi, A.; Schrappe, M.; De Lorenzo, P.; Castor, A.; Lucchini, G.; Gandemer, V.; Pieters, R.; Stary, J.; Escherich, G.; Campbell, M.; Li, C.K.; Vora, A.; Arico, M.; Rottgers, S.; Saha, V.; Valsecchi, M.G. Imatinib after induction for treatment of children and adolescents with Philadelphia-chromosome-positive acute lymphoblastic leukaemia (EsPhALL): a randomised, open-label, intergroup study. *Lancet Oncol.*, **2012**, *13*, 936-945.
- [123] Petain, A.; Kattygnarath, D.; Azard, J.; Chatelut, E.; Delbaldo, C.; Georger, B.; Barrois, M.; Seronie-Vivien, S.; LeCesne, A.; Vassal, G. Population pharmacokinetics and pharmacogenetics of imatinib in children and adults. *Clin. Cancer Res.*, **2008**, *14*, 7102-7109.
- [124] Peng, B.; Lloyd, P.; Schran, H. Clinical pharmacokinetics of imatinib. *Clin. Pharmacokinet.*, **2005**, *44*, 879-894.
- [125] Deininger, M.; Buchdunger, E.; Druker, B.J. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood*, **2005**, *105*, 2640-2653.
- [126] Savonarola, A.; Palmirotta, R.; Guadagni, F.; Silvestris, F. Pharmacogenetics and pharmacogenomics: role of mutational analysis in anti-cancer targeted therapy. *Pharmacogenomics J.*, **2012**, *12*, 277-286.
- [127] Dulucq, S.; Krajcinovic, M. The pharmacogenetics of imatinib. *Genome Med.*, **2010**, *2*, 85.
- [128] Angelini, S.; Soverini, S.; Ravegnini, G.; Barnett, M.; Turrini, E.; Thornquist, M.; Pane, F.; Hughes, T.P.; White, D.L.; Radich, J.; Kim, D.W.; Saglio, G.; Cilloni, D.; Iacobucci, I.; Perini, G.; Woodman, R.; Cantelli-Forti, G.; Baccarani, M.; Hrelia, P.; Martinelli, G. Association between imatinib transporters and metabolizing enzymes genotype and response in newly diagnosed chronic myeloid leukemia patients receiving imatinib therapy. *Haematologica*, **2012**, *98*, 193-200.
- [129] Gandhi, V.; Plunkett, W. Cellular and clinical pharmacology of fludarabine. *Clin. Pharmacokinet.*, **2002**, *41*, 93-103.
- [130] Rivero, A.; Rapado, I.; Tomas, J.F.; Montalban, C.; de Ona, R.; Paz-Carreira, J.; Canales, M.; Martinez, R.; Sanchez-Godoy, P.; de Sevilla, A.F.; de la Serna, J.; Martinez-Lopez, J. Relationship between deoxycytidine kinase (DCK) genotypic variants and fludarabine toxicity in patients with follicular lymphoma. *Leuk. Res.*, **2011**, *35*, 431-437.
- [131] Quarello, P.; Berger, M.; Rivetti, E.; Galletto, C.; Masetti, R.; Manicone, R.; Barisone, E.; Pession, A.; Fagioli, F. FLAG-liposomal doxorubicin (Myocet) regimen for refractory or relapsed acute leukemia pediatric patients. *J. Pediatr. Hematol. Oncol.*, **2012**, *34*, 208-216.
- [132] Lotfi, K.; Mansson, E.; Spasokoukotskaja, T.; Pettersson, B.; Liliemark, J.; Peterson, C.; Eriksson, S.; Albertioni, F. Biochemical pharmacology and resistance to 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, a novel analogue of cladribine in human leukemic cells. *Clin. Cancer Res.*, **1999**, *5*, 2438-2444.
- [133] Genini, D.; Adachi, S.; Chao, Q.; Rose, D.W.; Carrera, C.J.; Cottam, H.B.; Carson, D.A.; Leoni, L.M. Deoxyadenosine analogs induce programmed cell death in chronic lymphocytic leukemia cells by damaging the DNA and by directly affecting the mitochondria. *Blood*, **2000**, *96*, 3537-3543.
- [134] Bonate, P.L.; Arthaud, L.; Cantrell, W.R., Jr.; Stephenson, K.; Secrist, J.A., 3rd; Weitman, S. Discovery and development of clofarabine: a nucleoside analogue for treating cancer. *Nat. Rev. Drug Discov.*, **2006**, *5*, 855-863.
- [135] Hijiva, N.; Barry, E.; Arceci, R. Clofarabine in pediatric acute leukemia: current findings and issues. *Pediatric Blood Cancer*, **2012**, *59*, 417-422.
- [136] Zhang, Y.; Shahrari, M.; Zhang, J.; Ahmed, S.U.; Lim, S.H. Clofarabine induces hypomethylation of DNA and expression of Cancer-Testis antigens. *Leuk. Res.*, **2009**, *33*, 1678-1683.
- [137] Evans, W.E.; Relling, M.V. Moving towards individualized medicine with pharmacogenomics. *Nature*, **2004**, *429*, 464-468.
- [138] Link, E.; Parish, S.; Armitage, J.; Bowman, L.; Heath, S.; Matsuda, F.; Gut, I.; Lathrop, M.; Collins, R. SLCO1B1 variants and statin-induced myopathy—a genome-wide study. *N. Engl. J. Med.*, **2008**, *359*, 789-799.
- [139] Yang, J.J.; Cheng, C.; Yang, W.; Pei, D.; Cao, X.; Fan, Y.; Pounds, S.B.; Neale, G.; Trevino, L.R.; French, D.; Campana, D.; Downing, J.R.; Evans, W.E.; Pui, C.H.; Devidas, M.; Bowman, W.P.; Camitta, B.M.; Willman, C.L.; Davies, S.M.; Borowitz, M.J.; Carroll, W.L.; Hunger, S.P.; Relling, M.V. Genome-wide

- interrogation of germline genetic variation associated with treatment response in childhood acute lymphoblastic leukemia. *JAMA*, **2009**, *301*, 393-403.
- [140] Yang, J.J.; Cheng, C.; Devidas, M.; Cao, X.; Campana, D.; Yang, W.; Fan, Y.; Neale, G.; Cox, N.; Scheet, P.; Borowitz, M.J.; Winick, N.J.; Martin, P.L.; Bowman, P.; Camitta, B.; Reaman, G.H.; Carroll, W.L.; Willman, C.L.; Hunger, S.P.; Evans, W.E.; Pui, C.H.; Loh, M.; Relling, M.V. Genome-wide association study identifies germline polymorphisms associated with relapse of childhood acute lymphoblastic leukemia. *Blood*, **2012**, *120*, 4197-4204.
- [141] Pui, C.H.; Mullighan, C.G.; Evans, W.E.; Relling, M.V. Pediatric acute lymphoblastic leukemia: where are we going and how do we get there? *Blood*, **2012**, *120*, 1165-1174.
- [142] Mody, R.; Li, S.; Dover, D.C.; Sallan, S.; Leisenring, W.; Oeffinger, K.C.; Yasui, Y.; Robison, L.L.; Neglia, J.P. Twenty-five-year follow-up among survivors of childhood acute lymphoblastic leukemia: a report from the Childhood Cancer Survivor Study. *Blood*, **2008**, *111*, 5515-5523.
- [143] Pui, C.H.; Cheng, C.; Leung, W.; Rai, S.N.; Rivera, G.K.; Sandlund, J.T.; Ribeiro, R.C.; Relling, M.V.; Kun, L.E.; Evans, W.E.; Hudson, M.M. Extended follow-up of long-term survivors of childhood acute lymphoblastic leukemia. *N. Engl. J. Med.*, **2003**, *349*, 640-649.

# Deletion of Glutathione-S-Transferase M1 Reduces Azathioprine Metabolite Concentrations in Young Patients With Inflammatory Bowel Disease

Gabriele Stocco, PhD,\* Eva Cuzzoni, DSc,† Sara De Iudicibus, PhD,‡ Raffaella Franca, PhD,‡  
Diego Favretto, BSc,\* Noelia Malusà, DSc,§ Margherita Londero, MD,†|| Gabriele Cont, MD,‡  
Fiara Bartoli, MD,† Stefano Martelossi, MD,‡ Alessandro Ventura, MD,†‡  
and Giuliana Decorti, MD\*

**Goals:** To investigate, in young patients with inflammatory bowel disease (IBD) treated with azathioprine, the association between genetic polymorphisms of thiopurine-S-methyl-transferase (TPMT), inosine-triphosphate-pyrophosphatase (ITPA), and glutathione-S-transferases (GST), involved in azathioprine metabolism, the concentration of the main metabolites of azathioprine, thioguanine nucleotides (TGNs) and the methylated nucleotides (MMPN), and the dose of the medication.

**Background:** Azathioprine is widely used in IBD as an immunosuppressive agent, particularly to maintain remission in patients with steroid refractory disease. Azathioprine is a prodrug and requires conversion to its active form mercaptopurine, which has no intrinsic activity, and is activated by the enzymes of the purine salvage pathway to TGNs. Polymorphisms in genes of enzymes involved in azathioprine metabolism influence the efficacy and toxicity of treatment.

**Study:** Seventy-five young patients with IBD treated with azathioprine at least for 3 months were enrolled and genotyped for the selected genes; for these patients, TGN and MMPN metabolites were measured by high performance liquid chromatography in erythrocytes.

**Results:** GST-M1 deletion was associated with lower TGN/dose ratio ( $P = 0.0030$ ), higher azathioprine dose requirement ( $P = 0.022$ ), and reduced response to therapy ( $P = 0.0022$ ). TPMT variant genotype was associated with lower MMPN concentration ( $P = 0.0064$ ) and increased TGN/dose ratio ( $P = 0.0035$ ). ITPA C94A polymorphism resulted in an increased MMPN concentration ( $P = 0.037$ ).

**Conclusions:** This study describes the effect of candidate genetic polymorphisms in TPMT, ITPA, and GST-M1 on azathioprine pharmacokinetics in IBD patients, showing, for the first time, relevant effects of GST-M1 genotype on azathioprine metabolites concentration.

**Key Words:** azathioprine, inflammatory bowel disease, thiopurine-S-methyl-transferase, glutathione-S-transferases, pharmacogenetics (*J Clin Gastroenterol* 2013;00:000–000)

Received for publication October 27, 2012; accepted February 1, 2013. From the Departments of \*Life Sciences; †Medical, Surgical, and Health Sciences; ‡Scuola di Dottorato di Ricerca in Scienze della Riproduzione, University of Trieste; §Institute for Maternal and Child Health IRCCS Burlo Garofolo; and §Department of Prevention, Sanitary Services Agency Number 1, Trieste, Italy.

S.D.I. was supported by Associazione Azzurra and by a fellowship of IRCCS Burlo Garofolo. R.F. is supported by a fellowship of IRCCS Burlo Garofolo. This study was supported by "Fondazione Benefica Kathleen Foreman Casali."

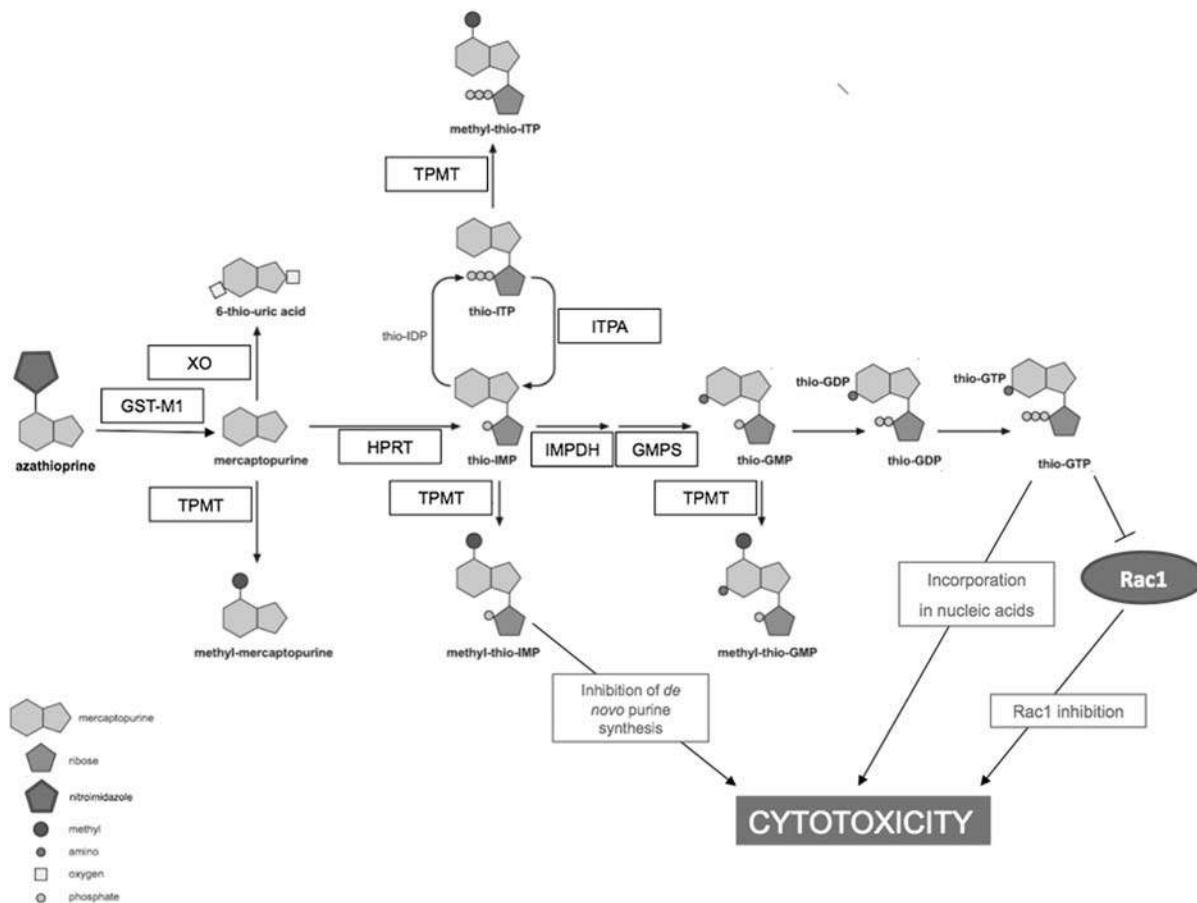
The authors declare that they have nothing to disclose.

Reprints: Giuliana Decorti, MD, Department of Life Sciences, University of Trieste, via Fleming 22, Trieste I-34127, Italy (e-mail: decorti@units.it).

Copyright © 2013 by Lippincott Williams & Wilkins

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract, which includes Crohn's disease (CD) and ulcerative colitis (UC); the goal of IBD pharmacotherapy is to induce and maintain remission.<sup>1,2</sup> Azathioprine is widely used as an immunosuppressive agent; although its efficacy in maintaining remission in IBD is largely accepted, adverse drug reactions to this agent occur in 15% to 38% of patients and often require the withdrawal of therapy.<sup>3,4</sup> Moreover, a significant proportion of patients does not respond to therapy with azathioprine.<sup>4,5</sup> Differences in azathioprine metabolism may explain this wide inter-individual variation in the clinical efficacy of this medication.<sup>6,7</sup> Azathioprine in fact is a prodrug and requires in vivo conversion to its active form (Fig. 1): the first step in its biotransformation involves conjugation with glutathione and the formation of mercaptopurine, which has no intrinsic activity.<sup>8</sup> The cytotoxic activity of mercaptopurine is mainly exerted after enzymatic conversion to thioguanine nucleotide (TGN) and the first step of this reaction is catalyzed by the enzyme hypoxanthine phosphoribosyltransferase.<sup>9</sup> The TGNs cause cytotoxicity by interfering with de novo purine biosynthesis and modification of DNA structure after their incorporation into nucleic acids, which produces an alteration of the function of DNA-processing enzymes.<sup>10</sup> In addition, TGN are structural analogues of ATP and GTP nucleotides, which are essential for intracellular messaging and energy-carrying processes and may therefore compete with these endogenous compounds in various biochemical pathways.<sup>11</sup> Indeed, it has been demonstrated that azathioprine-induced apoptosis in T lymphocytes is mediated by specific blockade of intracellular antiapoptotic pathways by TGN, resulting in the suppression of the overactive immune defense mechanism in IBD patients.<sup>12</sup>

Polymorphisms in genes for enzymes involved in the metabolism of azathioprine influence the efficacy and toxicity of the treatment.<sup>6</sup> Thiopurine-S-methyl-transferase (TPMT) influences thiopurines' metabolism, by methylation of the thiol moiety of mercaptopurine and consequent prevention of the activation to TGN metabolites.<sup>9,13</sup> Patients with inactivating mutations of TPMT have an increased production of the active TGN metabolites.<sup>14,15</sup> Another enzyme previously reported as involved in thiopurines' metabolism is inosine-triphosphate-pyrophosphatase (ITPA); this enzyme catalyzes the conversion of ITP to IMP and putatively influences thiopurines' effect by catalyzing the conversion of the thiolic analog thio-inosine-triphosphate (thioITP) to thio-inosine-monophosphate, therefore preventing the accumulation of the potentially toxic triphosphate metabolites. Polymorphisms in *ITPA*



**FIGURE 1.** Schematic representation of the metabolism of mercaptopurine and the enzymes involved. GDP indicates guanosine diphosphate; GMP, guanosine monophosphate; GMPS, guanosine monophosphate synthase; GST-M1, glutathione-S-transferase M1; GTP, guanosine triphosphate; HPRT, hypoxanthine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; IDP, inosine diphosphate; IMP, inosine monophosphate; ITP, inosine triphosphate; ITPA, inosine-triphosphate-pyrophosphatase; TPMT, thiopurine S-methyltransferase; XO, xanthine oxidase.

gene have been associated with azathioprine toxicity (recently reviewed by Stocco et al<sup>7</sup>) and increased concentrations of methylated nucleotides (MMPN),<sup>15,16</sup> additional metabolites of thiopurines, previously associated with de novo purine synthesis inhibition.<sup>17</sup> Glutathione-S-transferases (GST) may also have a role in azathioprine metabolism and toxicity: indeed, azathioprine is converted to mercaptopurine mainly through a reaction with glutathione and, although this reaction was considered to be a nonenzymatic conversion, recent studies have shown that GST might be involved.<sup>18,19</sup> Our group has shown that *GST-M1* polymorphism is associated with the incidence of adverse effects and lymphopenia during azathioprine therapy in young patients with IBD, whereas no association could be shown for *GST-P1* and *GST-T1*.<sup>19</sup>

The aim of this study was to investigate in a clinical setting treating young IBD patients with azathioprine, the correlation between candidate genetic polymorphisms of proteins involved in azathioprine metabolic activation and inactivation, (TPMT, ITPA, GST-M1, GST-P1, GST-T1), the concentration of the main azathioprine metabolites, TGN and MMPN and the dose of the medication.

Moreover, we checked retrospectively the association between disease activity at the time of metabolites measurement and the concentration of azathioprine metabolites.

**MATERIALS AND METHODS**

**Patients and Inclusion Criteria**

In this study, 75 patients with IBD were enrolled by the Gastroenterology Unit of the Pediatric Hospital “Burlo Garofolo” in Trieste, Italy between February 2004 and November 2010. The inclusion criteria were age less than 30 years, previous diagnosis of IBD, and treatment with azathioprine for at least 3 months. The patients enrolled are all the patients taking azathioprine at “Burlo Garofolo” in Trieste in the time-frame of the study. Blood samples for azathioprine metabolites measurement and for genotyping were taken at the appropriate clinic visit. Timing of metabolite level measurement was determined by the clinical setting of azathioprine administration at “Burlo Garofolo”: generally, azathioprine metabolites levels were measured after 3, 6, and 12 months of treatment and then every year. Patients were treated with a dose-escalating

strategy to reduce the risk of adverse events starting, however, from a relatively high dose (median of 2 mg/kg). At subsequent follow-up visits (every 3 mo), the dose was increased or reduced so as to obtain the optimal clinical response; the criteria used to increase or reduce the dose of azathioprine were the level of disease activity and laboratory parameters used to monitor azathioprine toxicity (in particular leukocyte, erythrocytes and platelets counts, hemoglobin concentration, mean corpuscular volume, liver enzymes alanine aminotransferase, aspartate aminotransferase and  $\gamma$ -glutamyltransferase, and amylase levels). According to current guidelines, genotyping information was shared with the clinicians only for patients presenting TPMT variant alleles, in order to allow increased monitoring of adverse events. The study was approved by the local ethical committee and appropriate informed consent was obtained from all patients or their parents or tutors.

### Definition of Remission

Clinical response was assessed using Pediatric Crohn's Disease Activity Index and Pediatric Ulcerative Colitis Activity Index, respectively, for CD<sup>20</sup> and UC patients,<sup>21</sup> at the time of blood sample collection for the first metabolites' measurement, which occurred at least 3 months since the beginning of therapy. Disease was considered inactive if the disease activity index was <10 at the time of sample collection.

### Measurement of Azathioprine Metabolites

Sample for azathioprine metabolites measurement were collected in patients taking an unchanged dose for at least 2 weeks.<sup>22-24</sup> Metabolites (TGN and MMPN) were measured in patients' erythrocytes using the high performance liquid chromatography assay by Dervieux and Bouliou<sup>25</sup> within few weeks from the sample' collection. The ratio between TGN and the dose of azathioprine was calculated considering, for each individual measurement of the metabolites, the dose the patients took the day the blood sample for the metabolites assessment was collected.

### Genotypes

Genomic DNA was extracted from peripheral blood samples using a commercial kit (Sigma, Milan, Italy), to characterize genetic polymorphisms in the candidate genes *GST-M1*, *GST-T1*, *GST-P1*, *TPMT*, and *ITPA*. The considered genotypes and method of analysis are described in Table 1.

### Statistical Analysis

Statistical analysis was performed using the software R (version 2.15).

The association between pharmacological phenotypes of interest (ie, TGN metabolites concentrations, MMPN metabolites concentrations, dose of azathioprine, ratio TGN/dose) and the considered demographic variables, IBD type, cotreatment or genotypes in a univariate analysis, was evaluated using 2 approaches. In the first approach, non-parametric statistical tests on the considered phenotype for each patient were applied: the effect of age or treatment duration was tested by Spearman rank test; for sex, IBD type or *TPMT*, *ITPA*, *GST-M1*, *GST-T1*, *GST-P1* genotypes, the effect was evaluated by Wilcoxon test; for cotreatment, Kruskal-Wallis test was applied. Univariate analysis was carried out even by considering for each phenotype and patient the individual observations and evaluating the effect of each covariate by calculating the *P*-value

**TABLE 1.** Assay Used for Genotyping of the Considered SNPs

Gene	rs Number	Polymorphism	
		Nucleotide Substitution	Genotyping Method
<i>TPMT</i>	rs1800462	G238C	PCR-ASO <sup>23</sup>
	rs1800460	G460A	PCR-RFLP <sup>23</sup>
	rs1142345	A719G	PCR-RFLP <sup>23</sup>
<i>GST-M1</i>	No rs number	Deletion	MULTIPLEX-PCR-ASO <sup>19</sup>
<i>GST-T1</i>	No rs number	Deletion	MULTIPLEX-PCR-ASO <sup>19</sup>
<i>GST-MP1</i>	rs1695	A1578G	PCR-RFLP <sup>19</sup>
<i>ITPA</i>	rs1127354	C94A	TaqMan SNP genotyping assay from Applied biosystems (C_27465000_10)
	rs7270101	IVS + 21 AC	TaqMan SNP genotyping assay from Applied biosystems (C_29168507_10)

*GST* indicates glutathione-S-transferase; *ITPA*, inosine-triphosphate-pyrophosphatase; SNP, single nucleotide polymorphism; *TPMT*, thio-purine-S-methyl transferase.

from a linear mixed effects model built using the phenotype as the dependent variable, each covariate as the fixed effect and the patients as the random effect in the model.

For the univariate analysis for clinical response, logistic regression was used. The dependent variable was disease status (active/inactive) at the first metabolites measurement and the independent variables were the TGN or MMPN concentrations or the ratio TGN/dose.

Multivariate analysis was carried out to test the independence of the effects of the genotypes significant in the univariate analysis on the phenotypes considered (ie, TGN or MMPN concentrations, dose of azathioprine, ratio TGN/dose); for this multivariate analysis generalized linear models of the Gaussian family were used considering individually each phenotype from the univariate analysis as the dependent variable and the genotypes significant in the univariate analysis as the independent variables. *P*-values corrected for multiple testing were calculated using Holm method.

For all parametric analysis (ie, linear mixed effects models used in the univariate analysis and the multivariate analysis), normality of the phenotype was tested by the Shapiro test and log10 transformation was applied if needed, in order to adjust the normality of the distribution.

## RESULTS

### Patients Enrolled and Samples Collected

The present study recruited 75 young patients with IBD. Clinical and demographic characteristics are reported in Table 2. From February 2004 to November 2010 a total of 150 samples of peripheral blood have been collected to measure azathioprine metabolites; on average, 2 samples for patient were collected (range, 1 to 7). Among these, 56 were obtained during treatment with azathioprine alone and 94 during treatment with azathioprine and other medications and in particular: 47 with an aminosalicilate, 15 with a glucocorticoid, 8 with an aminosalicilate and a glucocorticoid, 10 with infliximab, 7 with an antibiotic, and 7 with an aminosalicilate and an antibiotic. As a

**TABLE 2.** Demographic and Clinical Characteristics of the Patients Enrolled in the Study

	All Patients	GST-MI		P
		Deleted	Not Deleted	
Age (y) at time of sample collection	15.2, 2.7-28.1	15.4, 2.7-27.3	15.1, 2.9-28.1	0.74
Sex				0.23
Female (%)	36 (48)	18 (43)	18 (55)	
Male (%)	39 (52)	24 (57)	15 (45)	
Type of IBD				0.46
Crohn's disease (%)	46 (61)	28 (67)	18 (55)	
Ulcerative colitis (%)	29 (39)	14 (33)	15 (45)	
Dose (mg/kg) of azathioprine	2.0, 0.6-3.3	2.1, 1.5-3.3	1.9, 0.6-2.7	0.022
Length (d) of treatment with azathioprine	496, 102-2751	546, 114-2751	393, 102-1900	0.13

To report age, dose and length of treatment median and range are provided; for patients with more than one measurement of azathioprine metabolites, median age, dose and length of treatment were used.

GST indicates glutathione-S-transferase; IBD, inflammatory bowel disease.

consequence of the dose-escalation approach used in this clinical setting, the dose administered during the first 6 months of treatment was lower than the dose administered afterwards (median before 6 mo = 1.74 mg/kg, median after 6 mo = 2.07 mg/kg, Wilcoxon test  $P$ -value = 0.039, linear mixed effects:  $P$ -value = 0.082).

### Measurement of Azathioprine Metabolites

Summary of values of azathioprine dose and metabolites are reported in Table 3.

There was a significant correlation between TGN and MMPN concentrations (linear mixed effect  $P$ -value < 0.0001) and between azathioprine dose and metabolites concentrations (linear mixed effect  $P$ -value 0.019 for TGN and < 0.0001 for MMPN).

### Demographic and Clinical Covariates and Azathioprine Dose and Metabolites

For the demographic (sex and age) and clinical (type of IBD and treatment duration) covariates considered, none showed a fully significant effect on the median TGN or MMPN concentrations or azathioprine dose in a univariate analysis. For the ratio TGN/dose, a significant association was identified only for the type of IBD: indeed this ratio was higher in patients with UC than in patients with CD (Wilcoxon test  $P$ -value = 0.0033; linear mixed effects  $P$ -value = 0.015). The effect of comedications on metabolites levels and azathioprine dose was also evaluated; however, no statistically significant effect could be detected.

### Genotyping

Results of genotyping are reported in Table 4. All polymorphisms considered were respecting Hardy-Weinberg equilibrium and their distribution is comparable

to what has been reported in the literature for patients of white ethnicity.

### GST Genotypes and Azathioprine Dose and Metabolites

Patients with *GST-MI* null genotype have reduced TGN/azathioprine dose ratio (Wilcoxon test:  $P$ -value = 0.0030, linear mixed effects:  $P$ -value = 0.0002; Fig. 2D) compared to *GST-MI* normal genotype and were treated with higher doses of azathioprine (Wilcoxon test:  $P$ -value = 0.022, linear mixed effects:  $P$ -value = 0.017; Fig. 2C). Moreover, patients with *GST-MI* null genotype showed a trend toward a reduction in TGN nucleotides blood levels (Wilcoxon test:  $P$ -value = 0.063, linear mixed effects:  $P$ -value = 0.030; Fig. 2A). No significant effect for *GST-MI* was seen on MMPN metabolites concentrations (Fig. 2B).

The *GST-P1* and *GST-T1* genotypes considered had no significant effects on azathioprine metabolites or dose in this cohort of patients.

### Other Candidate Genotypes (TPMT and ITPA) and Azathioprine Dose and Metabolites

A significant effect of *TPMT* polymorphism was evident on TGN and MMPN concentration: indeed patients with variant genotype (3 with *TPMT\*3A* and 1 with *TPMT\*3C*) show higher TGN (Wilcoxon test:  $P$ -value = 0.034, linear mixed effects:  $P$ -value = 0.011) and lower MMPN levels compared to wild-type patients (Wilcoxon test:  $P$ -value = 0.0064, linear mixed effects:  $P$ -value = 0.0071). A fully significant association was also found with the ratio TGN metabolites/azathioprine dose (Wilcoxon test:  $P$ -value = 0.0035, linear mixed effects:  $P$  < 0.0001; Fig. 3A), whereas a trend was present for azathioprine dose, with patients with variant *TPMT* being treated with lower doses of the drug

**TABLE 3.** Summary of Azathioprine's Dose and Metabolites' Concentrations

	TGN (pmol/ 8×10 <sup>8</sup> Erythrocytes)	MMPN (pmol/ 8×10 <sup>8</sup> Erythrocytes)	Dose (mg/kg)	TGN/Dose
Mean	382.7	1868.7	2.0	206.6
Median	356.0	1078.9	2.0	181.1
SD	181.7	2210.7	0.5	113.5
Range	101.0-1259.0	84-12430.8	0.6-3.3	57.6-582.3

MMPN indicates methylated nucleotides; TGN, thioguanine nucleotides.

**TABLE 4.** Genotype Distribution in the 75 Patients Enrolled in the Study

Gene	Polymorphism		Genotyping Result				<i>P</i> Hardy-Weinberg
	rs Number	Nucleotide Substitution	Wild-Type	Heterozygous	Homozygous Variant	Not Available	
<i>TPMT</i>	rs1800462	G238C	73 (100%)	0	0	2	1.00
	rs1800460	G460A	70 (95.9%)	3 (4.1%)	0	2	0.86
	rs1142345	A719G	69 (94.5%)	4 (5.5%)	0	2	0.81
<i>GST-P1</i>	rs1695	A1578G	30 (44.1%)	31 (47.7%)	6 (8.2%)	8	0.77
<i>ITPA</i>	rs1127354	C94A	67 (94.4%)	4 (5.6%)	0	4	0.81
	rs7270101	IVS + 21 AC	55 (80.9%)	13 (19.1%)	0	7	0.79

Gene	Polymorphism	Genotyping Result		
		Not Deleted	Deleted	Not Available
<i>GST-M1</i>	Deletion	30 (41.7%)	42 (58.3%)	3
<i>GST-T1</i>	Deletion	57 (79.2%)	15 (20.8%)	3

*GST* indicates glutathione-S-transferase; *ITPA*, inosine-triphosphate-pyrophosphatase; *TPMT*, thiopurine-S-methyl transferase.

(Wilcoxon test: *P*-value = 0.092, linear mixed effects: *P*-value = 0.021).

An association between the C94A polymorphism of the *ITPA* gene and the concentration of MMPN (Wilcoxon test: *P*-value = 0.037, linear mixed effects: *P*-value = 0.049) was found: patients with variant *ITPA* genotype showed higher concentration of these metabolites compared to those with wild-type *ITPA*. No significant effect of *ITPA* genotype was seen on TGN concentrations, azathioprine dose, and the ratio TGN/dose (Fig. 3B). The additional *ITPA* SNP considered (IVS + 21 AC) did not have any significant effect on azathioprine metabolites or dose in these patients.

### Multivariate Analysis Evaluating Independence of the Effects of the Genotypes on Azathioprine Metabolites Concentrations or Dose

Analysis was performed by generalized linear models of the Gaussian family using the phenotype of interest as the dependent variable and the covariates significant in the univariate analysis as the independent ones. Detailed results of these multivariate analyses are reported in Table 5. For TGN metabolites concentration, no multivariate analysis was carried out, as only *TPMT* genotype had a fully significant effect in a univariate analysis. For MMPN metabolites concentration, the effect of *TPMT* was confirmed in the multivariate analysis, whereas *ITPA* showed a trend (*P*-value = 0.051). For the ratio TGN/dose, in the multivariate model *TPMT*, *GST-M1*, and the type of IBD maintained all full significance. These results were confirmed after correction for multiple testing.

### Azathioprine Metabolites and Clinical Response to Therapy

For this analysis, clinical response data were available at the first measurement of azathioprine metabolites for 68 of 75 patients; for patients in remission a higher TGN/dose ratio (logistic regression *P*-value = 0.0011) was observed, whereas a trend was present for the concentration of TGN metabolites (logistic regression *P*-value = 0.070). The concentration of MMPN metabolites was not associated with clinical response (logistic regression *P*-value = 0.52).

Considering the effect of the *GST*, *TPMT*, and *ITPA* polymorphisms on the clinical response to therapy at the

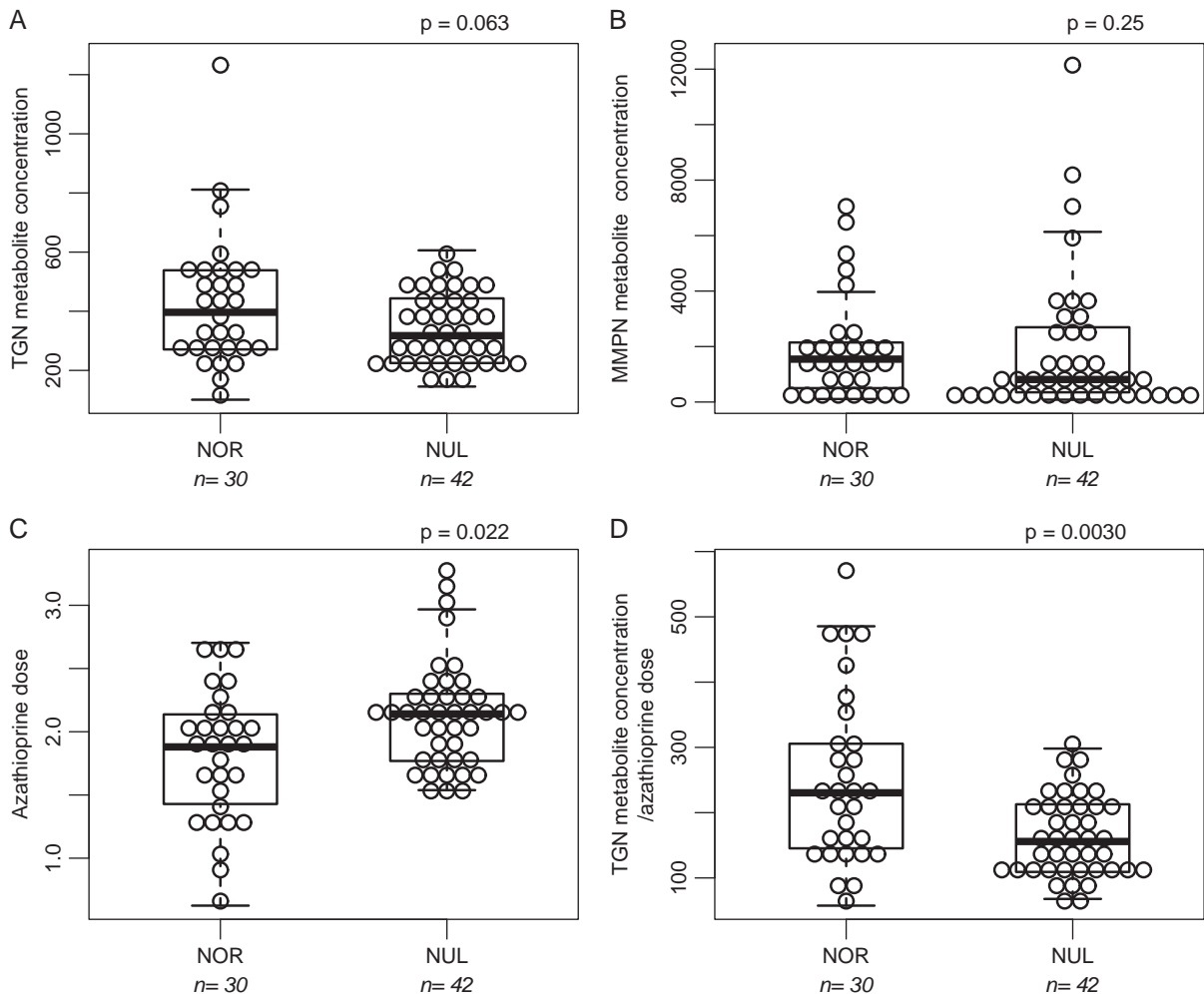
first measurement of azathioprine metabolites, only *GST-M1* was significant: indeed, among the patients with active disease, only 14.3% of patients presented a normal *GST-M1* genotype, whereas among the patients responding to azathioprine therapy, 52.3% had a normal *GST-M1* (logistic regression *P*-value = 0.0022, Fig. 4). To further confirm an effect of *GST-M1* on clinical response to azathioprine, patient status (remission or active) at a definite time point (6 mo) was determined; this analysis supported an effect of *GST-M1* on clinical response (logistic regression *P*-value = 0.053), although this result was not fully significant likely because of the reduced number of patients in this subgroup.

### DISCUSSION

Azathioprine is frequently used in IBD and is important to maintain remission; however, a significant proportion of patients does not respond to therapy or develops important adverse events. The causes for this inter-individual variability are not completely understood; however, a consistent amount of evidence relates variability in azathioprine response to interindividual differences in the metabolism of the medication, that are due, at least in part, to genetic polymorphisms of relevant enzymes.

Azathioprine has to be converted to mercaptopurine, in order to produce its pharmacological action, by a reaction involving reduced glutathione.<sup>8</sup> This reaction is in part nonenzymatic but it is even controlled, as demonstrated by recent publications, by the enzyme *GST*,<sup>18</sup> in particular by the isoforms A and M.<sup>18,26,27</sup> A recent study in our group, has analyzed the *GST-M1* polymorphic deletion, which leads to loss of enzymatic activity for this isoform, in relation to azathioprine-induced adverse events, showing that *GST-M1* deletion was associated to lower incidence of azathioprine-induced adverse events and lymphopenia.<sup>19</sup>

In our present analysis, a significant effect of *GST-M1* polymorphisms could be shown on azathioprine metabolites and dose: patients with the *GST-M1* gene deletion had lower concentrations of TGN metabolites and were treated with higher doses of the medication; indeed, a strong effect of the *GST-M1* genotype was seen for the ratio TGN/dose, showing that patients with a normal *GST-M1* produced 60% more TGN metabolite for unit of dose, than patients with *GST-M1* deletion and had a better clinical response considering the first metabolite measurement.



**FIGURE 2.** The box plots show patients with glutathione-S-transferase M1 wild-type (NORM) and with the deletion (NULL) of the gene: (A) thioguanine nucleotides (TGN) metabolites concentrations (pmol/ $8 \times 10^8$  erythrocytes); (B) methylated nucleotides (MMPN) metabolites concentrations (pmol/ $8 \times 10^8$  erythrocytes); (C) dose of azathioprine (mg/kg) and (D) ratio TGN/dose; *P*-value from Wilcoxon test. Each point represents the median value for the variable considered for each patient.

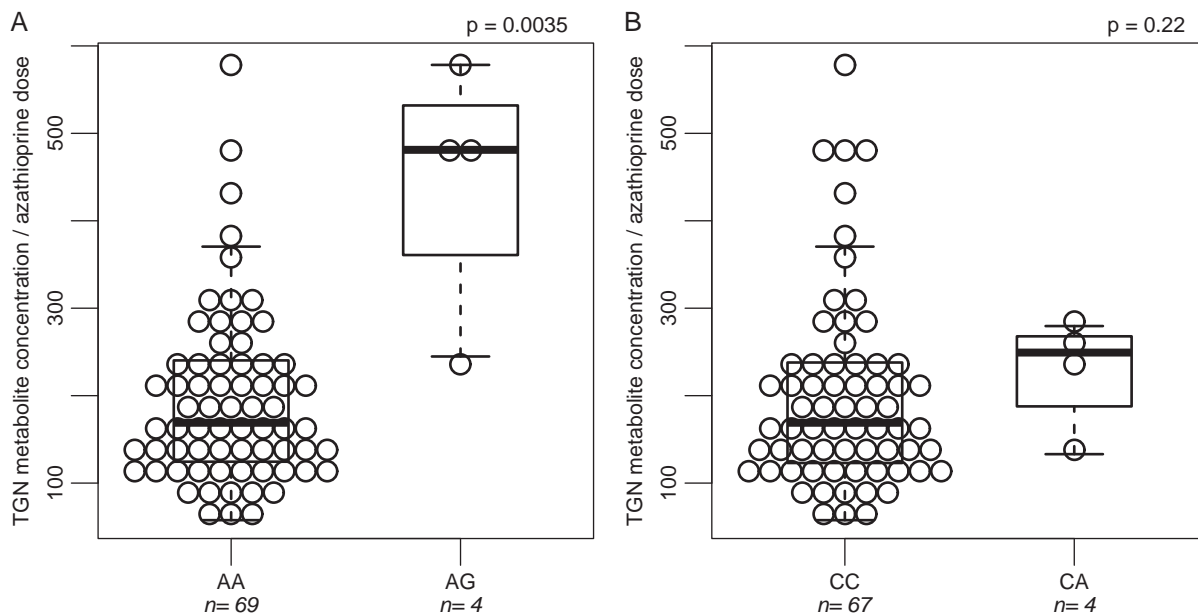
Besides the *M1* isoform of *GST*, even the *P1* and *T1* isoforms were evaluated: in our analysis no effect of genetic polymorphism of *GST-P1* and *T1* could be seen on azathioprine TGN and MMPN metabolites concentrations or on azathioprine dose. This is in agreement with previous reports that the *P1* and *T1* isoforms have no direct role in the activation of azathioprine to mercaptopurine<sup>18</sup> and their genetic polymorphisms do not seem related to azathioprine adverse events in young patients with IBD.<sup>19</sup>

For *TPMT*, our data substantially confirm previously published reports that clearly demonstrated an association between genetic polymorphisms of *TPMT* and an increased concentration of TGN metabolites during treatment with thiopurines.<sup>6,14</sup> In particular, the concentration of TGN metabolites measured in the erythrocytes showed a trend toward an increase in comparison with patients with a wild-type *TPMT* genotype. This increase was fully significant considering the ratio of TGN metabolites with the dose of azathioprine administered to the patients.<sup>28</sup> Patients with variant *TPMT* were treated with a dose of azathioprine that tended to be lower in comparison to

patients with wild-type genotype; this is probably because of the fact that, in these patients, azathioprine dose was adjusted by the clinician on the basis of efficacy, the appearance of lymphopenia or adverse events.<sup>29</sup> Moreover, as expected, the variant *TPMT* genotype was associated with a reduction of the concentration of the methylated metabolites.<sup>6,13</sup> For *ITPA*, in the present study, patients with the *ITPA C94A* polymorphism, which causes a reduction in *ITPA* activity, show increased concentrations of MMPN metabolites. This is in agreement with previous reports for patients with acute lymphoblastic leukemia treated with mercaptopurine.<sup>15</sup>

Multivariate analysis performed, even if limited by the relatively low number of patients for an extensive analysis with many covariates, in general confirmed that the effects of genetic variables on the azathioprine pharmacological phenotypes considered were independent from one another and therefore each one of the associations identified as significant has to be considered for further analysis.

Of note, patients with UC showed an higher TGN/dose ratio in our population, in comparison to patients with CD; this is likely because of the higher frequency of



**FIGURE 3.** The box plots show the ratio thioguanine nucleotides (TGN)/dose (pmol/8 × 10<sup>8</sup> erythrocytes/mg/kg azathioprine) according to thiopurine-S-methyl transferase A719G (A) and inosine-triphosphate-pyrophosphatase C94A (B) genotypes; *P*-value from Wilcoxon test. Each point represents the median value for the variable considered for each patient.

administration of aminosalicylates in patients with UC than CD (63% vs. 29%); indeed it is known that these medications increase the concentration of TGN during association therapy with azathioprine.<sup>30</sup>

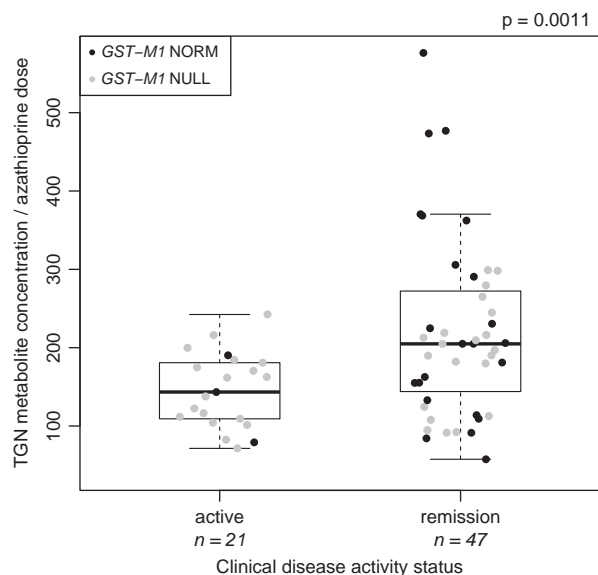
Moreover, our study confirms previous observations that patients responding to therapy have higher concentrations of the TGN metabolites,<sup>31-33</sup> highlighting that this relationship is of importance even in young patients with IBD and supporting the view that genetic markers of azathioprine metabolism, in particular those with effects on TGN concentration, may be useful to predict therapy response in this patient population. There has been conflicting data, with some prospective studies not showing an association between TGN concentration and clinical response to therapy,<sup>34</sup> but this may be due at least in part to assay methodology, patient inclusion bias, or

methodological inconsistencies.<sup>6</sup> Our study is the first one reporting a significant association of TGN concentration with response in young patients with IBD using the method by Dervieux and Boulieu.<sup>25</sup> This high performance liquid chromatography-based method has been reported to give values highly correlated with other methods used in the literature, such as the one from Lennard and colleagues.<sup>35,36</sup> However, a comparison with a method based on mass spectrometry has confirmed the reliability of the method used in the present study.<sup>37</sup> Several authors suggested that a significant methodological improvement, in the field of thiopurine metabolites measurement would be the discrimination between the different phosphorylation level of the TGN nucleotides, distinguishing in particular between relatively inactive monophosphate and the more biological active triphosphate TGNs.<sup>13,38</sup> Recently, an updated method

**TABLE 5.** Multivariate Analysis for Covariates With a Significant Effect in the Univariate Analysis

Azathioprine-related Pharmacological Phenotype (Dependent Variable)	Independent Variable in Multivariate Generalized Linear Model		Effect	<i>P</i>	<i>P</i> Corrected for Multiple Testing
	Comparison	Model			
MMPN metabolites concentration	<i>TPMT</i> variant genotype vs. wild-type	Heterozygous	-0.74	0.0081	0.016
	<i>ITPA</i> C94A genotype vs. wild-type	Heterozygous	0.47	0.051	0.051
Ratio TGN/dose	IBD type	UC vs. CD	0.96	0.043	0.043
	<i>TPMT</i> variant genotype vs. wild-type	Heterozygous	0.28	0.014	0.028
	<i>GST-M1</i> genotype	Deletion vs. normal	-0.14	0.0040	0.012

The effect size represents the increase (positive value) or decrease (negative value) in the value of the dependent variable for each independent variable listed. *P*-values adjusted for multiple testing were calculated using Holm method. *GST* indicates glutathione-S-transferase; IBD, inflammatory bowel disease; *ITPA*, inosine-triphosphate-pyrophosphatase; MMPN, methylated nucleotides; TGN, thioguanine nucleotides; *TPMT*, thiopurine-S-methyl transferase.



**FIGURE 4.** The box plot shows the ratio thioguanine nucleotides (TGN)/dose according to the clinical response to azathioprine treatment; *P*-value from logistic regression model. Each point represents the value of the variable considered at the first metabolite measurement. Patients with glutathione-S-transferase M1 (*GST-M1*) normal genotype are identified by a black dot, those with the deletion by a gray dot.

based on a novel highly specific and sensitive liquid chromatography—tandem mass spectrometry (LC-MS/MS) method for simultaneous quantification of eleven monophosphates, diphosphates, and triphosphates of thioinosine, methylthioinosine, methylthioguanosine, and thioinosine was published and the implementation of this or similar more detailed assessment of azathioprine metabolites looks promising as a significant improvement to clarify the relevance of therapeutic drug monitoring of azathioprine metabolites concentration in IBD.<sup>39</sup>

The most important and novel clinical finding of this report is the observation that *GST-M1* deletion correlates with reduced TGN/dose ratio and, consequently, azathioprine clinical efficacy: indeed, at a definite time point (6 mo of therapy), all patients with active disease had *GST-M1* deletion and, moreover, produced lower TGN levels after normalization for the azathioprine dose (Fig. 4). On this basis, it is likely that patients with *GST-M1* deletion may require a higher starting dose of azathioprine or a quicker dose-escalation strategy; moreover, in these patients, mercaptopurine could be considered as an alternative to azathioprine as its activity should not be influenced by conjugation with glutathione (Fig. 1). However, further prospective studies are needed to find the optimal starting dose for azathioprine and to evaluate the use of mercaptopurine as an alternative in patients with *GST-M1* deletion.

In conclusion, our analysis for the first time proves the effects of *GST-M1* polymorphism on azathioprine metabolism and dosage in young patients with IBD. As azathioprine metabolism and in particular TGN concentrations are related to patients' clinical response to the treatment, further studies should be carried out to validate the observation described in this paper and to develop strategies and clinical algorithms comprising these pharmacogenetics determinants, to optimize the response of these patients to azathioprine.

## REFERENCES

- Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. *Lancet*. 2007;369:1627–1640.
- Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet*. 2007;369:1641–1657.
- Barabino A, Torrente F, Ventura A, et al. Azathioprine in paediatric inflammatory bowel disease: an Italian multicentre survey. *Aliment Pharmacol Ther*. 2002;16:1125–1130.
- Prefontaine E, Macdonald JK, Sutherland LR. Azathioprine or 6-mercaptopurine for induction of remission in Crohn's disease. *Cochrane Database Syst Rev*. 2010;16:CD000545.
- Colombel JF, Sandborn WJ, Reinisch W, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med*. 2010;362:1383–1395.
- Chouchana L, Narjoz C, Beaune P, et al. Review article: the benefits of pharmacogenetics for improving thiopurine therapy in inflammatory bowel disease. *Aliment Pharmacol Ther*. 2012;35:15–36.
- Stocco G, De Iudicibus S, Addobbati R, et al. Personalized therapies in pediatric inflammatory and autoimmune diseases. *Curr Pharm Des*. 2012;18:5766–5775.
- Eliou GB. The purine path to chemotherapy. *Science*. 1989;244:41–47.
- Zaza G, Cheok M, Krynetskaia N, et al. Thiopurine pathway. *Pharmacogenet Genomics*. 2010;20:573–574.
- Somerville L, Krynetski EY, Krynetskaia NF, et al. Structure and dynamics of thioguanine-modified duplex DNA. *J Biol Chem*. 2003;278:1005–1011.
- Lennard L. TPMT in the treatment of Crohn's disease with azathioprine. *Gut*. 2002;51:143–146.
- Tiede I, Fritz G, Strand S, et al. CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4 + T lymphocytes. *J Clin Invest*. 2003;111:1133–1145.
- Stocco G, Crews KR, Evans WE. Genetic polymorphism of inosine-triphosphate-pyrophosphatase influences mercaptopurine metabolism and toxicity during treatment of acute lymphoblastic leukemia individualized for thiopurine-S-methyltransferase status. *Expert Opin Drug Saf*. 2010;9:23–37.
- 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;91:2001–2008.
- Stocco G, Cheok MH, Crews KR, et al. Genetic polymorphism of inosine triphosphate pyrophosphatase is a determinant of mercaptopurine metabolism and toxicity during treatment for acute lymphoblastic leukemia. *Clin Pharmacol Ther*. 2009;85:164–172.
- 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;71:575–584.
- Dervieux T, Blanco JG, Krynetski EY, et al. Differing contribution of thiopurine methyltransferase to mercaptopurine versus thioguanine effects in human leukemic cells. *Cancer Res*. 2001;61:5810–5816.
- Eklund BI, Moberg M, Bergquist J, et al. Divergent activities of human glutathione transferases in the bioactivation of azathioprine. *Mol Pharmacol*. 2006;70:747–754.
- Stocco G, Martellosi S, Barabino A, et al. Glutathione-S-transferase genotypes and the adverse effects of azathioprine in young patients with inflammatory bowel disease. *Inflamm Bowel Dis*. 2007;13:57–64.
- Hyams JS, Ferry GD, Mandel FS, et al. Development and validation of a Pediatric Crohn's Disease Activity Index. *J Pediatr Gastroenterol Nutr*. 1991;12:439–447.
- Turner D, Otley AR, Mack D, et al. Development, validation, and evaluation of a Pediatric Ulcerative Colitis Activity Index: a prospective multicenter study. *Gastroenterology*. 2007;133:423–432.

22. Carter MJ, Lobo AJ. Lack of effect of intravenous azathioprine on time to respond for steroid treated Crohn's disease. *Gut*. 2001;48:295–296.
23. Sandborn WJ, Tremaine WJ, Wolf DC, et al. Lack of effect of intravenous administration on time to respond to azathioprine for steroid-treated Crohn's disease. North American Azathioprine Study Group. *Gastroenterology*. 1999;117:527–535.
24. Lennard L, Lilleyman JS. Variable mercaptopurine metabolism and treatment outcome in childhood lymphoblastic leukemia. *J Clin Oncol*. 1989;7:1816–1823.
25. Dervieux T, Boulieu R. Simultaneous determination of 6-thioguanine and methyl 6-mercaptopurine nucleotides of azathioprine in red blood cells by HPLC. *Clin Chem*. 1998;44:551–555.
26. Zhang W, Moden O, Mannervik B. Differences among allelic variants of human glutathione transferase A2-2 in the activation of azathioprine. *Chem Biol Interact*. 2010;186:110–117.
27. Eklund BI, Mannervik B. Importance of a hypervariable active-site residue in human Mu class glutathione transferases catalyzing the bioactivation of chemotherapeutic thiopurine prodrugs. *Biochim Biophys Acta*. 2007;1770:1098–1103.
28. Gardiner SJ, Geary RB, Begg EJ, et al. Thiopurine dose in intermediate and normal metabolizers of thiopurine methyltransferase may differ three-fold. *Clin Gastroenterol Hepatol*. 2008;6:654–660; quiz 604.
29. Stocco G, Martelossi S, Barabino A, et al. TPMT genotype and the use of thiopurines in paediatric inflammatory bowel disease. *Dig Liver Dis*. 2005;37:940–945.
30. Stocco G, Martelossi S, Malusa N, et al. Interruption of mesalamine and reduction of the blood concentration of the active metabolites of azathioprine: possible causes of ulcerative colitis relapse. *Dig Dis Sci*. 2008;53:3246–3249.
31. Osterman MT, Kundu R, Lichtenstein GR, et al. Association of 6-thioguanine nucleotide levels and inflammatory bowel disease activity: a meta-analysis. *Gastroenterology*. 2006;130:1047–1053.
32. Dubinsky MC, Lamothe S, Yang HY, et al. Pharmacogenomics and metabolite measurement for 6-mercaptopurine therapy in inflammatory bowel disease. *Gastroenterology*. 2000;118:705–713.
33. Dubinsky MC, Yang H, Hassard PV, et al. 6-MP metabolite profiles provide a biochemical explanation for 6-MP resistance in patients with inflammatory bowel disease. *Gastroenterology*. 2002;122:904–915.
34. Gonzalez-Lama Y, Bermejo F, Lopez-Sanroman A, et al. Thiopurine methyl-transferase activity and azathioprine metabolite concentrations do not predict clinical outcome in thiopurine-treated inflammatory bowel disease patients. *Aliment Pharmacol Ther*. 2011;34:544–554.
35. Lennard L, Singleton HJ. High-performance liquid chromatographic assay of the methyl and nucleotide metabolites of 6-mercaptopurine: quantitation of red blood cell 6-thioguanine nucleotide, 6-thioinosinic acid and 6-methylmercaptopurine metabolites in a single sample. *J Chromatogr*. 1992;583:83–90.
36. Shipkova M, Armstrong VW, Wieland E, et al. Differences in nucleotide hydrolysis contribute to the differences between erythrocyte 6-thioguanine nucleotide concentrations determined by two widely used methods. *Clin Chem*. 2003;49:260–268.
37. Dervieux T, Meyer G, Barham R, et al. Liquid chromatography-tandem mass spectrometry analysis of erythrocyte thiopurine nucleotides and effect of thiopurine methyltransferase gene variants on these metabolites in patients receiving azathioprine/6-mercaptopurine therapy. *Clin Chem*. 2005;51:2074–2084.
38. Karner S, Shi S, Fischer C, et al. Determination of 6-thioguanosine diphosphate and triphosphate and nucleoside diphosphate kinase activity in erythrocytes: novel targets for thiopurine therapy? *Ther Drug Monit*. 2010;32:119–128.
39. Hofmann U, Heinkele G, Angelberger S, et al. Simultaneous quantification of eleven thiopurine nucleotides by liquid chromatography—tandem mass spectrometry. *Anal Chem*. 2012;84:1294–1301.