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Thiopurine methyltransferase genotype–phenotype discordance and thiopurine active metabolite formation in childhood acute lymphoblastic leukaemia

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Keywords
childhood leukaemia, genotype–phenotype discordance, mercaptopurine, thioguanine, thioguanine nucleotides, thiopurine methyltransferase

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- In healthy children and adults thiopurine methyltransferase (TPMT) activities have a trimodal frequency distribution. In adult populations the concordance between TPMT genotype and phenotype is over 98%.
- In childhood acute lymphoblastic leukaemia (ALL) the disease process and subsequent chemotherapy can influence TPMT activity measurements.

WHAT THIS STUDY ADDS

- In childhood ALL, at disease diagnosis, the extent of the reduction in measured TPMT activity and the resulting TPMT genotype–phenotype discordance, previously reported in small patient cohorts with low numbers of variant alleles, has been confirmed in a large population of children with ALL. TPMT activity should not be used to predict heterozygosity.
- During thiopurine chemotherapy, TPMT discordance is 45% in children with intermediate activity. TPMT genotype more accurately predicts mercaptopurine active metabolite accumulation and therefore should be used in preference to phenotyping for dosage recommendations.

AIMS

In children with acute lymphoblastic leukaemia (ALL) bone marrow activity can influence red blood cell (RBC) kinetics, the surrogate tissue for thiopurine methyltransferase (TPMT) measurements. The aim of this study was to investigate TPMT phenotype–genotype concordance in ALL, and the influence of TPMT on thiopurine metabolite formation.

METHODS

We measured TPMT (activity, as units ml^{-1} packed RBCs and genotype) at diagnosis ($n = 1150$) and TPMT and thioguanine nucleotide (TGN) and methylmercaptopurine nucleotide (MeMPN) metabolites (pmol/8 × 10^8 RBCs) during chemotherapy ($n = 1131$) in children randomized to thioguanine or mercaptopurine on the United Kingdom trial ALL97.

RESULTS

Median TPMT activity at diagnosis (8.5 units) was significantly lower than during chemotherapy (13.8 units, median difference 5.1 units, 95% confidence interval (CI) 4.8, 5.4, $P < 0.0001$). At diagnosis genotype–phenotype was discordant. During chemotherapy the overall concordance was 92%, but this fell to 55% in the intermediate activity cohort (45% had wild-type genotypes). For both thiopurines TGN concentrations differed by TPMT status. For mercaptopurine, median TGNs were higher in TPMT heterozygous genotype (754 pmol) than wild-type (360 pmol) patients (median difference 406 pmol, 95% CI 332, 478, $P < 0.0001$), whilst median MeMPNs, products of the TPMT reaction, were higher in wild-type (10 650 pmol) than heterozygous patients (3868 pmol) ($P < 0.0001$). In TPMT intermediate activity patients with a wild-type genotype, TGN (median 366 pmol) and MeMPN (median 8590 pmol) concentrations were similar to those in wild-type, high activity patients.

CONCLUSIONS

In childhood ALL, TPMT activity should not be used to predict heterozygosity particularly in blood samples obtained at disease diagnosis. Genotype is a better predictor of TGN accumulation during chemotherapy.

Introduction

Mercaptopurine forms the core of long term maintenance chemotherapy in childhood acute lymphoblastic leukaemia (ALL) whilst, conventionally, thioguanine (INN, tioguanine) is used in ALL intensive treatment blocks and in the myeloid leukaemias [1, 2]. The United Kingdom Medical Research Council (MRC) national trial ALL97 compared the efficiacy and toxicity of both thiopurines during the maintenance phases of chemotherapy.

Both drugs are directly inactivated by the polymorphic enzyme thiopurine methyltransferase (TPMT) [3] and both form cytotoxic thioguanine nucleotides (TGNs), metabolites linked to both treatment efficacy and toxicity [4, 5] However, mercaptopurine forms intermediate nucleotide metabolites prior to the TGNs (Figure 1). These mercaptopurine nucleotides are substrates for TPMT, and the resulting methylmercaptopurine nucleotides (MeMPNs) are formed at the expense of TGNs. Incorporation of drug derived TGN metabolites into DNA triggers cytotoxicity, a primary mode of action for thiopurine drugs [6, 7]. The TGNs can also promote cytotoxicity by inhibiting DNA methylation [8] and induce apoptotic cell death by inhibition of intracellular signalling pathways [9–11].

Life-threatening bone marrow toxicity, due to excess production of TGN metabolites, occurs in the 0.3% of patients who are TPMT deficient [5, 12–14]. Less severe myelosuppression can develop in TPMT heterozygotes, approximately 11% of patients, when taking standard doses of mercaptopurine [15, 16]. Most protocols incorporate routine assessment of TPMT status at the start of thiopurine treatment by phenotype [17] or genotype [18]. The phenotype concords with genotype in >98% of healthy adults [19]. Screening for the *TPMT*3* family of variant alleles (>92% of low activity alleles) and the *TPMT*2* variant will detect >95% of TPMT deficient alleles [19, 20].

The clinical results of the ALL97 thiopurine randomization have been previously reported and showed no difference in efficacy between the two thiopurines. A significant reduction in central nervous system relapse with thioguanine was balanced by increased risks of death in remission and vascular liver disease [21]. The latter toxicity was associated with TPMT heterozygosity [22]. In this paper we report on the thiopurine metabolism studies within ALL97. The aim of these studies was to investigate the phenotype–genotype TPMT concordance in children with ALL and the influence of TPMT status on the inter- and intra-patient variability in thiopurine metabolism. Analyses of the association between thiopurine metabolism and clinical efficacy are on-going and will be reported separately.

Methods

Patients and chemotherapy

MRC ALL97 (registration number ISRCTN26727615) compared the efficacy and toxicity of dexamethasone and thioguanine (experimental arms) with prednisone and mercaptopurine (standard approach). Treatment centres in the UK and Ireland recruited patients aged 1 to 18 years with ALL diagnosed between January 1997 and June 2002. Treatment centres obtained local ethics committee approval and informed consent from patients and/or parents before entering children into the trial. The trial had an add-on pharmacogenetic and drug metabolism study to investigate inter- and intra-patient variability in response to oral thiopurines.

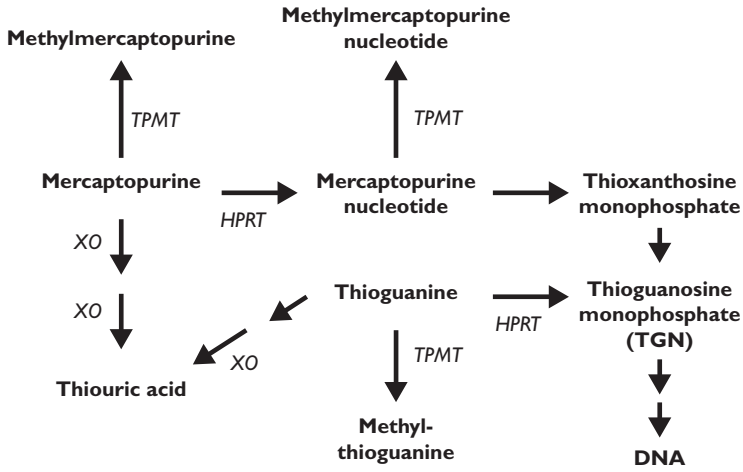

Figure 1

Thiopurine metabolism. Both mercaptopurine and thioguanine are methylated by thiopurine methyltransferase (TPMT), as is mercaptopurine nucleotide (thioinosine monophosphate). Nucleotide formation is catalyzed by hypoxanthine phosphoribosyltransferase (HPRT). Oxidation is catalyzed by xanthine oxidase (XO). Thioguanine requires deamination by guanase before catabolic oxidation. The thioguanine nucleotides (TGNs) are the mono-, di- and triphosphates of thioguanosine

ALL97 underwent several modifications, details of which have been previously reported [21, 22]. The steroid and thiopurine randomizations were retained and maintenance chemotherapy, upon which this study is based, remained the same. During maintenance patients received daily oral randomized thiopurine, weekly methotrexate and monthly intravenous vincristine followed by 5 days of randomized steroid. The thiopurine dose was titrated to toxicity from a standard protocol dose (thioguanine 40 mg m^{-2} and mercaptopurine 75 mg m^{-2}, 100% protocol dose). The dosage titrations have been previously described [22].

The trial showed a survival benefit with dexamethasone [23] but an excess of thioguanine-related veno-occlusive disease [21, 22]. At closure it was recommended that children randomized to thioguanine, who had not finished maintenance chemotherapy, should be transferred to the mercaptopurine arm.

Thiopurine studies

TPMT phenotype and genotype were measured in a diagnostic lithium heparin blood sample (5 ml). During thiopurine maintenance chemotherapy, TPMT and thiopurine metabolite concentrations were measured in blood samples taken immediately before a monthly vincristine injection. Samples were requested after at least 7 days at the standard protocol dose of thiopurine, or the maximum tolerated dose, but not within 2 months of a red cell transfusion. The first sample was requested at the earliest convenient point when the criteria above were met. A second sample was requested to be taken at the start of year 2 and a third sample at the end of year 2. If the patient's treatment had been reduced or interrupted at any time point, the sample was taken on recovery of cell counts during the next cycle of thiopurine treatment as per the criteria stated above.

Thiopurine assays

Thiopurine metabolite concentrations and TPMT activities were measured by high performance liquid chromatography [22, 24, 25]. Metabolite assays are stated in the text in pmol which represent pmol/8×10^8 red blood cells (RBCs) and TPMT activities are stated in units which represent units ml^{-1} packed RBCs [24]. The lower limit of detection and quantitation for TGN metabolites were 6 pmol and 30 pmol (coefficient of variation, CV, 8.2%), respectively and for MeMPNs 15 pmol and 60 pmol (CV 9.8%), respectively. The interassay coefficients of variation, for 25 assays over a 12 month period, for the thiopurine quality control samples at 300, 3000 and 12 000 pmol/8×10^8 RBCs were 4.6%, 3.8% and 5%, respectively with an accuracy of 1.3%, 1.6% and 3.3%. RBC TPMT activity assay [24] was modified to measure directly the methylmercaptopurine reaction product [22] with no interference from methylmercaptopurine nucleotide or nucleoside drug metabolites. The lower limit of detection and quantitation was 6 pmol (0.75 units TPMT, CV 9%) with a control patient quality control CV of 7.8%.

Routinely, blood samples were genotyped for *TPMT*3A, TPMT*3B* and *TPMT*3C,* by amplification of exons 7 and 10 as previously described [22]. *TPMT*3A* is an exon 7 and 10 double mutant. Aliquots (10 µl) of exon 7 (460G > A) and exon 10 (719A > G) PCR product were digested with the restriction enzymes *Mwo1* for 4 h at 60°C and *Acc1* for 4 h at 37°C, respectively, and analyzed on a 4% polyacrylamide gel. Exon 7 (*TPMT*3B*) gave wild-type fragments of 226 bp and 100 bp. The variant allele, 326 bp, was not digested. Exon 10 (*TPMT*3C*) gave variant allele fragments of 268 bp and 169 bp. The wild-type, 437 bp, was not digested. *TPMT*2* was detected by sequencing exon 5 of the TPMT gene [20]. To identify novel sequence variations, the TPMT open reading frame from exon 3 to exon 10 was sequenced using intron based primers [20] on an ABI 3730 capillary sequencer, using dye-primer technology (Applied Biosystems, Warrington, UK).

Stability studies

To investigate the stability of TGN and MeMPN metabolite concentrations in the samples forwarded from UK treatment centres, blood samples arriving in the laboratory were left at room temperature (22°C) over several days. Aliquots of whole blood were taken at day 1 (24 h post-sampling), day 3, day 6 and day 9, and washed resuspended RBCs prepared for metabolite assays. The then established TGN assay used washed RBCs [25]. In addition, if the sample volume permitted, an aliquot of whole blood was removed at each time interval. From both the washed RBC and whole blood samples a 200 µl aliquot was removed for RBC counting prior to freezing at –30°C. Stability studies on TPMT have been previously reported [26].

Statistical analysis

The Anderson-Darling test was used to examine the fit of observations to a normal distribution. Differences between groups were compared by the Chi-square statistic, the Kruskal–Wallis one way analysis of variance, Wilcoxon matched pairs test or the Mann–Whitney test. Metabolite and TPMT activity values are stated as median and range. The median differences (and the 95% confidence interval, CI) were calculated from the point estimates of all the median differences. Correlations were assessed using the Spearman-rank correlation coefficient (r_s).

Results

Blood samples

At least one sample was obtained from 1565 children (81% of children in the trial) entered into ALL97 (n = 803) and ALL97/99 (n = 762). Not all samples, as requested in the study protocol, were provided for each child. In addition,

Table 1

Blood samples available for thiopurine analysis

	Total	TG	MP
Individual children	1565		
Individual TPMT genotypes	1320 (84%)		
Individual children at disease diagnosis			
TPMT activity measured	1150		
TPMT genotypes	950 (83%)		
Individual children during chemotherapy			
TPMT activity measured	1131	428	703
TPMT activity and genotype	1117 (99%)	423	694
TPMT activity and metabolites	1114 (98%)	424	690
TPMT activity, genotype and metabolites	1100 (97%)	419	681
TPMT activities measured at diagnosis and during chemotherapy	755 (67%)		
TPMT genotypes available in the above cohort	741 (66%)		
Children with multiple samples at the protocol directed time intervals	378 (33%)	134	244

MP, mercaptopurine cohort; TG, thioguanine cohort; TPMT, thiopurine methyltransferase. TPMT activity was used in the analysis if it was measured in blood samples processed within 5 days of sampling. Metabolite values were used in analysis if they were measured in whole blood samples up to 6 days old or, for washed red cells, up to 3 days old. For chemotherapy samples the total number is split between those taking TG or taking MP. Two TPMT deficient children are included in this table, one randomized to TG and one to MP.

due to the small sample volume forwarded from some children, TPMT phenotype, genotype and drug metabolites could not be measured in every sample. Table 1 summarizes the samples available for analysis.

A number of children switched from thioguanine to mercaptopurine but only had samples whilst taking mercaptopurine. Samples were taken from 754 children when taking mercaptopurine (667 randomized to mercaptopurine, 87 after switching to mercaptopurine from thioguanine) and 457 when taking thioguanine (456 randomized to thioguanine, one given thioguanine but randomized to mercaptopurine). The first blood sample that met the study criteria was used as the representative sample in the subsequent analysis of thiopurine metabolism. The representative samples for mercaptopurine were taken at a median of 12.7 months after the start of induction therapy (range 1.6–39.6 months). For thioguanine samples the representative sample was at a median of 6.2 months (range 1.9–32.9 months).

Stability studies

TGN and MeMPN metabolites were measured in washed RBCs from 53 children (25 mercaptopurine, 28 thioguanine). Matched comparisons showed no loss of metabolites between the day 1 and day 3 samples. By day 6, thioguanine derived TGNs had decreased significantly compared with day 1 (day 1 median 1892 pmol, day 6 median 1054 pmol; median difference 502 pmol, 95% CI 283, 737, $P = 0.002$). Similarly, by day 6, mercaptopurine derived TGNs had fallen by 35% compared with day 1 (day 1 median 360 pmol, day 6 median 234 pmol; median difference 153 pmol, 95% CI 90, 251, $P < 0.001$), and mercaptopurine derived MeMPNs had fallen by 20% (day 1 median 11 084 pmol, day 6 median 8886 pmol, median difference 1704 pmol, 95% CI 1194, 4615, $P < 0.001$).

Whole blood samples were available from 20 children (15 mercaptopurine, 5 thioguanine), there was no significant difference between metabolite values measured at day 1, 3 or 6. The day 6 analysis showed a non-significant decline of 1.1% for thioguanine derived TGNs and 0.1% for mercaptopurine derived TGNs and 9.3% for MeMPN. Ten children, taking mercaptopurine, had a blood sample available at day 9. TGNs had decreased by 7.2% (day 1 median 335 pmol, day 9 median 311 pmol; median difference 30 pmol, 95% CI −6.5, 60, $P = 0.154$) and MeMPNs by 30% (day 1 median 12 289 pmol, day 9 median 8723 pmol; median difference 2060, 95% CI 699, 4874, $P = 0.053$).

Drug metabolites were stable for at least 6 days when measured in whole blood. If the blood sample volume received was low (<2 ml), metabolites were measured in washed RBCs (a preparation also used for the production of TPMT lysates), and samples processed within 3 days of sampling were used in data analysis.

TPMT phenotype

TPMT activities were measured in 1131 children during maintenance chemotherapy. There was no significant difference between the range of TPMT activities measured in children taking thioguanine (0 to 23 units, median 13.8, $n = 428$) compared with children taking mercaptopurine (0 to 26 units, median 13.8, $n = 703$). There was no gender difference in TPMT activities. TPMT activities measured in 1150 children at diagnosis (range 0 to 38 units, median 8.5) were significantly lower than those during chemotherapy (median difference 5.1 units, 95% CI 4.8, 5.4, $P < 0.0001$). TPMT activities were available for 755 children both at diagnosis (median 8.5 units, range 0 to 38) and on thiopurine (median 13.8 units, range 0–26.3). The disparity in TPMT activities remained when restricting the analysis to this matched cohort (median difference 5.3 units, 95% CI 5.0, 5.6, $P < 0.0001$).

Blood samples were received from four children who were TPMT deficient. Two of these children received randomized thiopurine on the trial protocol and were included in this analysis. Their TPMT activities at diagnosis and during chemotherapy were <0.75 units.

TPMT genotype

TPMT genotype was available for 1320 children, 1149 were white Caucasian and 171 belonged to other ethnic groups (71 Asian, 44 mixed race, 19 Black, 6 Oriental and 31

unknown or non-Caucasian). One hundred and twenty-three children had variant alleles (100 *TPMT*1/*3A*, 17 *TPMT1/*3C*, four *TPMT*1/*2*, one compound heterozygote *TPMT*2/*3A* and one homozygous *TPMT*3A/*3A*). The *TPMT*3B* allele was not detected. The *TPMT*3* family allele frequency was 5.2% in white Caucasians (4.5% *3A*, 0.7% *3C*) and 1.75% in other ethnic groups (0.58% *3A*, 1.17% *3C*). *TPMT*2* was only detected in white Caucasians (allele frequency 0.22%).

Concordance between genotype and phenotypic activity

TPMT genotypes were available for 1117 of the 1131 children with TPMT activities measured during chemotherapy. The break point between the 'intermediate' and 'high' TPMT activity ranges was examined at 9.5, 10.5 and 11.5 units, values based on the nadir of the TPMT frequency distributions in healthy control adults and children [3, 15] and in children taking chemotherapy [15]. At 9.5 units the sensitivity for the detection of the variant allele was 78% (specificity 97%), at 10.5 units the sensitivity was 92% (specificity 92%) and at 11.5 units the sensitivity was 94% (specificity 85%). A 10.5 unit break point was used for data analysis. The overall concordance was 92%, but the specificity of 92% results in 8% of wild-type alleles (79 alleles) in the intermediate activity cohort and a concordance of 55% in this group (i.e. 45% of intermediate activity patients had a wild-type genotype). The concordance in the high activity group (above 10.5 units) was 99% (Figure 2). It should be noted that the intermediate activity cohort contained 16.5% of children rather than the 11% predicted by the TPMT polymorphism [3, 15].

TPMT genotypes were available for 950 of the 1150 children with TPMT activities measured at disease diagnosis. The distribution of TPMT activities was unimodal, with a negative skew, with overlapping activities for heterozygous and wild-type children. Of the 92 variant alleles, 81 were below the median TPMT activity of 8.5 units. At this cut-off the sensitivity for detecting the heterozygous genotype was 88%, but the specificity for wild-type (*TPMT*1/*1*) was only 53%. Other than for the detection of TPMT deficiency, TPMT activities at diagnosis *per se* had a poor predictive value with respect to heterozygosity and functional activity during chemotherapy. This is illustrated for the matched group of 755 children in whom 741 genotypes were available (Figure 3).

Sequencing non-concordant samples

Sequencing of the TPMT open reading frame in children with TPMT activities <12 units without *TPMT*2* or *TPMT*3* variant alleles (n = 186; 79 children with a wild-type genotype and ≤10.5 units TPMT i.e. intermediate activity) revealed five children with sequence variations: *TPMT*1/*9* (7.4 units, mercaptopurine derived TGNs 502 pmol at 67 mg m^{-2} mercaptopurine), *TPMT*1/*21* (6.8 units, thioguanine derived TGNs 4060 pmol at 41 mg m^{-2} thioguanine) and three novel variants *TPMT*1/*32* (exon 5, 340G > A, Glu114Lys, rs115106679; activity 9.7 units, mercaptopurine derived TGNs 694 pmol at 75 mg m^{-2} mercaptopurine), *TPMT*1/*33* (exon 7, 487C > T, Arg163Cys, rs112339338; activity 8.5 units, mercaptopurine derived TGNs 634 pmol at 54 mg m^{-2} mercaptopurine), *TPMT*1/*34* (exon 5, 244C > T, Arg82Trp, rs111901354; activity 8.0 units, thioguanine derived TGNs 3006 pmol at 44 mg m^{-2} thioguanine).

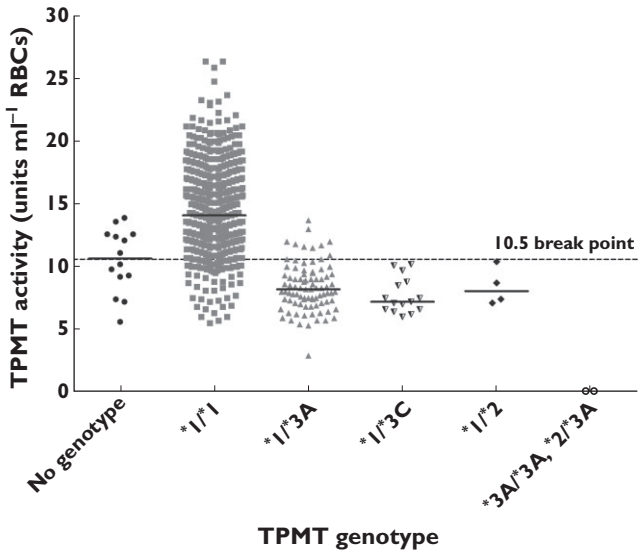

Figure 2

TPMT activities for 1131 children during thiopurine maintenance chemotherapy (median 13.8 units, range 0–26). Thiopurine genotype was available for 1117 children (1009 wild-type, 87 *TPMT*1/*3A*, 15 *TPMT*1/*3C*, four *TPMT*1/*2* and two TPMT deficient children *TPMT*3A/*3A, TPMT*2/*3A*). At the 10.5 unit break point (dotted line) the sensitivity for the *TPMT*3* variant allele was 92% (specificity for wild-type 93%). Solid line = median TPMT activities

TPMT status and thiopurine metabolism

Two TPMT deficient children were randomized for thiopurine, one to thioguanine and one to mercaptopurine. An initial thioguanine dose of 4 mg m^{-2} produced 3544 pmol TGNs, dose reduction to 2.5 mg m^{-2} daily produced 2252 pmol TGNs. The mercaptopurine child was treated on 7.5 mg m^{-2} on alternate days and TGNs were 1670 pmol. The mercaptopurine treated child lacked MeMPN metabolites.

For children with TPMT activity, thioguanine derived TGNs ranged from 36–6874 pmol, median 1940, at a median thioguanine dose of 40 mg m^{-2} (range 10–78) and mercaptopurine derived TGNs ranged from 0–2228 pmol, median 372, at a median mercaptopurine dose of 75 mg m^{-2} (range 6–125). Mercaptopurine derived MeMPNs ranged from 0–141 772 pmol, median 9590. There was no difference in drug dose or metabolite concentrations when analyzed by steroid group or gender.

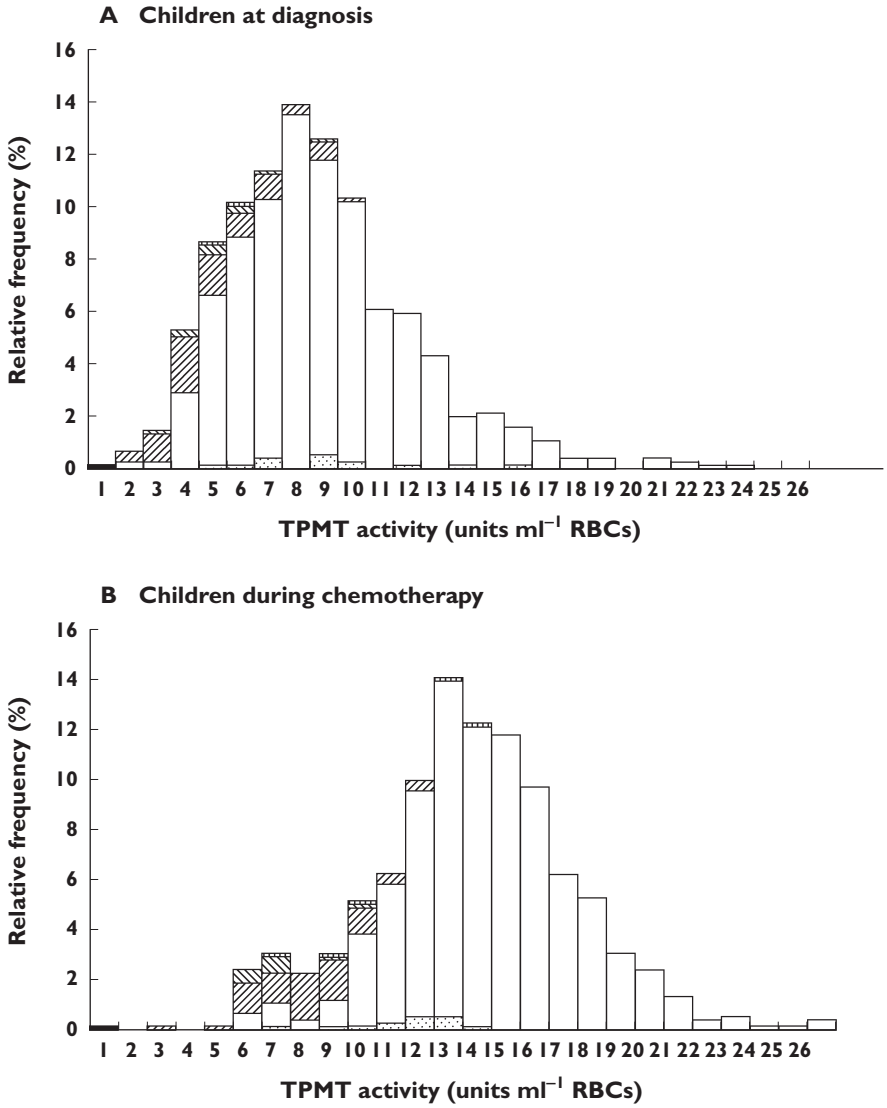

Figure 3

TPMT frequency distribution histograms for 755 children at disease diagnosis (median 8.5 units, range 0–38) and for those same children during thiopurine maintenance chemotherapy (median 13.8 units, range 0–26). The histograms are plotted to 26 units, histogram (A) omits one child who had an at diagnosis TPMT activity of 38 units. Thiopurine genotypes were available for 741 of the 755 children. ■, *TPMT*3A/*3A*; ▯, *TPMT*1/*2*; ▧, *TPMT*1/*3C*; ▨, *TPMT*1/*3A*; □, *TPMT*1/*1*; ⬚, no genotype

TGN concentrations negatively (thioguanine $r_s = -0.187$, $P < 0.001$; mercaptopurine $r_s = -0.23$, $P < 0.001$) correlated with TPMT activity. Mercaptopurine derived MeMPNs showed a positive correlation ($r_s = 0.104$, $P = 0.006$) to TPMT.

Analysis of metabolites by genotype ($n = 418$ thioguanine, $n = 680$ mercaptopurine) indicated that, for both thiopurines, TGN concentrations were significantly higher in heterozygotes whilst mercaptopurine derived MeMPNs were significantly lower in the TPMT heterozygous cohort (Table 2).

Within the intermediate activity cohort (TPMT ≤10.5 units), metabolites were compared between children with a wild-type and heterozygous genotype (Table 3). For children taking thioguanine there was no difference in TGN formation. However, mercaptopurine derived TGNs were significantly higher, and MeMPNs were significantly lower, in the heterozygous genotype children compared with the intermediate activity/wild-type genotype children. For both thiopurines, TPMT activities were lower in the heterozygous genotype children.

Comparisons between the heterozygote *TPMT*1/*3A* and *TPMT*1/*3C* children were not possible with thioguanine (32 *TPMT*1/*3A*, four *TPMT*1/*3C*), but within the mercaptopurine cohort, *TPMT *1/*3A* children ($n = 53$) had significantly higher TGN and MeMPN concentrations than *TPMT *1/*3C* children ($n = 11$) (Table 4), despite having similar drug dosages and TPMT activities.

Table 2

Thiopurine methyltransferase genotype and metabolite formation

	Wild-type *TPMT*1/*1*	Heterozygous *TPMT *1/*3, *1/*2*	Median difference (95%CI)
TG			
Patients	381	37	
Dose (mg m^{-2})	40 (10–78)	40 (18–38)	–1.0 (–3, –0.1), $P = 0.009$
TG-TGNs (pmol)	1 904 (36–4 337)	2 468 (174–6 730)	478 (180, 772), $P = 0.0026$
TPMT units	14 (5.4–22.8)	8.1 (5.2–12.9)	–5.8 (–6.5, –5), $P < 0.0001$
MP			
Patients	613	67	
Dose (mg m^{-2})	75 (6–125)	74 (14–113)	–1.0 (–3.0, 0.002), $P = 0.09$, **NS**
MP-TGNs (pmol)	360 (0–1 216)	754 (132–2 228)	406 (332, 478), $P < 0.0001$
MeMPNs (pmol)	10 650 (0–141 772)	3 868 (60–38 386)	–5524 (–7396, –3868), $P < 0.0001$
TPMT (units)	14 (5.6–26.3)	7.9 (2.8–13.6)	–6.3 (–6.9, –5.7), $P < 0.0001$)

MeMPNs, methylmercaptopurine nucleotides; MP, mercaptopurine cohort; TG, thioguanine cohort; TGNs, thioguanine nucleotides; TPMT, thiopurine methyltransferase. TGN and MeMPN units are pmol/8 × 10^8 red blood cells. TPMT units are units ml^{-1} packed red blood cells. The TPMT variant alleles quantified were *TPMT *1/*2* (MP $n = 3$; TG $n = 1$), *TPMT *1/*3A* (MP $n = 53$; TG $n = 34$) and *TPMT *1/*3C* (MP $n = 11$; TG $n = 4$). Values are given as median (range), NS, not significant. The analysis excludes TPMT deficient children.

Table 3

Metabolite accumulation within the thiopurine methyltransferase intermediate activity cohort; wild-type genotype and heterozygous genotype compared

TPMT ≤10.5 units	Wild-type genotype	Heterozygous genotype	Median difference (95% CI)
TG patients	35	33	
TG-TGNs (pmol)	2 360 (910–4 060)	2 626 (174–6 730)	246 (–214, 664), $P = 0.29$, **NS**
TPMT (units)	9.9 (5.4–10.5)	7.7 (5.2–10.5)	–1.7 (–2.4, –1.0), $P < 0.0001$
MP patients	44	63	
TG-TGNs (pmol)	366 (0–1 216)	754 (132–2 228)	369 (250, 502), $P < 0.0001$
MeMPNs (pmol)	8 590 (0–96 964)	3 825 (60–38 386)	–4822 (–7278, –2518), $P = 0.0001$
TPMT (units)	9.7 (5.6–10.5)	7.8 (2.8–10.5)	–1.4 (–2.0, –0.7), $P < 0.0001$

MeMPNs, methylmercaptopurine nucleotides; MP, mercaptopurine cohort; TG, thioguanine cohort; TGNs, thioguanine nucleotides; TPMT, thiopurine methyltransferase. TGN and MeMPN units are pmol/8 × 10^8 red blood cells. TPMT units are units ml^{-1} packed red blood cells. For the intermediate activity cohort (TG $n = 68$, MP $n = 114$) median TG derived TGNs = 2492 pmol, MP derived TGNs = 562 pmol, MeMPNs = 5542 pmol. TPMT genotypes were available for all 68 children on thioguanine but only for 107 of the 114 children on mercaptopurine. The analysis excludes TPMT deficient children. Values are given as median (range).

Table 4

Mercaptopurine metabolite accumulation compared within the heterozygous genotypes *TPMT *1/*3A* and *TPMT*1/*3C*

Mercaptopurine	*TPMT *1/*3A*	*TPMT *1/*3C*	Median difference (95% CI)
Patients	53	11	
MP-TGNs (pmol)	802 (132–2 228)	492 (288–910)	288 (23, 445), $P = 0.031$
MeMPNs (pmol)	4 542 (84–38 386)	1 832 (60–10 746)	2458 (129, 5605), $P = 0.034$

MeMPNs, methylmercaptopurine nucleotides; MP, mercaptopurine; TGNs, thioguanine nucleotides; TPMT, thiopurine methyltransferase. TGN and MeMPN units are pmol/8 × 10^8 red blood cells. Values are given as median (range).

Multiple assays

For the investigation of thiopurine metabolism over time blood samples were requested during year 1, at the start of year 2 and towards the end of year 2. Of the 766 children from whom multiple samples were received only 465 children (221 thioguanine, 244 mercaptopurine) provided samples at the requested time intervals. Of these, 87 children randomized to thioguanine switched to mercaptopurine during maintenance therapy and were excluded from the analysis.

Thioguanine Multiple assays on thioguanine maintenance therapy were available for 134 children. Kruskal–Wallis analysis indicated that the thioguanine dose prescribed was lower at the end of therapy than at the start ($P = 0.029$) but there was no difference in TGN

Table 5

Thiopurine metabolism in the year 1 and in the end of year 2 assays. (A) Thioguanine analyzed by genotype, (B) Mercaptopurine analyzed by genotype

	Year 1	End of year 2	Wilcoxon difference (95% CI)
(A) TG, wild-type TPMT, *n* = 114			
TG dose (mg m^{-2})	40 (23–67)	40 (8–114)	–2.5 (–5.0, –1.0), *P* = 0.005
TG-TGNs (pmol)	1 898 (36–5 366)	1 952 (408–5 376)	54 (–114, 204), *P* = 0.52, **NS**
TG, heterozygous TPMT, *n* = 18			
TG dose (mg m^{-2})	38 (20–42)	35 (8–60)	–3 (–8.5, 2.5), *P* = 0.23, **NS**
TG-TGNs (pmol)	2 375 (174–6 730)	2 026 (1 282–4 216)	–172 (–806, 899), *P* = 0.70, **NS**
(B) MP, wild-type TPMT, *n* = 225			
MP dose (mg m^{-2})	75 (26–107)	75 (23–116)	0 (–1.5, 0.5), *P* = 0.59, **NS**
MP-TGNs (pmol)	372 (18–994)	324 (36–742)	–45 (–66, –24), *P* < 0.001
MeMPNs (pmol)	9 916 (0–55 952)	14 747 (506–88 134)	3877 (2286, 5541), *P* < 0.001
TPMT (units)	14.9 (10.6–26.3)	13.9 (9.8–20.2)	–1.0 (–1.8, –0.5), *P* < 0.001
MP, heterozygous TPMT, *n* = 18			
MP dose (mg m^{-2})	73 (37–77)	73 (34–78)	0 (–1.0, 1.0), *P* = 0.92, **NS**
MP-TGNs (pmol)	644 (360–1 928)	802 (48–1 258)	82 (–155, 311), *P* = 0.37, **NS**
MeMPNs (pmol)	4 310 (247–41 084)	4 886 (0–29 048)	931 (–2858, 4317), *P* = 0.66, **NS**
TPMT (units)	8.9 (6.0–13.6)	7.4 (6.1–13.6)	0.33 (–2.15, 3.4), *P* = 0.79, **NS**

MeMPNs, methylmercaptopurine nucleotides; MP, mercaptopurine cohort; NS, not-significant; TG, thioguanine cohort; TGNs, thioguanine nucleotides; TPMT, thiopurine methyltransferase. TGN and MeMPN units are pmol/8 × 10^8 red blood cells. TPMT units are units ml^{-1} packed red blood cells. Table 5A Thioguanine *n* = 134, genotypes available for 132 children (17 TPMT*1/*3A, one TPMT*1/*3C). One wild-type had overt compliance problems and was prescribed thioguanine doses of >200% protocol by the end of year 2. If this child was removed from the analysis the maximum prescribed dose was 68 mg m^{-2} and the median dose difference at the end of treatment was –3.0 mg (–5.0 to –1.0), *P* = 0.002. Table 5B Mercaptopurine *n* = 244, genotypes available for 243 children (16 TPMT*1/*3A, one *1/*3C, one *1/*2). Values are presented as the median (range).

concentrations or TPMT activities. Matched analysis of the year 1 assay with the end of year 2 assay indicated a decrease of 2.5 mg m^{-2} in thioguanine dose over time, a similar decrease was observed in both genotype groups (Table 5A).

Mercaptopurine Kruskal–Wallis analysis indicated that TGN concentrations were higher at the start of therapy ($P < 0.001$), whilst MeMPN concentrations were higher at the end ($P < 0.001$). TPMT activities were lower at the end of year 2 ($P < 0.001$), a difference (1.0 units TPMT) that was clinically not-significant. There was no significant difference in mercaptopurine dosage.

Matched analysis of the year 1 assay with the end of year 2 assay indicated a marginal decrease in TGN concentrations and TPMT activities over time but a profound increase in MeMPN concentrations. Splitting the data by genotype (225 wild-type TPMT, 18 heterozyotes) clearly showed that the increase in MeMPN production was in the wild-type cohort (Table 5B). Further analysis of the wild-type cohort indicated that MeMPNs increased throughout maintenance therapy. Comparing the year 1 assay *vs.* the start of year 2 assay gave median MeMPN concentrations of 9916 pmol *vs.* 11 084 pmol respectively, (median difference 2199 pmol, 95% CI 494, 4052, $P < 0.001$).

Compliance problems

A number of children were suspected of taking their thiopurine doses intermittently or not at all. Blood cell counts remained high despite long term thiopurines at the protocol standard, or higher, dose. Mercaptopurine derived TGNs and MeMPNs accumulate slowly and uniformly in a population of red cells over a period of days and weeks [27]. Twenty children (2.7% of the 744 cohort) prescribed long term ≥100% mercaptopurine had metabolite concentrations persistently at or below the lower limit of detection. Thioguanine derived TGNs accumulate rapidly in the red cell after an oral dose. After a single 40 mg m^{-2} dose the inter-patient variation in TGNs concentrations at 6 h post-dose is 144 to 574 pmol [28, 29] and, after seven daily doses, 959 to 2361 pmol [28]. Thus, TGNs <750 pmol whilst taking ≥100% thioguanine could be regarded as resulting from non-compliance. Twenty-eight children had thioguanine TGNs of <750 pmol (6.2% of the 450 children taking thioguanine). Twelve of these children (2.7%) had TGNs <500 pmol.

Discussion

We report, in children with ALL, that an intermediate TPMT phenotype, based on enzyme activity measurements, cannot be categorically defined either at disease diagnosis or during chemotherapy. At disease diagnosis TPMT activities are much reduced and are not reflective of on-therapy activities. This reduction has been previously reported [26], along with the resulting TPMT genotype–phenotype discordance, in small patient cohorts with low numbers of variant alleles [30, 31], and is now confirmed in a large population of children with ALL. The reduction in TPMT activities is well below the ranges recorded for healthy children [15, 30] and has been attributed to the

disease process and the resulting anaemia of ALL with the associated measurement of a decayed TPMT enzyme [26]. During chemotherapy TPMT activities increase to levels well above the range recorded for healthy children [15, 30, 32]. These sizable leukaemia- and treatment-related changes in TPMT activities are not reported to occur in other clinical situations where thiopurine drugs are widely used [33]. Any disease-dependent TPMT activity distribution fluctuation is clinically insignificant [34]. For children with ALL, particularly at disease diagnosis, this is not so. TPMT reference ranges derived from healthy individuals, or patients on thiopurine immunosuppression, should not be used to derive presumed heterozygosity.

The elevation of RBC TPMT activities during chemotherapy may reflect the influence of therapy on red cell kinetics and life-spans. Bone marrow suppression, and the subsequent activation of erythropoiesis, has been shown to elevate TPMT activity [35], young red cells having higher TPMT activities than old cells [26]. Elevated TPMT activities are also observed in healthy neonates, (who have excess reticulocytes and nucleated red cells), compared with children [36] and in younger children compared with older children and adults [37]. We could detect no influence of age on TPMT activities in our study, any age effect perhaps masked by the underlying chemotherapy. Despite these possible influences, the TPMT activity ranges measured during chemotherapy were similar for both thiopurines and, when measured under standard conditions, TPMT activities were reproducible over time. However, genotype–phenotype concordance in the intermediate activity range was poor with 45% of children having a wild-type genotype. Sequencing revealed only two rare and three novel TPMT alleles. This discordance in the intermediate activity range has previously been reported in small patient cohorts and healthy subjects [19, 31, 38]. The first large scale TPMT genotype–phenotype concordance study in healthy blood donors ($n = 1214$) reported a clearly defined trimodal distribution and an overall concordance of >98% yet, after sequencing all discordant samples, reported 13 of 111 (11.7%) individuals with an intermediate activity wild-type in the open-reading frame [19]. A systematic review [39] of gastroenterology patients prescribed thiopurines reported the near-perfect specificity of variant allele genotyping for identifying patients with intermediate TPMT enzyme activities but, in agreement with our study, the converse was not true. An intermediate activity range, defined by sensitivity and specificity analysis, contains a high proportion of false positives i.e. wild-type genotype.

Polymorphic tandem repeats in the TPMT promoter can modulate TPMT activity [38] but larger studies ($n = 1211$) have shown these effects to be quantitatively small [40]. However, this may not be so for the influence of underlying chemotherapy [30]. Methotrexate mediated inhibition of intracellular methylation could limit the supply of the methyl cofactor for the TPMT reaction. An association between the *MTHFR 677C > T* variant (reduced methylation, up to 10% of the population) and heterozygous TPMT phenotypes with wild-type genotypes has been reported [41].

The fact that genotype is a better indicator of constitutive TPMT function than phenotype, in children with ALL, is illustrated by mercaptopurine metabolite accumulation in the intermediate activity cohort. The wild-type genotype/intermediate activity children have similar TGN concentrations to children with TPMT high activity and wild-type genotype. If TPMT assessment is restricted to genotyping the *TPMT*3* family and *TPMT*2* alleles, the risks of misclassification of a TPMT deficient child have been reported to be 1 in 7416 patients for a result of *TPMT*1/*3* or *TPMT*1/*2* and 1 in 14 832 patients for a *TPMT*1/*1* result [42]. Measurement of TPMT activity in heterozygous genotype children at disease diagnosis would detect the former whilst the use of activity measurements alongside genotype, for all children, would be required for the latter.

TGN concentrations in the TPMT deficient children were within the same range as those previously reported [12–14, 16]. TGN concentrations in the deficient child taking thioguanine were similar to those for mercaptopurine. For both thiopurines, heterozygous children accumulated significantly higher TGN concentrations compared with the wild-type cohort. This difference in TGN accumulation with respect to TPMT status has been reported for mercaptopurine [16], but not thioguanine, and is a reflection of increased drug methylation by higher TPMT activities. For mercaptopurine, TGNs were effective at much lower concentrations, some three to five-fold lower, than thioguanine derived TGNs. This effect has been attributed to the ability of MeMPNs to inhibit *de novo* purine synthesis [43]. Thus the TGN competitive inhibitor is successful at lower concentrations. The difference in mercaptopurine metabolite accumulation between *TPMT*1/*3A* and *TPMT*1/*3C* children has not been previously reported and may be due to differences in the degradation half-lives of the mutant variant alleles [44, 45]. Alternatively these observations may be due to problems with tablet taking or the small sample size of the latter cohort. In agreement with previous reports [46], the TPMT allele frequency differed between ethnic groups with white Caucasians having the highest frequency of low activity alleles and *TPMT*3A* predominating.

The analysis of thiopurine metabolism over time revealed potentially clinically significant findings. For thioguanine, the dose required to keep cell counts within the target range decreased, but the range recorded was wide and possible non-compliance with oral chemotherapy hinders the interpretation of these findings. Judged by very low, or absent, metabolite concentrations 2.7% of children were non-compliant, which may impact on treatment outcome. The use of drug metabolite ratios has estimated that there is strong evidence of non or partial compliance in 10% of ALL children [47], and electronic tablet counting

has estimated that up to 17% of ALL children take less than 80% of their prescribed pills [48]. The latter has been confirmed in a recent study and linked to a higher incidence of disease relapse [49].

For mercaptopurine, despite similar dosages and TGN concentrations, MeMPN concentrations increased as maintenance therapy progressed. There is some debate as to whether excess MeMPN accumulation is a cause, or a reflection, of hepatotoxic events [50, 51]. MeMPN concentrations above 5000 pmol/8 $\times$ 10^8 RBCs, measured in the first year of therapy in children with ALL, have been associated with hepatotoxicity [52], MeMPNs concentrations approximately half the group median concentration reported in the present UK study. However, a degree of hepatotoxicity, as demonstrated by elevated liver function tests, is associated with a lower relapse risk [53]. Whatever the mechanism underlying the increased MeMPN formation over time observed in the current study, the clinical significance warrants further investigation.

The data reported here will allow a more informed use of TPMT measurements in children and young adults with ALL. In this patient group genotype is superior to phenotype in the classification of TPMT heterozygosity. Previous observations with respect to TGN accumulation and TPMT status have been confirmed and extended to thioguanine, findings which may help guide the use of thiopurine drugs. The effective delivery of thiopurines is important in the successful treatment of ALL [15, 49]. It is of some concern that we observed overt non-compliance with oral thiopurines in about 3% of children and this was a confounding factor in the interpretation of our data. These findings indicate a possible role for metabolite monitoring, or compliance surveillance, throughout maintenance.

Competing Interests

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

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