ORIGINAL ARTICLE

Methotrexate polyglutamate levels and co-distributions in childhood acute lymphoblastic leukemia maintenance therapy

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Abstract

Purpose Methotrexate polyglutamates (MTXpg) facilitate incorporation of thioguanine nucleotides into DNA (DNA-TG, the primary cytotoxic thiopurine metabolite and outcome determinant in MTX/6-mercaptopurine treatment of childhood ALL). We hypothesized that mapping erythrocyte levels of MTXpg with 1–6 glutamates and their associations with DNA-TG formation would facilitate future guidelines for maintenance therapy dosing.

Methods and results Summed MTX with 1–6 glutamates resolved by LCMS [median (interquartile): 5.47 (3.58–7.69) nmol/mmol hemoglobin] was in agreement with total MTX by radio ligand assay. In 16,389 blood samples from 1426 ALL maintenance therapy patients, MTXpg3 21.0 (15.2–27.4)% was the predominant metabolite, and MTXpg1 (the maternal drug) constituted 38.6 (27.2–50.2)% of MTXpg1–6. All subsets correlated; the strongest associations were between metabolites with similar polyglutamate lengths. Correlations of MTXpg1 with MTXpg2 and MTXpg3,4,5,6 were $r_s = 0.68$ and $r_s = 0.25$–0.42, respectively. Intercorrelations of MTXpg3,4,5,6 were all $r_s \geq 0.51$. MTXpg4 accounted for 29.8 (24.7–33.3)% of MTXpg3–6, yet explained 96% of the summed MTXpg3–6 variation. MTXpg1–4, MTXpg1–6, MTXpg2–6 and MTXpg3 were all associated with DNA-TG levels ($p < 0.00001$), but collinearity precluded identification of the most informative subset.

Conclusions Measuring erythrocyte MTXpg4 simplifies and can replace longer chain MTXpg monitoring. Resolving individual MTXpg identifies samples that are unsuitable for dose guidance due to high levels of MTXpg1 remaining in the plasma fraction because of recent MTX intake. All tested MTXpg subsets correlated with DNA-TG and may be used for ALL maintenance therapy dose adjustments, but the most informative subset remains to be identified.

Abbreviations

DHFR	Dihydrofolate reductase
DNA-TG	DNA (-incorporated) thioguanine nucleotides
Ery-	Erythrocyte
G	Guanine
HD	High dose

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HR   High-risk
IR   Intermediate-risk
LCMS   Liquid chromatography tandem mass
          spectrometry
MP   Mercaptopurine
MT1/2   Early and late maintenance therapy phases
MTX   Methotrexate
MTXpgX   Polyglytamated MTX with X γ-linked
          glutamates
SR   Standard-risk
MTX_{tot} Total MTX determined by radio ligand assay
RLA   Radio ligand assay
TG   Thioguanine
TGN   Thioguanine nucleotides
WBC   White blood cell count

Introduction

The antifolate methotrexate (MTX) is widely used in childhood ALL as high-dose MTX (HD-MTX) and in oral maintenance therapy in combination with mercaptopurine (MP) [1]. The direct anticancer effect of MTX involves depletion of reduced folates, which in turn reduces de novo synthesis of thymidine and purine nucleotides. Further, the effect of MP is dependent on the formation and subsequent DNA incorporation of thioguanine nucleotides (TGN), and since endogenous purine nucleotides compete with TGN in the latter step, MTX augments the DNA incorporation and the anticancer effect of co-administered thiopurines [2, 3].

The low therapeutic index and high inter-patient variation in drug disposition challenge MTX dosing [1, 4]. During therapy, blood cell counts are related to thiopurine metabolite levels, and in most contemporary childhood ALL treatment protocols MTX/MP is adjusted according to a fixed white blood cell (WBC) count. However, since the patients' natural WBC deviate from this common target by an amount unknown to the physicians until therapy cessation, this does not correspond to identical degrees of bone marrow suppression [5], and therapeutic drug monitoring (TDM) may improve therapy adjustment.

Intracellular enzymatic glutamyl synthetase and -hydrolase activities, respectively, add and remove γ-linked glutamic acid residues to MTX and its metabolites. This balance determines the polyglutamate status of intracellular MTX, which ranges from MTXpg1 (the mother pro-drug) to MTXpg7 with a total of 7 γ-linked glutamic acid residues. Longer chain variants are retained longer within cells and have higher affinities to folate metabolism target enzymes (e.g. dihydrofolate reductase, DHFR). Thus, the molar antifolate activity for longer chain (pg ≥ 3) MTX metabolites is higher [6]. Moreover, since MTX half-life in blood plasma is 4–6 h, TDM has used erythrocyte (Ery-) levels as a surrogate marker for MTX treatment intensity as this is an easily accessible cell population, where MTXpg can be measured weeks after MTX administration [4]. During erythropoiesis, accumulation of MTX ceases at the erythroblast stage. Thus, MTXpg in mature erythrocytes reflect exposure at the bone marrow precursor stage, and MTX accumulation is delayed approximately 2 weeks corresponding to their maturation and migration into peripheral blood [7]. We have recently shown that cytosolic thiopurine and total MTXpg2–6 synergize in DNA-TG formation and that DNA-TG is associated with relapse risk independent of on-therapy WBC [8].

In 2015, a liquid chromatography tandem mass spectrometry (LCMS) assay for erythrocyte MTX metabolites resolved into polyglutamate variants was introduced in our laboratory to replace our radio ligand assay (RLA) that does not distinguish between polyglutamate chain lengths [9]. In the transition period, 3134 samples were measured with both methods. Since in this multicenter study, we did only have dosing information from our local ward, and since the plasma fraction of blood drawn shortly after MTX administration contains interfering MTXpg1, a major limitation with RLA method was to exclude these samples from being used in TDM [9]. For the most extreme samples this problem persisted even with the technical precautions implemented with LCMS analysis as outlined in the section "Erythrocyte methotrexate assays". With the samples analyzed with both LCMS and RLA, we developed an algorithm to exclude those with interfering plasma MTXpg1. With this we here report MTXpg metabolite levels and co-distributions in 16,389 blood samples from 1426 Nordic/Baltic patients. In a patient subpopulation with close MTX and thiopurine metabolite monitoring we investigated the effect of selected MTXpg subsets on DNA-TG formation to explore the TDM potential of MTXpg.

Materials and methods

Therapy and patients

The NOPHO ALL2008 treatment protocol has been described [8, 10]. Briefly, maintenance therapy comprises oral MP at starting doses dependent on thiopurine methyltransferase status (deficient: 10; heterozygous: 50; wild-type 75 mg/m²/day) and MTX 20 mg/m²/week. Doses are subsequently adjusted to achieve a WBC target of $1.5\text{–}3 \times 10^9$/L. The patient risk group allocation [11] dictates onset and duration of maintenance therapy, which for standard- and intermediate-risk (SR, IR) patients during the first year is supplemented with alternate courses at 4-week intervals of vincristine/dexamethasone or HD-MTX (5 g/m²/24 h) with intrathecal (it) until five HD-MTX have been given (MT1). In addition, pegylated *E. coli* asparaginase is given at 2- or

6-week intervals (NOPHO 2008 randomized clinical trial) during the first months of MT1 [10]. The subsequent MT2 until treatment week 130 consists of oral 6MP/MTX, supplemented in IR patients only with intrathecal MTX at 8-week intervals. In high-risk (HR) patients the multidrug MT1 and delayed intensification phases (weeks 36–104) are followed by a short MT2 phase (weeks 105–130) with oral 6MP and oral/it MTX.

For the method comparison and MTXpg co-distribution parts of this study, we retrieved from our database 1432 Nordic/Baltic NOPHO-2008 ALL patients 045 years of age who had MTXpg analyzed with LC–MSMS median (interquartile) 9 (4–16) times, corresponding to a total of 16,795 samples collected during MT1 or MT2. Among these, 3134 samples from 462 patients were also analyzed for total MTX (MTX$_{tot}$) using RLA with median 4 (2–10) determinations. In the patient subset with the most intensive metabolite monitoring we assessed the association of DNA-TG levels with free thiopurine and MTXpg metabolites during MT2. Briefly, 338 SR- and IR-patients 1–18 years of age, who did not experience an event (relapse, death, second tumor), and with at least five metabolite determinations were included as described in [5, 8].

Erythrocyte methotrexate assays

Blood samples were to be taken 2 and 14 days after oral and HD-MTX, respectively, to avoid contribution from plasma MTXpg1 even in the event of delayed elimination; with the RLA assay, violation of these guideline was suspected in samples with high MTX$_{tot}$ levels. To alleviate this, washing the erythrocytes prior to analysis was implemented as part of the LCMS analysis.

For Ery-MTX measurements we used RLA based on displacement by sample MTX of radio-labelled MTX binding to DHFR [9]. This assay does not discriminate between MTXpg variants, thus total MTX (MTX$_{tot}$) is assayed. Moreover, due to the competitive nature of RLA, the standard curve plateaus, which compromise detection accuracy at high levels. Briefly, whole blood was diluted with PBS and incubated at 100 °C for 10 min to release cellular MTX. After centrifugation the clear supernatant was stored at − 20 °C until analysis. Competitive binding of MTX-metabolites and ^{3}H-MTX to DHFR [partially purified from chicken liver by sequential $(NH_4)_2SO_4$ precipitations] was performed in parallel with standards with known MTXpg1 concentrations. After precipitation of free ^{3}H-MTX with a charcoal/dextran mixture, radioactivity remaining in the supernatant was determined with liquid scintillation. Increasing unlabeled MTX beyond "complete" ^{3}H-MTX displacement from DHFR has no further effect on ^{3}H-MTX precipitation leading to the plateau described above. In replicate determinations RSD < 5% was obtained with MTX$_{tot}$ up

to ~ 50 nmol/L in working dilutions. Samples exceeding this level were designated "MTX$_{high}$" to indicate unknown but high MTX$_{tot}$ concentrations, and suspected contribution by extracellular MTXpg1. MTX levels were normalized by the sample hemoglobin (Hgb, Abacus hematology analyzer) to produce the MTX$_{tot}$ reporting unit nmol/mmol Hgb. Hence, with normal hematocrit values the assay range is approximately 0.6–45 nmol/mmol. Samples with lower levels were registered as 0 nmol/mmol.

With the recently published LCMS method individual erythrocyte MTXpg variants are quantified [12]. While being as sensitive as the RLA, the detection response with this technology does not plateau at high analyte concentrations. We adapted this analysis to run on an Acquity UPLC system coupled with a triple quadropole mass spectrometry detector (TQD) (Waters, Milford, MA, USA). Briefly, EDTA blood (0.5 ml/sample) was washed with (~ 5 ml) Dulbecco's PBS before freezing. Using stable-isotope-labelled IS (^{13}C, D$_3$) MTX-pg1 (Alsachim, Illkirch-Graffenstaden, France) and (^{13}C$_5$,^{15}N) MTXpg2–6 (Pepscan, Lelystad, The Netherlands) the analyte range was extended to include MTXpg6. At the time of analysis 400 μl water was added to the erythrocyte pellet, and 550 μl of the lysate was mixed with 100 μl internal standard (IS) solution in a separate tube before protein precipitation with 75 μl 70% perchloric acid. Chromatography was as described [12]. In line with the report of van Haandel et al. [13] we observed double charged (M + 2) parent ions of longer chain MTXpg and for MTXpg4–6 we used these in the multiple reaction monitoring transitions. Thus, transitions for MTXpg1,2,3,4,5 and 6 were 455.2/308.2, 584.3/308.2, 713.4/308.2, 421.8/175.2, 486.4/175.2, 550.9/175.2, and for the corresponding IS 459.2/312.2, 590.2/308.2, 719.4/308.2, 424.8/175.2, 489.3/175.2, 553.9/175.2, respectively. To enhance electrospray + ionization a post-column 0.3 ml/min methanol flow was introduced throughout the elution period using a Reagent Manager pump (Waters). The mass spectrometer was tuned by infusing MTXpg1–6 and was comparable with reported settings [12]. Calibrators were blank matrix with known amounts of MTXpg1–6. For all analytes, the RSDs of the low and high controls were between 10.0 and 17.0% and 9.3 and 10.9%, respectively. Hemoglobin concentration in the lysate tube was determined at A$_{541}$ using the short light path of a NanoDrop 1000 spectrophotometer to normalize MTXpg levels as described for the RLA method.

DNA-TG analysis

DNA-TG in 1–2 μg whole blood DNA was measured essentially as described by Jacobsen et al. [14], except that stable isotope internal standards (8-^{13}C-7,9-^{15}N$_2$ guanine (G, Cambridge Isotope Laboratories) and 4,5-^{13}C$_2$-7-^{15}N thioguanine (TG, Sigma-Aldrich, custom synthesis)) were included

during the ethno (ε)-derivatization step with chloroacetaldehyde. After solid phase extraction and hydrophilic interaction liquid chromatography tandem mass spectrometry, the response (peak areas normalized with their respective internal standard) ratios, εTG/εG were used to calculate levels (unit: fmol TGN/μg DNA) using calibrators with known amounts of TG spiked into blank DNA. The RSDs of low, medium and high controls were lower than 7.0, 5.4 and 3.3%, respectively.

Erythrocyte TGN and -methylated MP analysis

Erythrocyte levels of free thioguanine nucleotides (Ery-TGN) and methylated MP metabolites (Ery-MMP) were analyzed in EDTA-anticoagulated whole blood essentially as described by Shipkova et al. [15]. Briefly, proteins were precipitated with perchloric acid and the glycosidic bonds were hydrolyzed by incubation of the supernatants at 100 °C. Subsequently the released thioguanine bases and a methylated mercaptopurine derivative were quantified using reverse phase ultra-performance liquid chromatography (UPLC) with diode array detector. Metabolite levels were normalized to the sample's hemoglobin content as for the MTX RLA analysis to produce the nmol/mmol Hgb reporting unit.

Software and statistics

Unless otherwise specified, the levels of individual MTX metabolite were calculated as medians with interquartile ranges in parentheses, and their associations were evaluated with Spearman's rank correlation coefficient r_s or with Pearson's coefficient of determination r_p^2.

We used "R" (R version 3.4.3: a language and environment for statistical computing, R Core Team) and multiple linear mixed effect model for analysis of change in DNA-TG per increase in Ery-TGN, Ery-methylated mercaptopurine metabolites, Ery-MTXpg2–6, sex, age, and number of days in MT2 as fixed effects and with an individual (random) patient intercept. The analyses included 3853 measurements during MT2 in 338 patients with five or more simultaneous determinations of MTXpg, Ery-TGN, and Ery-methylated mercaptopurine metabolites (Ery-MMP) as described [5, 8].

Ethics

The study was approved by the Regional Ethical Committee of the Region of Copenhagen (H-2-2010-002). Written consent was obtained from all participants or their legal guardians.

Results

In the 3134 patient samples with Ery-MTX concentrations determined with both the RLA and LCMS, three subsets were defined: samples with MTX_{tot} above highest standard in the RLA assay [$n=81$ (2.58%)] were designated "RLA_{high}". Further, by visual inspection (Fig. 1a) of MTXpg1–6 vs MTX_{tot} co-distributions a line was defined that divided the remaining samples into another two subsets: valid sample drawing in accordance with our guidelines was expected for 2894 (92.3%) "RLA_{val}" samples above the division line, but for the remaining 159 (5.07%) "RLA_{inv}" samples, contribution of plasma MTXpg1 was suspected (as was also the case for "RLA_{high}" samples). In support of this, the relative contribution of MTXpg1 to MTXpg1–6 was higher among RLA_{inv} and in particular in RLA_{high} samples (Fig. 1b). In contrast, the absolute level of MTXpg2–6 was comparable for all three subsets (Fig. 1b).

Washing the blood prior to LCMS analysis removes approximately 98% of the sample plasma. Accordingly, LCMS provides valid results with most RLA_{inv} samples, and even with those (unknown) RLA_{high} samples that are only marginally above the highest RLA standard, because the contribution from remaining extracellular MTXpg1 relative to intracellular MTXpg1–6 is small. From the RLA subgrouping and MTXpg2–6 vs MTXpg1–6 co-distribution (Fig. 1b) an algorithm based exclusively on LCMS was designed whereby samples with MTXpg1–6 more than $2.7 \cdot MTXpg2–6 + 2.5$ were considered invalid ($LCMS_{inv}$). Overall, 96.6% (3027/3134) of the samples below or on this line were scored valid ($LCMS_{val}$), and this subset contained 98.9, 93.7 and 18.5% of the RLA_{val}, RLA_{inv} and RLA_{high} samples, respectively.

Expanding the dataset with 13,661 ALL patient samples analyzed with LCMS only, overall 97.6% (16,389/16,795) were scored $LCMS_{val}$. These samples were used for investigating the levels and co-distributions of individual MTXpg fractions (Fig. 2). Median summed MTXpg1–6 was 5.472 (3.576–5.932) nmol/mmol Hgb. Despite the omission of the $LCMS_{inv}$ samples, MTXpg1 constituted a median of 38.6% of total MTXpg1–6, and median contributions of MTXpg2 to MTXpg5 were all between 7.3 and 21.0% of MTXpg1–6. Details and absolute levels are shown in Fig. 2. MTXpg6 constituted less than 1% of total MTXpg1–6 as reported elsewhere [16].

All MTXpg were pairwise positively correlated with the closest correlations between MTXpg species with similar polyglutamate chain lengths (closest to the diagonal in Fig. 2), especially between longer chain MTXpg species. MTXpg4 tightly correlated ($r_s=0.98$, $r_p^2=0.965$) with total MTXpg3–6. Arguably this, to some extent, reflects autocorrelation of MTXpg4 included in both variables. However,
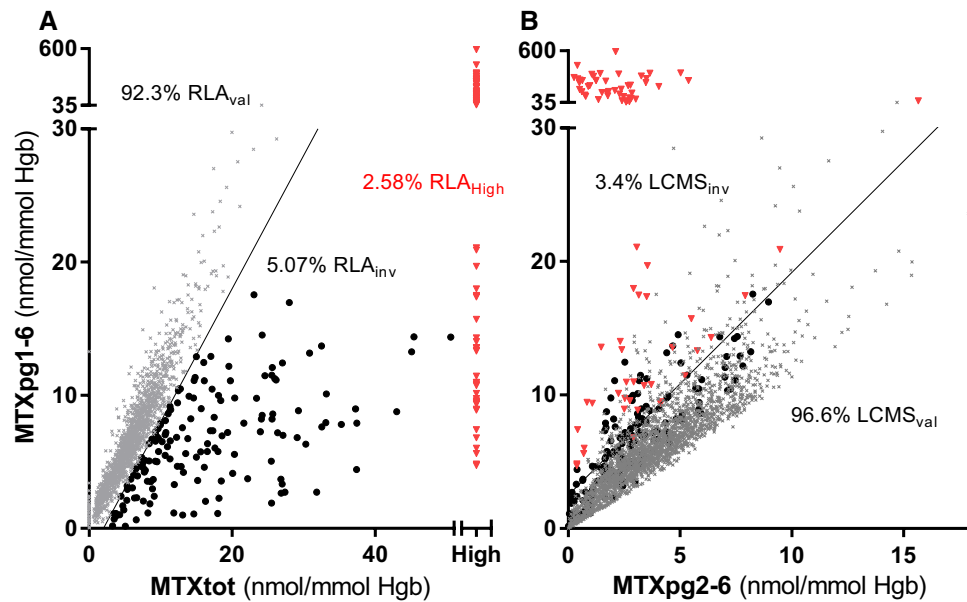
Fig. 1 Methotrexate metabolite levels by RLA and LCMS. **a** Co-distribution of MTXpg1–6 (by LCMS) vs MTX$_{tot}$ (by RLA) in 3134 samples from 462 patients analyzed median (interquatile) 4 (2–10) times with both RLA and LCMS. For the 92.3% RLA$_{val}$ samples (grey crosses), above the line ($y = x - 2$) the coefficients in weighed (1/[MTXpg1–6]) linear regression were $y = 1.07x + 0.16$. Invalid results due to high contributions of extracellular MTXpg1 was suspected in the 5.07% RLA$_{inv}$ (black circles) and 2.58% RLA$_{high}$ (red triangles) samples. **b** Co-distribution of MTXpg1–6 vs MTXpg2–6 (both by LCMS). With the LCMS method more samples were considered valid (96.6% LCMS$_{val}$ below the line $y = 2.7x + 2.5$), and the relative contribution of MTXpg1 to total MTXpg1–6 in these samples was lower as compared to the RLA$_{high}$ and RLA$_{inv}$ sample subsets. The RLA validity of the samples is indicated with the same coding as in the **a**

MTXpg4 contributed median 29.8 (24.7–33.3)% to total MTXpg3–6 only, and correlations of MTXpg4 with the remaining MTXpg3,5,6 were all $r_s = 0.74$–0.92 in the 16,353 samples with detectable MTXpg3–6 out of total 16,389 LCMS$_{val}$ samples (99.8%).

Using our recently published linear mixed model for associations of erythrocyte MTX/6MP metabolite levels with DNA-TG [5, 8] we compared the effects of MTXpg1–4, MTXpg1–6, MTXpg2–6, and MTXpg3 (Table 1). However, since the effect estimates and *p* values for these subsets in multiple regression models were very similar, the most informative MTXpg subset could not be determined.

Discussion and conclusions

Resolving individual MTXpg with LCMS identifies samples drawn too close to MTX administration and provides valid data for more samples than our RLA. Indeed, the calculated 92.2% RLA$_{val}$ vs 96.6% LCMS$_{val}$ underestimates the quality difference of the methods in that the distinction between RLA$_{val}$ and RLA$_{inv}$ for a substantial fraction of the samples requires concurrent measurement with LCMS (Fig. 1a). Recognizing that the measurements with RLA and LCMS were not comparable (due to the washing step in the latter

method) we did not attempt a formal Bland–Altman type assay comparison. Nonetheless, for the RLA$_{val}$ samples the two methods are in good agreement (Fig. 1a). Admittedly, the designation into RLA$_{val}$ and RLA$_{inv}$ samples subsets is subjective and somewhat arbitrary. However, it is corroborated by the higher contribution to total MTX metabolites of MTXpg1 among RLA$_{inv}$ samples, and the inclusion or non-inclusion of the small number of LCMS$_{inv}$ samples (Fig. 1b) will not substantially influence the median metabolite levels or r_s correlation estimates.

The close association between MTXpg4 and MTXpg3–6 (Fig. 2) in more than 1426 SR-, IR-, or HR-patients over time and across sex and age is noteworthy and may at least in part be explained by the decreasing folylpoly-gamma-glutamate synthetase activity with substrates with longer polyglutamate chains [17]. Regardless of the cause, MTXpg4 captured more than 96.5% (Pearson's r^2) of the variation in estimated total MTXpg3–6 and may therefore replace and simplify total longer chain MTXpg monitoring without appreciable information loss. Moreover, omitting (the most hydrophilic) MTXpg5–6 allows steeper gradients and more organic solvents with shorter run times and better peak shape and LCMS sensitivity in the reverse phase separation, which is operated at the lower extreme with respect to organic eluent.
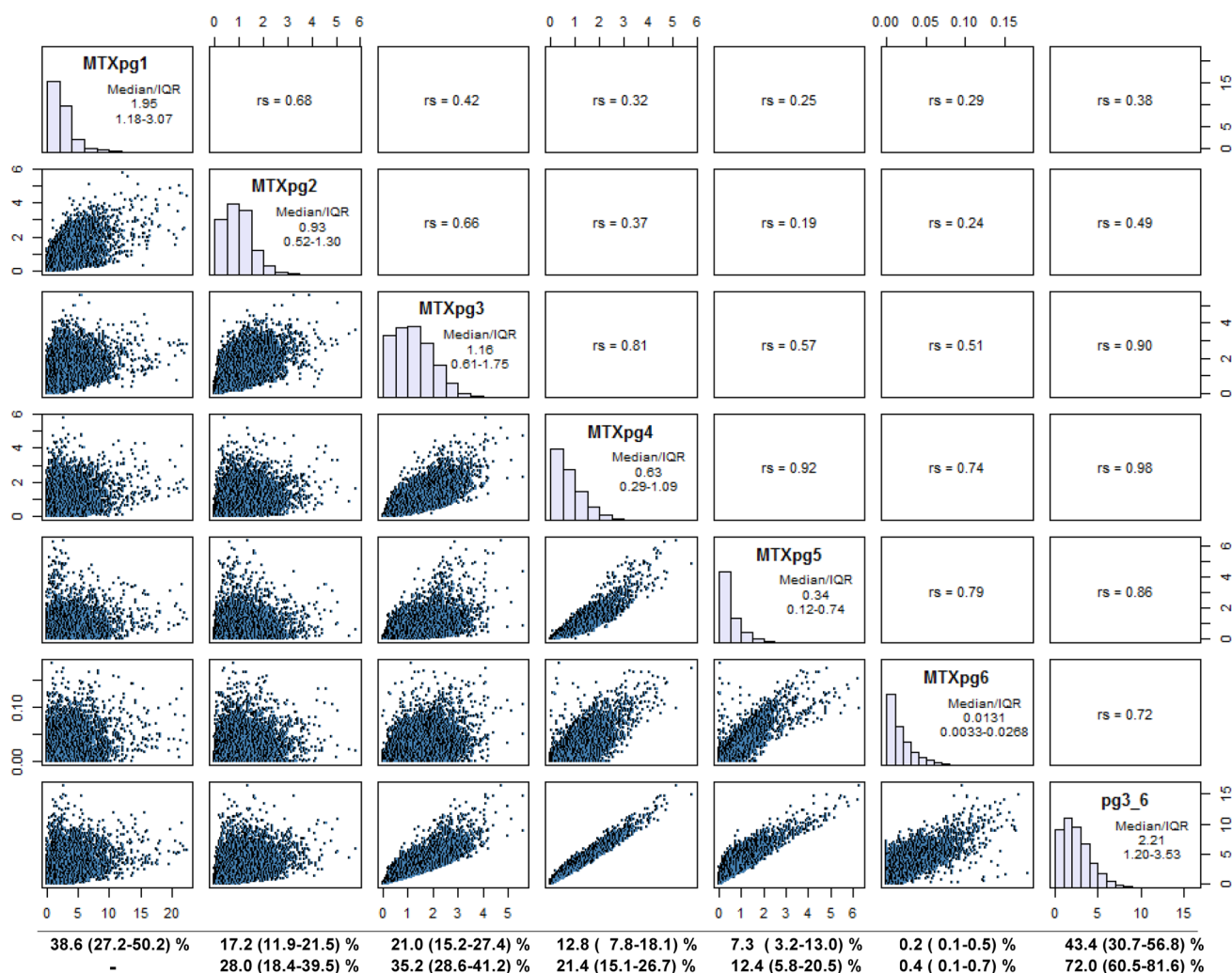
Fig. 2 MTX metabolite co-distributions. MTXpg levels (nmol/mmol Hgb) in 16,389 LCMS$_{val}$ samples from 1426 patients with median (interquartile range IQR) 8 (4–16) determinations. Distributions of individual MTXpg metabolites are shown in the panel diagonal. Dot plots with MTXpg co-distributions are shown in the lower left panels with Spearman's r_s in the corresponding upper right panels. Below the panels the median (IQR) contributions of individual subsets to MTXpg1–6 and MTXpg2–6 are shown in the upper and lower row, respectively

Since our linear mixed models for DNA-TG formation (Table 1) are not nested within each other, formal comparison to identify the most informative MTXpg metabolite subset could not be performed. However, all models produced the expected positive main effect estimates for all MTX and MP metabolites, and the negative EryMMP:MTXpg interaction term can be explained by the diminishing effect of either of these purine de novo synthesis inhibitors in the presence of the other. Thus, optimal TDM could employ the total MTXpg2–6 pool, which best reflects concurrent antifolate activity and exposure yet not too sensitive to the short-term fluctuations associated with MTX administration. Associations of longer chain MTXpg with outcomes in malignant and autoimmune disease have been reported [18–20], but its superiority to total MTX metabolite monitoring remains to be demonstrated in childhood ALL, as does the underlying cause of any such effect. Indeed, identification of samples with interfering plasma MTXpg1 is the only gain by resolving individual MTXpg that is supported by our data. Nonetheless, MTX monitoring may supplement maintenance therapy dosing by WBC, and although no direct effect of MTX on survival could be demonstrated [8], MTX monitoring may aid correction in patients with suboptimal levels of free and DNA-incorporated TGN [1]. Thus, although equipment and expertise for MTX monitoring are not available at every treating center, the dynamics and analyte stabilities of MTX metabolites makes monitoring feasible and possible worthwhile [8].

Table 1 Associations of MTXpg metabolites with DNA-TG formation

	MTXpg subset				
	MTXpg1–4	MTXpg1–6	MTXpg2–6	MTXpg3	All models
	Effect estimate				p value range
Ery-TGN per 100	1.1724	1.17304	1.17449	1.17159	< 0.00001
Ery-MMP per 1000	1.04416	1.04388	1.04395	1.04824	< 0.00001
MTXpg subset	1.0776	1.06875	1.09934	1.29148	< 0.00001
MT2day per 100	1.08863	1.08974	1.0872	1.08131	< 0.00001
Age at diagnosis	0.99492	0.99453	0.99472	0.99541	0.28–0.36
Female Sex	0.89082	0.89135	0.89417	0.89169	0.004–0.006
EryMMP per 1000*MTXpg subset	0.99636	0.99666	0.99486	0.98493	< 0.00001

Linear mixed models with random intercept by patient of DNA-TG (fmol/microgram DNA) with MTXpg subsets: MTXpg1–4, MTXpg1–6, MTXpg2–6, and MTXpg3. The models include 338 SR-, IR- or HR-patients with no event during therapy and with five or more simultaneous measurements of MTXpg, Ery-TGN, and Ery-methylated mercaptopurine metabolites (Ery-MMP) (both in nmol/mmol Hgb), corresponding to 3853 measurements during MT2. The effect estimates are the relative change in DNA-TG per increase in thiopurine/MTX metabolites, time (days) in MT2, age in years at diagnosis, and Sex. Thus, DNA-TG increases approx. 17% per 100 nmol/mmol Hgb increments in Ery-TGN in all the models. Ranges of p value across all four models are shown in the right column. Upper- and lower 95% confidence intervals for all main effects and for the (EryMMP per 1000)*MTXpg subset interaction were within ±1.5% of the effect estimates except for Sex (±7.6%) and MTXpg subset in the MTXpg3 model (±4.7%). For all models the intraclass correlation coefficient [the ratio of the inter-patient variance to the total variance (inter-plus intrapatient variance)] was 0.28–0.29

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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