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ORIGINAL ARTICLE

Dynamics of leucocyte DNA thioguanine nucleotide levels during maintenance therapy of childhood acute lymphoblastic leukemia

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Abstract

Purpose Methotrexate (MTX)/6-Mercaptopurine (6MP)-based maintenance therapy is crucial to cure childhood acute lymphoblastic leukemia (ALL). Cytotoxicity is mediated by incorporation of thioguanine nucleotides (TGN) into DNA (DNA-TG) with higher levels in leucocytes being associated with reduced relapse risk. To further understand the dynamics of DNA-TG formation, we measured DNA-TG levels in leucocyte subsets during maintenance therapy and in the months following its discontinuation.

Methods DNA-TG levels were measured in leucocytes (DNA-TG_{Total}), polymorph nucleated granulocytes (neutrophils, eosinophils, basophils [DNA-TG_{PMN}]) and mononucleated cells (lymphocytes, monocytes [DNA-TG_{MNC}]) in 1013 samples from 52 patients on ALL maintenance therapy (951 samples during therapy and 62 samples after therapy discontinuation, respectively).

Results Median DNA-TG_{Total}, DNA-TG_{PMN} and DNA-TG_{MNC} during maintenance therapy were 539, 563 and 384 fmol/µg DNA, respectively. DNA-TG_{PMN} displayed more pronounced fluctuation than DNA-TG_{MNC} (range 0–3084 [interquartile range IQR 271–881] versus 30–1411 [IQR 270–509] fmol/µg DNA). DNA-TG_{Total} was more strongly correlated with DNA-TG_{PMN} ($r_S = 0.95$, $p < 0.0001$) than DNA-TG_{MNC} ($r_S = 0.73$, $p < 0.0001$). DNA-TG_{PMN} correlated less with DNA-TG_{MNC} ($r_S = 0.64$, $p < 0.0001$) and to a much lesser extent with absolute neutrophil count ($r_S = 0.35$, $p < 0.0001$). Following discontinuation of therapy, DNA-TG_{PMN} was rapidly eliminated, and not measurable beyond day 22 after discontinuation, whereas DNA-TG_{MNC} was slowly eliminated, and five patients demonstrated a measurable DNA-TG_{MNC} more than 365 days after therapy discontinuation.

Conclusion Fluctuations in DNA-TG_{Total} are predominantly caused by corresponding fluctuations in DNA-TG_{PMN}, thus DNA-TG_{Total} measures recent TGN incorporation in these short-lived cells. Measurement of DNA-TG_{Total} at 2–4 weeks intervals provides a reliable profile of DNA-TG levels.

Keywords Acute lymphoblastic leukemia · Mercaptopurine · Methotrexate · Pharmacokinetics · Thioguanine nucleotides · Maintenance therapy

Introduction

Survival rate of childhood acute lymphoblastic leukemia (ALL) has improved tremendously over the last decades and now surpasses 90% in children receiving the best contemporary therapy [1, 2]. Methotrexate (MTX) and 6-Mercaptopurine (6MP)-based maintenance therapy constitutes a key element of ALL therapy [3]. To adjust for the substantial inter-individual variability in MTX and 6MP pharmacokinetics, maintenance therapy doses are guided by degree of myelosuppression, i.e. by white blood cell count (WBC) or

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absolute neutrophil count (ANC), which have been associated with relapse rates [4–9]. WBC is, however, confounded by substantial natural variation with gender, age, circadian fluctuations and ethnicity, and is therefore a weak surrogate parameter for MTX/6MP treatment intensity [10–12]. Insufficient treatment intensity during maintenance therapy has alongside shortening of maintenance therapy been related to a poorer ALL outcome, thus emphasizing the importance of effective maintenance therapy and warranting identification of a better indicator of MTX/6MP treatment efficacy [3, 13–16]. Thioguanine nucleotides (TGN) are the primary mediators of 6MP cytotoxicity and compete with guanine for incorporation into DNA (DNA-TG) [17]. Incorporated TGN undergo random methylation, which favors mismatch between TGN and thymidine. This results in repetitive but futile attempts of mismatch repair, which ultimately leads to cell death catalyzed by the mismatch repair system [18–20]. Both methylated 6MP metabolites and MTX inhibit purine de novo synthesis, and concomitant administration of MTX therefore enhances DNA-TG formation, due to decreased guanine availability [17, 21–24].

Higher leucocyte DNA-TG levels during maintenance therapy have been shown to be associated with decreased relapse rates [15]. This argues for a potential advantage of adjusting maintenance therapy according to DNA-TG level, thus aiming to reduce risk of relapse through increased DNA-TG. To further understand the dynamics of DNA-TG incorporation, we studied DNA-TG levels in leucocytes and leucocyte subsets during maintenance therapy and in the months following its discontinuation.

Materials and methods

Study participants

Study patients were aged 1–18 years at ALL diagnosis and included from May 2016 to November 2018. Patients were treated according to the Nordic Society of Pediatric Hematology and Oncology (NOPHO) ALL2008 protocol at the Department of Pediatrics and Adolescent Medicine, Rigshospitalet, University of Copenhagen, Denmark. Risk grouping and therapy details of the NOPHO ALL2008 protocol have been published previously elsewhere [1, 25, 26]. Eligible patients were those who had started maintenance therapy in first remission and had at least one DNA-TG measurement available in leucocytes and subsets during maintenance therapy.

The study was approved by the Ethical Committee of the Capital Region of Denmark (H-2.2010-002), and written informed consent was secured from all participants or their legal guardians.

Maintenance therapy, NOPHO ALL2008 protocol

Maintenance therapy is divided into two therapy phases: Maintenance-I and maintenance-II. The fundament of both phases of maintenance therapy is daily oral 6MP and weekly oral MTX irrespective of risk group stratification, and dose adjustments during maintenance therapy is targeted to achieve a WBC target of $1.5–3.0 \times 10^9$/L. Patients with standard risk (SR), intermediate risk (IR) and high risk (HR) ALL start maintenance-I in week 20, 22 and 36 following diagnosis, respectively. For patients with SR and IR ALL maintenance-I contains alternating pulses every fourth week with either vincristine (2.0 mg/m^2 once; total five times for SR, and four times for IR patients) and dexamethasone (6 mg/m^2 for 5 days) or high-dose MTX (5.0 g/m^2/24 h iv, five times). Patients were further randomized as part of a NOPHO ALL2008 phase 3 study to receive intramuscular pegylated-asparaginase administrations either every second (total of ten administrations) or sixth week (total of three administrations) until week 33. Results of this study have been published in detail elsewhere [27, 28]. Patients with HR ALL receive three high-dose intravenous MTX administrations at 24-week intervals during maintenance-I.

Moreover, patients receive intrathecal MTX during maintenance-I, which for patients with IR ALL is supplemented with prednisolone and cytarabine in case of central nervous system leukemia at diagnosis (triple intrathecal therapy [TIT]), and patients with HR ALL receive alternating TIT and intrathecal MTX during maintenance-I.

After maintenance-I, patients with SR ALL continue directly to maintenance-II, which is the last phase of therapy in the NOPHO ALL2008 protocol. Patients with SR ALL enter maintenance-II in week 58 following diagnosis, whereas patients with IR and HR ALL receive 6 weeks of delayed intensification before entering maintenance-II in week 66 and 105, respectively. Patients with IR and HR ALL continue to receive intrathecal chemotherapy in addition to oral 6MP and oral MTX during maintenance-II until discontinuation of antileukemic therapy 2.5 years after diagnosis.

DNA-TG quantification

Blood samples for metabolite measurements were only drawn, when blood samples were otherwise indicated to evaluate ALL therapy. On the same day, patients had complete blood counts evaluated, including WBC, ANC, absolute lymphocyte count (ALC), monocytes, eosinophils, basophils, and thrombocytes.

DNA-TG level was quantified as a total in circulating leucocytes (DNA-TG$_{Total}$), and furthermore in polymorph nucleated granulocytes, i.e. neutrophils, eosinophils and

basophils (DNA-TG_{PMN}) and in mononucleated cells, i.e. lymphocytes and monocytes (DNA-TG_{MNC}). The gradient medium, Lymphoprep™ (Abbott) 1.077 ± 0.001 g/mL was used for separation into polymorph nucleated granulocytes and mononucleated cells.

For the quantification of DNA-TG, the same method was applied to determine DNA-TG_{Total}, DNA-TG_{PMN} and DNA-TG_{MNC}; 1 − 2 µg DNA was purified, de-purinized and subsequently ethenoderivatized using chloroacetaldehyde. Following normalization with the respective isotope internal standards, the ratios of thioguanine and guanine were calculated. Ratios were quantified with ultra-performance liquid chromatography tandem mass spectrometry and reported as fmol TGN/µg DNA. With 1 µg DNA per sample, the limit of detection was 4.2 fmol/µg DNA and the limit of quantification 14.1 fmol/µg DNA, with intraday and interday relative standard deviations of less than 11%, and an analytical linearity up to a minimum of 10,000 fmol/µg [29].

For the analysis, DNA-TG values in the range 1–29 fmol/µg DNA were set to 30 fmol/µg DNA, i.e. approximately twice the lowest quantifiable level, as this was the lowest standard in the applied DNA-TG analysis. A DNA-TG of 100 fmol/µg DNA corresponds approximately to a median ratio of incorporation of 1:30,000 nucleobases.

Outcomes

The objectives of this study were to (1) identify the primary determinator of DNA-TG_{Total} among DNA-TG_{PMN} and DNA-TG_{MNC}, (2) describe fluctuations in DNA-TG_{Total} and in subsets during maintenance therapy, (3) investigate the quality of separation into DNA-TG_{PMN} and DNA-TG_{MNC}, and (4) describe DNA-TG elimination after cessation of therapy as a surrogate parameter for the dynamics of DNA-TG incorporation.

Statistical analysis

Spearman correlations between DNA-TG measurements and blood counts were estimated with *p*-values based on cluster-robust standard errors to account for multiple measurements per patient. Patient levels of DNA-TG were calculated as medians of each patient's DNA-TG measurements, and the distributions of these were compared between independent groups with Mann–Whitney test. Patient coefficients of variation were calculated for each patient as the ratio of the standard deviation to the mean and were compared between DNA-TG subgroups with Wilcoxon signed-rank test.

To evaluate the quality of our separation into polymorph nucleated granulocytes and mononucleated cells, we calculated DNA-TG_{Total} from DNA-TG_{PMN}, DNA-TG_{MNC} and the blood counts, when all measurements were available from the same day for the same patient as:

$$\text{DNA-TG}_{\text{Total}} = \text{DNA-TG}_{\text{PMN}} \times \frac{\text{neutrophils} + \text{eosinophils} + \text{basophils}}{\text{leucocytes}} + \text{DNA-TG}_{\text{MNC}} \times \frac{\text{lymfocytes} + \text{monocytes}}{\text{leucocytes}}.$$

These were compared with the measured DNA-TG_{Total} with Wilcoxon signed-rank test.

Statistical analyses were performed using R version 4.0.0.

Results

A total of 52 patients were included (patient characteristics are summarized in Table 1), and a total of 1013 samples were available for analysis. Of the 1013 samples, 951 samples were taken during maintenance-I and maintenance-II (median 15.5 per patient, range 2–50), and 62 samples were taken after discontinuation of therapy.

All samples taken during maintenance therapy did not include all blood counts (as shown in Online Resource 1) and/or DNA-TG measurements (Table 2); hence, a total of 939 DNA-TG_{Total}, 947 DNA-TG_{PMN}, and 937 DNA-TG_{MNC} measurements taken during maintenance therapy were available. Neutrophils and lymphocytes constituted by far the largest proportion of polymorph nucleated granulocytes and mononucleated cells, respectively. Hence, DNA-TG_{PMN} and DNA-TG_{MNC} are regarded to predominantly reflect DNA-TG

Table 1 Demographics and patient characteristics

Sex, *n* (%)	
Male	33 (63)
Female	19 (37)
Age (years) at ALL diagnosis	
Median (range)	4.4 (1.6–17.1)
ALL Immunophenotype, *n* (%)	
Precursor B-cell	47 (91)
T-cell	4 (8)
Bi-lineage	1 (2)
Risk group stratification (day 79), *n* (%)	
Standard risk	26 (50)
Intermediate risk	24 (46)
High risk	2 (4)

ALL acute lymphoblastic leukemia

Table 2 Characteristics of DNA-TG levels during maintenance therapy (maintenance-I + II)

	Median	Interquartile range	Range	Number of samples
DNA-TG_{Total}	539	318–809	31–2888	939
DNA-TG_{PMN}	563	271–881	0–3084	947
DNA-TG_{MNC}	384	270–509	30–1411	937

Level of thioguanine nucleotides incorporated into DNA (DNA-TG) during maintenance therapy. DNA-TG_{Total}, DNA-TG level in leucocytes; DNA-TG_{PMN}, DNA-TG level in polymorph nucleated granulocytes (neutrophils, eosinophils, basophils); DNA-TG_{MNC}, DNA-TG level in mononucleated cells (lymphocytes, monocytes). All DNA-TG levels are reported in fmol/µg DNA

levels in neutrophils and in lymphocytes, respectively (Online Resource 1).

Median DNA-TG_{Total}, median DNA-TG_{PMN}, and median DNA-TG_{MNC} of all samples during maintenance therapy were 539, 563, and 384 fmol/µg DNA, respectively (Fig. 1a–c, Table 2).

DNA-TG_{Total} was more strongly correlated with DNA-TG_{PMN} ($r_s = 0.95$) than DNA-TG_{MNC} ($r_s = 0.73$, both $p < 0.0001$) Fig. 2a, b), whereas DNA-TG_{PMN} to a lesser extent was correlated with DNA-TG_{MNC} ($r_s = 0.64$). DNA-TG_{PMN} was to a much lesser extent correlated with ANC ($r_s = 0.35$, $p < 0.0001$, Fig. 2c). A comparable poor correlation was also observed between DNA-TG_{MNC} and ALC ($r_s = -0.22$, $p < 0.01$, Fig. 2d).

DNA-TG_{PMN} fluctuated with a range of 0–3084 fmol/µg DNA during maintenance therapy, and DNA-TG_{MNC} with a range of 30–1411 fmol/µg DNA. Median patient coefficients of variation (i.e. one coefficient of variation from each patient including all of their respective measurements) for DNA-TG_{Total}, DNA-TG_{PMN} and DNA-TG_{MNC} were 0.55 (interquartile range [IQR] 0.45–0.62), 0.67 (IQR 0.53–0.77) and 0.36 (IQR 0.29–0.44), respectively. The distribution of coefficients of variation for DNA-TG_{PMN} differed significantly from the distribution for DNA-TG_{MNC} ($p < 0.0001$).

The distributions of patient levels from all patients are summarized for the SR and IR groups and according to maintenance therapy phase in Table 3. The HR risk group was not reported here due to small patient numbers.

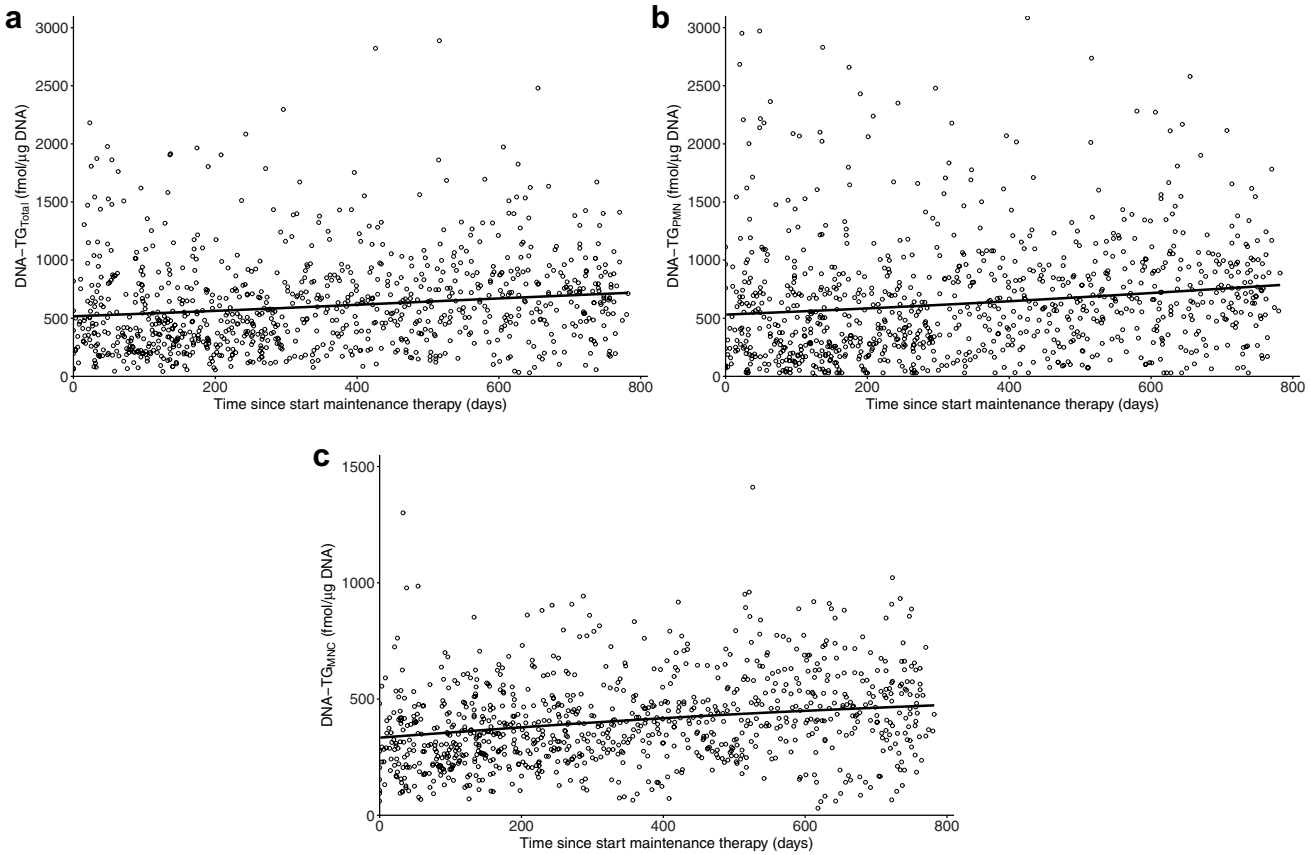

Fig. 1 **a–c** Level of thioguanine nucleotides incorporated into DNA (DNA-TG) during maintenance therapy. **a** DNA-TG_{Total}, DNA-TG level in leucocytes. **b** DNA-TG_{PMN}, DNA-TG level in polymorph nucleated granulocytes (neutrophils, eosinophils, basophils). **c** DNA-TG_{MNC}, DNA-TG level in mononucleated cells (lymphocytes, monocytes). All DNA-TG levels are reported in fmol/µg DNA

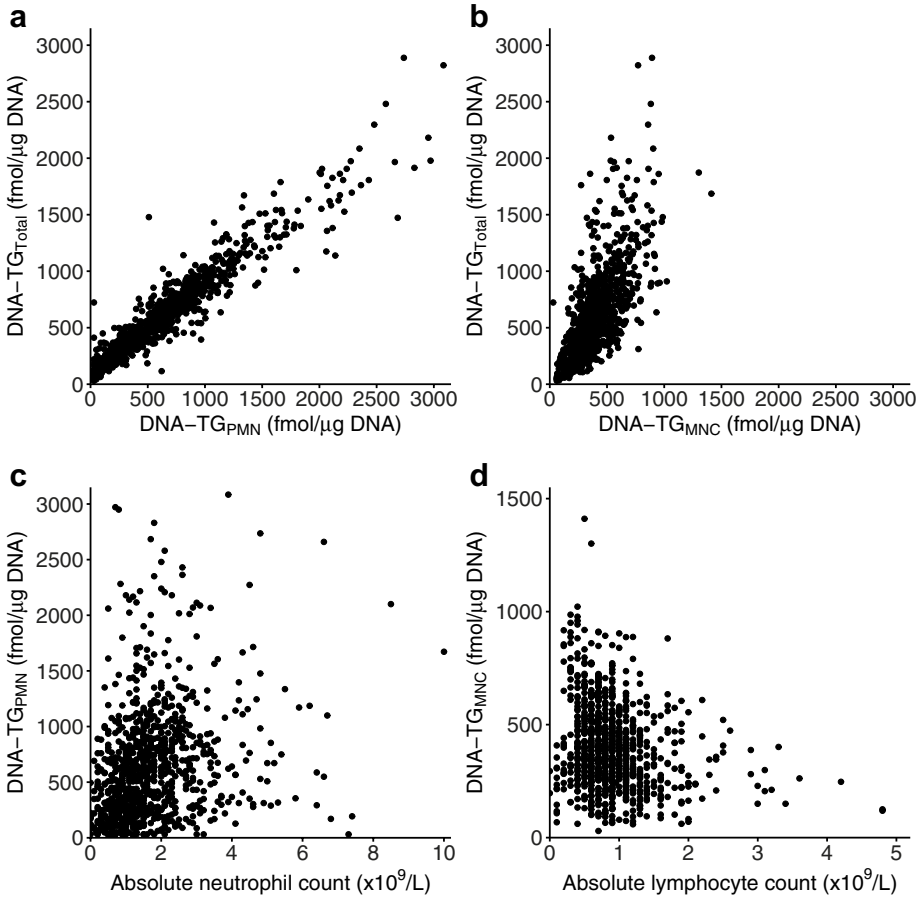

Fig. 2 **a–d** Correlation plots of levels of thioguanine nucleotides incorporated into DNA (DNA-TG) during maintenance therapy. DNA-TG_{Total}, DNA-TG level in leucocytes; DNA-$TG_{PMN,}$ DNA-TG level in polymorph nucleated granulocytes (neutrophils, eosinophils, basophils); DNA-TG_{MNC}, DNA-TG level in mononucleated cells (lymphocytes, monocytes). **a** DNA-TG_{Total} versus DNA-TG_{PMN}. **b** DNA-TG_{Total} versus DNA-TG_{MNC}. **c** DNA-TG_{PMN} versus absolute neutrophil count (ANC). **d** DNA-TG_{MNC} versus absolute lymphocyte count (ALC). All DNA-TG levels are reported in fmol/µg DNA

Including all patients, DNA-TG_{PMN} was significantly higher in patients with SR ALL compared with patients with IR ALL during maintenance-I (median 494 versus 275 fmol/µg DNA, respectively, $p = 0.03$). Age did not differ between patients in the two risk groups ($p = 0.98$). There were no other significant differences in DNA-TG levels, when comparing patients with SR ALL with patients with IR ALL.

A subset of 18 patients had at least one DNA-TG measurement available from both maintenance-I and maintenance-II. Comparison of DNA-TG levels from these patients showed that DNA-TG_{Total} was significantly higher in maintenance-II compared with maintenance-I ($p < 0.001$). In this subset of patients, the median DNA-TG_{Total} was 335 fmol/µg DNA during maintenance-I, and 719 fmol/µg DNA during maintenance-II.

Confirming the quality of separation into polymorph nucleated granulocytes and mononucleated cells, we found that measured DNA-TG_{Total} was strongly correlated with the calculated DNA-TG_{Total} ($r_s = 0.96$). The measured DNA-TG_{Total} was on average 7.7% higher than the calculated DNA-TG_{Total} (IQR 0.7–16.7%).

To describe DNA-TG elimination as a surrogate parameter for incorporation dynamics, a total of 62 samples from a median of 122 days after discontinuation of therapy (range 1–619, IQR 37–247) were available from 20 patients (11 males) with 1–7 DNA-TG_{Total} samples per patient (median 3). Of the total 62 samples, six samples from five patients demonstrated a measurable DNA-TG_{PMN} after discontinuation of therapy (i.e. above 30 fmol/µg DNA). These samples were all taken between 1 and 22 days after discontinuation of therapy and the patients had subsequently a sample of 30 or below. Beyond day 22 after discontinuation of therapy, no patients demonstrated a measurable DNA-TG_{PMN}. In comparison, DNA-TG_{MNC} was very slowly eliminated. Six patients had a DNA-TG_{MNC} sample available more than 365 days after discontinuation of therapy, of which five patients demonstrated a measurable DNA-TG_{MNC} at this time point (range 35–110 fmol/µg DNA).

Table 3 Distribution of patient medians from all patients according to risk group assignment and therapy phase

	Maintenance-I			Maintenance-II		
	Median	IQR	Range	Median	IQR	Range
All patients						
DNA-TG_{Total}	353	307–597	129–825	682	539–848	169–1412
DNA-TG_{PMN}	366	264–597	76–874	707	592–926	172–1561
DNA-TG_{MNC}	338	260–398	147–585	426	323–596	142–792
Standard risk						
DNA-TG_{Total}	517	332–597	222–825	695	564–831	279–1412
DNA-TG_{PMN}	494	363–726	188–874	700	603–872	243–1561
DNA-TG_{MNC}	348	281–411	208–585	429	382–531	171–792
Intermediate risk						
DNA-TG_{Total}	324	243–592	129–782	749	580–890	217–955
DNA-TG_{PMN}	275	245–523	76–753	725	622–940	172–1047
DNA-TG_{MNC}	316	257–363	147–580	486	358–604	236–753

Level of thioguanine nucleotides incorporated into DNA (DNA-TG) during maintenance-I and maintenance-II and according to risk group (i.e. standard risk and intermediate risk). Table shows distribution of patient medians (i.e. one median for all patients of the all their respective samples). Data from maintenance-I contained 500 samples from 33 patients. Of these 17 patients/243 samples were from patients with standard risk ALL, and 14 patients/203 samples were from patients with intermediate risk ALL

Data from maintenance-II contained 451 samples from 37 patients. Of these 18 patients/272 samples were from patients with standard risk ALL, and 17 patients/157 samples were from patients with intermediate risk ALL. High risk patients were included in the reporting of therapy phase characteristics, but were excluded when analyzing risk group characteristics, due to low patient number ($n=2$). DNA-TG_{Total}, DNA-TG level in leucocytes; DNA-TG_{PMN}, DNA-TG level in polymorph nucleated granulocytes (neutrophils, eosinophils, basophils); DNA-TG_{MNC}, DNA-TG level in mononucleated cells (lymphocytes, monocytes). All DNA-TG levels are reported in fmol/µg DNA. *IQR* Interquartile range

Discussion

Sufficient treatment intensity during maintenance therapy is crucial to ensure lasting remission of ALL. The persistent challenge has been to identify an optimal parameter to monitor for MTX/6MP treatment intensity. Historical strategies have been based on WBC or ANC, as sustained myelosuppression has been strongly associated with relapse rates [4, 7–9]. The inherent weakness of WBC to reflect MTX/6MP cytotoxicity (WBC fluctuates according to gender, ethnicity, age, infection and circadian rhythms [10–12]) is, however, a tremendous challenge to this strategy.

The cytotoxicity of maintenance therapy is based on formation of DNA-TG [17]. In the NOPHO ALL2008 maintenance therapy sub-study, it was shown that higher leucocyte DNA-TG levels during maintenance therapy were associated with decreased relapse rates [15]. The relapse hazard was reduced by 28% for every 100 fmol/µg DNA increase in DNA-TG, without signs of plateauing of the effect [15]. It was further demonstrated that DNA-TG levels were neither associated with MTX/6MP doses, nor associated with ANC or ALC during maintenance therapy [15]. MTX/6MP dose intensity and degree of myelosuppression can therefore not predict the DNA-TG level [15], which is consistent with the poor correlations we observed between DNA-TG_{PMN} and ANC, as well as between DNA-TG_{MNC} and ALC.

We observed that DNA-TG_{PMN} was significantly higher during maintenance-I in patients with SR ALL compared with patients with IR ALL. Potential explanations could be that treatment prior to maintenance-I is notably more intensive for patients with IR ALL compared with patients with SR ALL. This could render the bone-marrow of IR patients with a different turnover, thus resulting in an altered pattern of DNA-TG incorporation. Further, the behavior of the treating physician might be influenced by the more intensive treatment for patients with IR ALL prior to maintenance-I, thus making their dosing strategy during maintenance-I less "aggressive" to obtain WBC target. Patients with IR ALL "catch up" during maintenance-II, where no significant difference was observed with regards to DNA-TG level.

DNA-TG level seems to represent an integrated measure of MTX/6MP cytotoxicity, and titration of MTX/6MP therapy according to DNA-TG level could therefore be a superior alternative to WBC/ANC aiming to reduce risk of relapse through increased DNA-TG. Thus, DNA-TG level holds the potential to serve as a dose adjustment parameter during maintenance therapy, and exploration of this potential in a therapeutic drug monitoring setting is currently investigated in the Thiopurine Enhanced ALL Maintenance therapy study (clinicaltrials.gov NCT02912676).

Present results highlighted the strong correlation between DNA-TG_{Total} and DNA-TG_{PMN}, and for multicenter studies

DNA-TG_{Total} can be used as a surrogate biomarker for the most recent build-up of DNA-TG in dividing cells due to the stability of DNA and additionally without the need to perform cell separation. Further, DNA-TG_{Total} and DNA-TG_{PMN} displayed similar fluctuations during maintenance therapy, whereas DNA-TG_{MNC} was much less variable. This was emphasized by the coefficients of variation, and further underlined by DNA-TG_{MNC} displaying an IQR nearly half the size of the corresponding interval for DNA-TG_{Total} and DNA-TG_{PMN}.

As lifespan for neutrophils is 0.5–1 day versus months to years for lymphocytes, the results of the present study are well explained. Due to the short lifespan of neutrophils (constituting the majority of the PMN fraction), the turnover of DNA-TG will be rapid, and thus prone to fluctuations, and due to the strong correlation between DNA-TG_{Total} and DNA-TG_{PMN}, these wide fluctuations are also observed in DNA-TG_{Total}. The short neutrophil turnover results in a rapid TGN incorporation, which was also confirmed by the observed very rapid elimination of DNA-TG_{PMN} after discontinuation of therapy.

Further, we hypothesized that elimination of DNA-TG after discontinuation of therapy was a surrogate parameter for the dynamics of DNA-TG incorporation. This is an approximation as lifespan of cells can change. However, elimination of DNA-TG was shown to be a two compartmental model, with a very rapid elimination in neutrophils and a far more prolonged elimination of DNA-TG in lymphocytes. Due to origin of the malignant clone, a clinical significance of both a higher DNA-TG_{MNC} after completion of therapy as well as a prolonged elimination could be speculated. Data were however too limited to address this.

Conclusion

Fluctuations in DNA-TG_{Total} are predominantly caused by corresponding fluctuations in DNA-TG_{PMN}. Measurements of DNA-TG_{Total} during maintenance therapy therefore primarily reflect recent TGN incorporation in the short-lived neutrophils. Due to this association and based on the observed elimination characteristics of DNA-TG_{PMN} after discontinuation of therapy, measurement of DNA-TG_{Total} at 2–4 weeks intervals will provide a reliable profile of DNA-TG level during maintenance therapy.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00280-020-04219-5.

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Author contributions RHL coordinated the study, compiled data, and drafted the manuscript. JN and MD performed DNA-TG analyses. KG supervised the study and performed the statistical analysis. LH and BAN supervised the study. KS initiated and supervised the study and had responsibility for the final submission for publication. All authors approved the final manuscript.

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Compliance with ethical standards

Conflict of interest Kjeld Schmiegelow has received speaker and/or advisory board honoraria from Jazz Pharmaceuticals and Servier; speaker fee from Amgen and Medscape; Educational grant from Servier.

Ethics approval The study was approved by the Ethical Committee of the Capital Region of Denmark (H-2.2010–002).

Informed consent Written informed consent was obtained from all participants or legal guardians.

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