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IMPACT OF MERCAPTOPURINE METABOLITES ON DISEASE OUTCOME IN THE AIEOP-BFM 2009 PROTOCOL FOR ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

In the maintenance phase of AIEOP-BFM acute lymphoblastic leukemia (ALL) 2009 protocol, mercaptopurine (MP) is given at the planned dose of 50 mg/m2/day; however, dose adjustments are routinely performed to target patients' white blood cells to the optimal range of 2000-3000 cells/μl. ALL pediatric patients (n=290, age: median (1st-3rd quartile): 4.8 (3.0-8.1) years; male: 56.9%) were enrolled mainly in four medium-large Italian pediatric hospitals; 14.1% of patients relapsed after a median (1st-3rd quartile) follow-up time of 4.43 (3.82-5.46) years from maintenance beginning. MP metabolites (thionucleotide (TGN) and methyl-derivatives (MMPN)) were measured in the erythrocytes of 387 blood samples of 200 patients by HPLC-UV. SNPs (rs1800462, rs1800460 and rs1142345 in TPMT gene, rs116855232 in NUDT15, rs1127354, rs7270101, rs6051702 in ITPA and rs2413739 in PACSIN2) were characterized by Taqman SNP genotyping assays. Cox proportional hazard models did not show an impact TGN levels and variability on relapse. In contrast, after multivariate analysis, relapse hazard ratio (HR) increased in ALL children of the intermediate risk arm compared to those in standard risk arm (3.44, 95% confidence interval (CI), 1.31-9.05, p= 0.012), and in carriers of the PACSIN2 rs2413739 T allele compared to those with the CC genotype (heterozygotes CT: HR, 2.32; 95% CI, 0.90-5.97; p=0.081; homozygous TT: HR, 4.14; 95% CI, 1.54-11.11; p=0.005). Future studies are needed to confirm the lack of impact of TGN levels and variability on relapse in the AIEOP-BFM ALL trials, and to clarify the mechanism of PACSIN2 rs2413739 on outcome.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common haematological cancer in children (<18 years), with a 90% 5-year survival rate in developed countries[1]. Regardless of risk and with the exception of patients with no adequate chemotherapy response and indication to hematopoietic stem cell transplantation, ALL protocols usually consist of earlier intensive phases to clear malignant leukemic cells from the body and restore normal hematopoiesis, followed by a prolonged mild bone marrow suppression to maintain clinical remission[2]. The importance of the maintenance phase has been demonstrated since the early 80s, with an increased incidence of relapse in undertreated patients after reinduction[3-5]. It is now generally accepted that a total therapy duration of at least 24 months is as necessary as an early remission for sustained event-free survival[6]. Administration of antimetabolite drugs such as daily mercaptopurine (MP) and weekly methotrexate (MTX), both given orally in outpatient care, is commonly used during maintenance. To exert their antileukemic effect, MP and MTX require a complex multistep intracellular enzymatic conversion (Figure S1); thus, these drugs are susceptible to intra- and inter-individual variations in their therapeutic response[7, 8]. MP is converted into pharmacologically active thioguanine nucleotides (TGN), including the triphosphate thionucleotides (dTGTP/TGTP) that compete with conventional dGTP/GTP nucleotides for incorporation into nucleic acids thus altering DNA base-pair stability and dynamics, impairing the mismatch repair system and causing cell cycle arrest and apoptosis[9]. Moreover, TGTP interfere with the GTPase Rac1, a Rho GTPase that regulates T-lymphocyte proliferation and represses immune responses[10]. Parental MP and TGN intermediates are methylated by the thiopurine methyltransferase (TPMT) enzyme. Methylated metabolites (MMPN) compete with the formation of TGN; the methylthioinosine monophosphate (meTIMP) is an effective inhibitor of the purine *de novo* biosynthesis pathway thus indirectly contributing to antiproliferative effects in the white blood cells (WBC), target cells of MP[9, 11]. Therefore, the overall balance between TGNs and MMPNs is to account for MP response and cytotoxicity. Patients carrying genetic low-activity variants in *TPMT* gene are at risk of hematological adverse events because of higher TGN levels when treated with full MP doses[12]. Similarly, low-activity genetic variants in nudix hydrolase 15 *(NUDT15),* a gene encoding for a pyrophosphatase that converts oxidized GTP to its monophosphate form thus preventing the dTGTP incorporation into DNA, lead to higher TGN levels and increased risk of myelosuppression[13]. These variants are now accepted as pharmacogenetic markers of MP-related toxicities and pharmacogenetically-based dose adjustment guidelines are available[14]. Additional single nucleotide polymorphisms (SNPs) in the inosine triphosphate pyro-phosphatase (*ITPA*) and in the protein kinase C and casein kinase substrate in neurons 2 (*PACSIN2)* genes are under investigation for their contribution to MP efficacy and safety[15, 16]. ITPA degrades TITP into TIMP regenerating an intermediate of reaction required for the synthesis of TGN; PACSIN2 is a protein involved in the regulation of cell cytoskeleton, intracellular trafficking and signaling, and autophagy[17, 18]. MTX is conjugated intracellularly to polyglutamates and thus remains trapped inside the cells. As an antifolate, MTX inhibits several folate-dependent enzymes whose function is central to thymidylate and *de novo* purine biosynthesis and in one-carbon metabolism, affecting amino acid metabolism, and

mitochondrial protein synthesis[19-21]. Ideally, the combination of MP and MTX in maintenance is synergic and safe to provide the required long-term mild suppression with minimal adverse effects on bone marrow. Planned MP and MTX doses vary according to international protocols (e.g., MP: 50 mg/m^2/day in the Associazione Italiana di Ematologia e Oncologia Pediatrica (AIEOP)- Berlin-Frankfurt-Muenster (BFM) and 75 mg/m^2/day in St. Jude Children's Research Hospital (SJCRH) Total XVII and Children's Oncology Group (COG) trials). An international consensus on the degree of myelosuppression to achieve is missing. Generally, a range between 1500-3500/µl is proposed when WBC are considered to prevent excessive myelosuppression[22, 23]; however, this approach remains a questionable parameter for tailoring MP/MTX because the degree of leukopenia reflects the pharmacological effects of both drugs (plus other pulses of additional drugs in some protocols) and other additional confounding factors (e.g., WBC inter-individual variations due to age, ethnicity, circadian and seasonal rhythms and concurrent infections)[24, 25]. The therapeutic drug monitoring (TDM) of thiopurines, measured as TGN levels in patients' red blood cells (RBC), could be proposed as an alternative method to guide MP dose adjustment to optimize therapeutic response while minimizing toxicity. The rationale of quantifying thiopurine metabolites in RBC relays on relative abundance and stability compared to whole blood and WBC[26]; moreover, thiopurine treatment in ALL reduces WBC, therefore quantification in RBC is more representative of the exposure to the drug[13]. The benefit of such pharmacological approach comes also from the opportunity of timely identifying patients who are non-compliant or clinically under dosed. However, the impact of erythrocyte TGN levels on relapse risk was not confirmed in all SJRCH/COG studies, whereas the importance of steady TGN systemic exposure to MP along maintenance arose only recently[27-30]. In this pilot study, we measured RBC-TGN and MMPN levels to explore thiopurine systemic exposure and variability during maintenance therapy and their impact on outcome in the AIEOP-BFM ALL 2009 protocol. Here, we also evaluated the contribution of candidate SNPs in genes involved in MP metabolic pathway (rs1800462, rs1800460 and rs1142345 in *TPMT* gene, rs116855232 in *NUDT15,* rs1127354, rs7270101, rs6051702 in *ITPA* and rs2413739 in *PACSIN2*) on outcome for the first time in the AIEOP-BFM ALL trial.

MATERIALS AND METHODS

Study design and population

Newly diagnosed Philadelphia-negative pediatric ALL patients treated according to the AIEOP-BFM ALL 2009 trial (ClinicalTrials.gov identifier: NCT01117441, http://clinicaltrials.gov) were enrolled mainly in four medium-large Italian AIEOP affiliated pediatric hospitals (n=290). Patients received a risk-adapted polychemotherapy. High-risk criteria were one of the following: presence of t(4;11) translocation (*MLL/AF4* transcript), prednisone poor response (PPR), minimal residual disease (MRD) on day 15 measured by flow cytometry (FCM; blasts in bone marrow ≥10%), no complete remission at day 33, PCR-MRD in bone marrow with markers with sensitivity ≥10^{-3} at days 33 and 78, hypoploidia (< 45 chromosome) Patients not meeting any of the high-risk criteria were stratified as standard risk according to PCR-MRD at days 33 and 78 (if negative at both time points with at least one marker with sensitivity 10^{-4}) or, in second option, to FCM-

MRD results (<0.1% at day 15). Patients not matching criteria for high or standard risk were classified as intermediate risk. According to protocol, maintenance consisted in the oral administration of 50 mg/m^2 daily MP and 20 mg/m^2 MTX once a week, until week 104 from diagnosis, regardless the risk class. The actual dosing of both MP and MTX by protocol was based on the maximum tolerated dose to maintain WBC counts between a protocol-specific target range of 2000-3000/µl, to balance risks of inadequate myelosuppression with those of severe pancytopenia. MP and MTX were reduced by 50% when WBC are between 1000-2000/µl; both drugs were withheld if the WBC drop below 1000/µl. According to protocol, planned dose of MP should begin with 25% of full MP dose in *TPMT* homozygous variant patients.

Peripheral blood samples were collected at the 3rd, 9th and 15th month of maintenance therapy in EDTA and immediately added with 1 mg of the antioxidant dithiothreitol (DTT, Sigma-Aldrich, Milan, Italy) to preserve the free thiol moiety of thiopurines. Blood samples were sent at 4 °C to the University of Trieste to be processed within 24 hours for pharmacogenetic and pharmacokinetic analysis (Figure S2). Pediatricians collected clinical data and biological samples blindly from pharmacological results. The study was approved by the Institutional Review Board (or Ethics Committee) of IRCCS Burlo Garofolo (Protocol number CE/V 135; March 5th 2012). Informed consent was obtained from all subjects involved in the study.

DNA samples and genotyping

Germline DNA was extracted from the first available blood sample of ALL patients, while in remission, by Gene Elute Blood Genomic-DNA-kit (Sigma-Aldrich, Milan, Italy). SNPs in genes of interest (rs1800462, rs1800460 and rs1142345 in *TPMT* gene, rs116855232 in *NUDT15,* rs1127354, rs7270101, rs6051702 in *ITPA* and rs2413739 in *PACSIN2)* were characterized by Taqman® SNP genotyping assays (Applied Biosystem, USA). Sample genotyping was repeated twice with no inconsistent results. Some patients (~60% of the study population) were included in a previous validation study on the role of *PACSIN2* SNP on thiopurine pharmacokinetics[16].

Measurement of thiopurine metabolites in patients' RBC

Thiopurine metabolites were quantified in patients' red blood cells (RBC), as previously described[16]. MP systemic exposure was assessed as average pmol/8x10^8 RBC per patient. Intra-individual variability in MP exposure was expressed as coefficient of variation (CV-TGN); 321 TGN levels in 134 patients were considered; the proportion of children with 2, 3 and 4 repeated TGN measurements was 61.9% (n=83), 36.6% (n=49) and 1.5% (n=2), respectively.

Clinical data collection

Clinical data of the study cohort and outcome were retrieved from the AIEOP central database on February 2022. At February 2019, all patients were out of therapy. Patients were followed since the beginning of maintenance up to relapse (defined according to standard clinical practice), death or last available follow up. Hematological (HEM) and gastrointestinal (GI) toxicities data were reported by pediatricians for 47 and

52 patients, respectively, as already published[16]. Data regarding MP dose intensity, cumulative MP doses, concomitant treatments, therapy time-off during maintenance were not included in the database and were not available,

Statistical analysis

Statistical analyses were performed using the software R version 3.5.2. Metabolite levels were grouped according to blood samples collection time. They were distinguished in early TGN measurements ("3rd month" group, including blood samples collected between the 2nd and the 5th month of maintenance), intermediate TGN measurements ("9th month" group, between the 6th and11th) and late TGN measurements ("15th month" group, since 12th month up to therapy conclusion). MP systemic exposure was considered as the average TGN measurement per patient, and treated both as continuous variable and as quartiles (i.e: $TGN_{Q1\text{-}Q4}$; TGN_{Q4} representing the group with the highest MP systemic exposure). Variability in thiopurine exposure was assessed as intra-patient coefficient of variation (CV-TGN) for TGN measurements and considered both as continuous variable and as quartiles (i.e: $CV\text{-}TGN_{Q1\text{-}Q4}$; $CV\text{-}TGN_{Q4}$ representing the group with highest varying values). The association between TGN/MMPN metabolites (dependent variable) and pharmacogenomic and demographic variables (independent variables) was examined using linear models of the Gaussian family and linear mixed effect models; for the association between TGN/MMPN metabolites (dependent variable) and time of blood sampling for TDM (independent variable), a non parametric test was used. When CV-TGN was the dependent variable, non parametric tests were used. For statistical analysis, all pharmacogenomic analyses evaluated an additive effect of the genotype on the phenotype of interest.

The follow-up time was calculated from the maintenance beginning to the date of last contact available or the date of an event (relapse, death or second neoplasm), whichever came first. The Cox proportion hazard model was adopted for evaluating the impact of MP systemic exposure, CV-TG and SNP genotypes on relapse. Information of other concomitant medications used in maintenance was not available.

Toxicities were classified based on severity, and dichotomized as absent ("no", grade 0-2) and present ("yes", grade 3-5). The association between genotypes and MP exposure ($TGN_{Q1\text{-}Q4}$ and $CV\text{-}TGN_{Q1\text{-}Q4}$ was analyzed by χ^2 test or Fisher's exact test.

RESULTS

Study population and g*enotype distribution of candidate SNPs*

Demographic and clinical characteristics of the study population are shown in Table 1, genotyping results in Table 2. No patient was homozygous variant for *TPMT*; the incidence of heterozygous *TPMT*3A* (with variant allele in both rs1800460 and rs1142345) was 5.6% and was less than the 10% expected in Caucasians but in line with what had been reported for ALL patients enrolled in the previous AIEOP-BFM LLA 2000 protocol (~5%). *NUDT15* rs116855232 is very rare in Caucasians and only one patient of the study population carried one variant allele. Distributions of *ITPA* rs1127354, rs7270101, rs6051702 and

PACSIN2 rs2413739 variant genotypes were as expected for Caucasians. Genotype frequencies were in Hardy–Weinberg equilibrium (HWE, p>0.05).

Thiopurine exposure and intra-individual variability in the study population

TGN/MMPN levels were measured in 387 blood samples collected during the long-term maintenance therapy of 200 patients (Table S1). A significant decrease in TGN levels was observed towards the end of therapy compared to earlier time points (TGN "15th month" *versus* TGN "3rd month": p=2.04 x10^{-7}, TGN "15th month" *versus* TGN "9th month": p= 1.67 x10^{-4}, Kruskal-Wallis test, Dunn post-test, Figure S3A). In contrast, MMPN levels remained stable over time (Figure S3B). Intra-individual variability in thiopurine exposure assessed as CV-TGN could be calculated in 134 patients with multiple TGN measurements (median (IQR): 46.35 (25.94-74.12), min: 1.28; max: 141.37). The intra-individual variability observed within TGN measurements in the first year of maintenance was lower than that observed in blood samples at later time points of maintenance therapy (median (IQR): 33.32 (17.32- 54.11) *versus* 49.44 (21.64- 72.51) respectively, Wilcoxon test: p= 0.049, Figure S4).

A negative correlation was found between thiopurine exposure and intra-individual variability when treated as a continuous variables (Spearman correlation, p=0.02, r= -0.201, Figure S5). When CV-TGN were divided in quartiles (CV-TGN$_{Q1}$: median (IQR): 13.06 (7.94-20.25), n=34; CV-TGN$_{Q2}$: 35.67 (31.71-40.12), n=33, CV-TGN$_{Q3}$: 54.75 (50.88-62.61), n=33; CV-TGN$_{Q4}$: 93.81 (82.69-127.46), n=34), children with greater variability among TGN measurements over time (i.e.: CV-TGN$_{Q4}$) showed also lower MP systemic exposure compared to patients with more stable values (CV-TGN$_{Q1-Q3}$, Kruskal-Wallis test, p= 0.022); MMPN values were instead comparable (Figure S6).

Demographic and genetic contribution of candidate SNPs on thiopurine pharmacokinetics in the study population

Demographic and genetic contributions on MP systemic exposure has been investigated in univariate analysis (Table 3). In comparison to younger ALL patients, older children showed a tendency towards higher TGN and MMPN levels (linear mixed-effect model, p-value= 0.048 and p= 0.047, Figure S7, panels A and B, respectively) whereas metabolites did not differ according to gender. The presence of *TPMT* low-activity alleles significantly increased the concentration of TGN (p<10^{-4}, Figure S7C) and decreased that of MMPN (p<0.018) in patients' RBC (Figure S7D) whereas *PACSIN2* SNP rs2413739 genotypes had no impact. An effect of *ITPA* rs1127354 (p= 0.041, Figure S7E) and a borderline contribution of rs7270101 variant alleles on TGN (p= 0.054, Figure S7F) was also observed, with opposite direction for the variant alleles; *ITPA* SNPs did not affect MMPN. Multivariate analysis considered only fully significant variables in univariate. *TPMT* low-activity allele remained significantly associated with higher TGN levels (increase of 739.71±71.68 pmol/8x10^{8} RBC in heterozygous patients compared to wild type, p<10^{-4}, 378 blood samples of 194 patients analysed) and with lower MMPN (decrease of 4944.93 ± 1994.61 pmol/8x10^{8} RBC, p= 0.014, 384 blood samples of 197 patients analysed). Age maintained a borderline contribution on TGN

levels (increase of 8.46±4.27 pmol/8x10^8 RBC for each year, p=0.049) and MMPN levels (increase of 242.98±118.90 pmol/8x10^8 RBC for each year, p=0.042).

Demographic and genetic factors did not affect CV-TGN (Table 3). Although intra-individual variability of thiopurine exposure was not affected by *PACSIN2* SNP rs2413739 genotypes (Figure 1A), 7 of CC carriers (14.6%) fell in the CV-TGN_{Q4} group *versus* 17 of CT (27.0%) and 10 of TT carriers (43.5%) indicating a higher variability in MP exposure in T allele carriers (p-value Fisher test: 0.03, Figure 1B).

Contribution of pharmacological parameters on clinical outcome

On univariate analysis, risk class and the genetic variables *PACSIN2* rs2413739 and *ITPA* rs7270101 proved to have an effect on relapse risk; risk class and *PACSIN2* rs2413739 remained significant also after multivariate Cox analysis (Table 4, Figure 2). Compared to patients in standard arm, relapse risk increased in ALL children with disease of the intermediate risk (hazard ratio (HR) of 3.44, 95% confidence interval (CI), 1.31-9.05, p= 0.012); hazard risk was comparable for high-risk patients (2.76; 95% CI, 0.66-11.64, p= 0.166). Carriers of the *PACSIN2* rs2413739 T allele showed higher risk for relapse than those with the CC genotype (heterozygotes CT: HR, 2.32; 95% CI, 0.90-5.97; p = 0.081; homozygous TT: HR, 4.13; 95% CI, 1.54-11.11; p = 0.005). Patients were classified as having low systemic exposure to MP if their average erythrocyte TGN concentration fell in the lower quartile (TGN_{Q1} group), and as having high intra-individual variability in MP if their CV-TGN fell in the upper quartile (CV-TGN_{Q4}). Relapse risk was not affected by thiopurine exposure and metabolites variability during maintenance (Table 4, Figure 2).

HEM toxicities data were available for 47 patients. Severe HEM episodes were reported in 5 out of 15 (33.3%) of patients in the low systemic exposure group (TGN_{Q1-Q2} group) and in 26 out of 32 (81.2%) of those in the TGN_{Q3-Q4} group (p-value chi square= 0.004, Figure 3). GI toxicities data were available for 52 patients. Higher incidence of severe GI episodes was observed in TGN_{Q1} group compared to others (p-value chi square= 0.032, Figure 3): indeed, 2 patients with gastrointestinal adverse effects out of 6 (33.3%) fell in the TGN_{Q1} and only 1 out of 46 (2.2%) in the TGN_{Q2-Q4}. The three patients who experienced severe GI episodes experienced severe HEM toxicity as well. Thiopurine intra-individual variability was calculated in 34 patients with HEM toxicity data. HEM occurrence was comparable within groups (CV-TGN_{Q1} 57.1%, CV-TGN_{Q2} 66.7%, CV-TGN_{Q3} 77.8% and CV-TGN_{Q4} 77.8%, p-value chi square= 0.9). CV-TGN data were available for 38 patients with GI toxicity data, none of them experiencing severe adverse effects.

None of the SNPs of interest had an impact on toxicities.

DISCUSSION

In recent years, improvement in survival of ALL pediatric patients has occurred. However, important challenges still need to be faced, including cure rates of specific patients' subsets, e.g., adolescents and young adults (5 years survival: 60-70%) and relapsed children (5 years survival: ~40-50%), as well as the

reduction of severe chemotherapy-related toxicities, both in the short- and in the long-term[31, 32]. Advances in ALL therapy includes the development of targeted therapy, such as tyrosine kinase inhibitors, and the introduction of immunotherapies[1]. The promise of these novel approaches is to improve cure rates, to overcome chemotherapy resistance and, being target-specific, to confer limited toxicity to normal tissues[33-36]. Pharmacological strategies, including the analysis of the proactive monitoring of drug metabolites and important predictive pharmacogenetic markers, could represent additional valuable tools for a rational use of "old" drugs to personalize therapy with the same purposes.

The AIEOP-BFM ALL 2009 trial was conducted as collaborative international study between October 1st 2010 and December 31st 2016; results on the whole population enrolled are not published yet because follow-up time is too short to report data on relapse incidence or survival. Risk-oriented stratification was accomplished based on biological features of the leukemia (specific chromosomal aberrations) and treatment response during induction phase (prednisone response, MRD monitoring). In our study population, medium risk patients, who did not fulfill the AIEOP-BFM ALL 2009 high-risk criteria, had a significant ~3.4-fold higher risk of relapse compared to those with the best prognostic factors in the standard risk-arm. Patients in the intermediate arm account for ~35-40% of the whole population treated according to the AIEOP-BFM ALL 2009 protocol whereas those in high-risk arm account for ~20-25%[37]. Distribution of risk classes in our study population is thus not completely superimposable to that of the entire cohort, with an overrepresentation of children in the intermediate class (~ 50%) and an underrepresentation of high-risk ALL children (~ 10%).

MP and MTX are antimetabolites that constitute the backbone of the maintenance phase for all patients. Our report is the first showing the course of RBC-TGN during maintenance in the AIEOP-BFM ALL 2009 protocol, and is the first investigating in an explorative way the impact of adequate and stable MP exposure on outcome in this therapeutic context. Benefit of TDM for thiopurines as predictor of relapse is still uncertain in ALL patients. In our study cohort, MP systemic exposure and variability did not affect the cumulative risk of relapse. Similarly, a recent study of the COG international group did not confirm the association between RBC-TGN and relapse of previous reports, although it highlighted that a constant MP exposure (meant as high adherence rate to the prescribed MP regimen) is mandatory for therapeutic success[27, 32]. The importance of steady exposure is assessed by several studies[27, 29, 38-43]. In ALL, the RBC-TGN therapeutic window is not clearly defined as in other thiopurine-treated diseases, likely because MP dosages vary according to protocols and other chemotherapeutic drugs used in combination. It is generally acknowledged that TGN values above 1000 pmol/8×10^8 RBC showed greater incidence of hematological adverse events, and a MMPN threshold of 5000 pmol/8×10^8 RBC was associated with an increased risk of hepatotoxicity[44]. Moreover, since RBC are not the drug-target cells, erythrocytes TGN levels could not be considered entirely representative for the pharmacodynamic effects of MP in WBC. Recent studies proposed to measure the DNA-incorporated 6-thioguanine (DNA-TG) in ALL patients' lymphocytes as routinely monitoring marker, using LC-MS/MS techniques[43, 45]. An adequate level of DNA-TG in ALL patients' lymphocytes during

maintenance significantly associated to relapse-free survival[43]. However, these analyses are only available in few clinical centers, and are currently less standardized than HPLC measurements of RBC-TGN.

In our study cohort, we observed an inverse correlation between average TGN and intra-individual variability in RBC-TGN, RBC-TGN levels remained stable within the first year of maintenance, whereas they were reduced afterwards when also an increase CV-TGN was observed. Although a clear interpretation of these data is hampered by the fact that TGN could not be normalized to MP dose intensities, this observation could suggest poor patients' compliance towards the end of therapy. Non-adherence to MP/MTX oral medications has been associated with increased relapse risk, particularly in some sociodemographic group[41, 46]. Routine measurements of MP metabolites could thus represent an objective tool for monitoring patients' compliance and could become part of a monitoring plan of adherence, currently missing in the AIEOP-BFM ALL trial, which could provide additional relevant information for therapy optimization. However, to be effective, an important issue to address is how to define non-adherent patients according to MP metabolites.

Patients who showed higher MP exposure were those more prone to develop episodes of severe hematological toxicities while poorly exposed patients experienced more episodes of severe gastrointestinal toxicity. Our results were generated in a small-size subset of patients and are not sufficient to draw definitive conclusions; a full characterization of the cohort is required. However, if confirmed, the results suggest that monitoring TGN levels could become a helpful strategy in fine-tuning an efficient therapy, limiting the occurrence of toxicities. Again, an important issue to address is the reference TGN values to achieve.

In this study, we also investigated the role of common genetic polymorphisms in *TPMT*, *NUDT15*, *ITPA* and *PACSIN2* genes involved in MP metabolism to evaluate their impact on MP systemic and intra-individual exposure and disease outcome. Pharmacogenomic factors have been identified also for MTX although genetic contributions have not yet been well quantified[47], and were not evaluated in this study. According to CPIC guidelines, *TPMT*2*, *TPMT*3A* and *NUDT15* rs116855232 are "no function" alleles, and homozygous variant and heterozygous patients are classified as "poor" and "intermediate" metabolizers, respectively, presenting higher TGN values and higher risk of toxicities when exposed to full doses of MP [14]. We thus investigated whether heterozygous carriers of these variant alleles could be more susceptible to fluctuations in RBC-TGN, since in the AIEOP protocol they were treated as wild type patients with regard to MP planned dose and adjustments. Our results suggest that *TPMT* variant allele carriers did not represent a concern in terms of inadequate or inconstant thiopurine exposure during maintenance in the AIEOP-BFM ALL 2009 protocol. This could be related to the standard dose of MP used in this protocol (50 mg/m^2) that is lower also for heterozygous patients than the limit of 60 mg/m^2 suggested by clinical CPIC guidelines. SNP rs116855232 is one of the few gene variants of *NUDT15* found in Caucasians, and one of the most extensively studied for clinical implementation; it was too rare in the study population to be conclusive on its contribution on MP pharmacokinetics or outcome[14]. An interesting result arose from the

PACSIN2 non-coding SNP rs2413739, with the presence of T allele increasing the risk of relapse over time compared to CC patients. SNP rs2413739 has been proposed as an example of a low penetrance genetic factor affecting TPMT activity, with a potential clinical significance as biomarker of adverse drug reactions, in particular on gastrointestinal tissues[48]. This SNP was consistently associated to a reduced TPMT activity in ALL independent cohorts, but not in adolescents affected by non-malignant condition under long-term thiopurine treatment[16 49]. *PACSIN2* genotypes did not affect average RBC-TGN or their variation over time. However, the proportion of patients who fell in the higher intra-individual variability group (CV-TGN_{Q4}) increased when the T variant allele was present. Current data available in this study did not provide insight on the reasons of such higher variability (whether it is due to lack of efficacy or increased toxicity), neither on its direction (whether RBC-TGN are increasing or decreasing over time). PACSIN2 is involved in other biological processes such as endocytosis, cell spreading and migration, bacterial cell-to-cell spread and viral propagation, among others[50,51,52, 53]. Since this SNP is clinical irrelevant in terms of thiopurine metabolites, we can suggest that the impact of *PACSIN2* SNP on outcome and TGN intra-individual variability could be due to TPMT-independent factors, such as predisposition to severe infections or GI complications. In univariate analysis, a borderline impact of *ITPA* SNPs was also observed on measured RBC-TGN and on relapse; however, after multivariate analysis, significance were lost. The pharmacogenetic role of *ITPA* in the therapy personalization of thiopurines is still under discussion. The *ITPA* SNPs rs1127354 and rs7270101 reduce ITPA function to different degree, with unclear contribution on thiopurine metabolism, dose, efficacy and toxicity.[54] Two recently published systemic reviews and meta-analysis reinforced the role of *ITPA* genotyping for the prediction of MP-induced myelosuppression in ALL patients, in particular for the missense SNP rs1127354 [55, 56].

In conclusion, this study is the first report focusing on RBC-TGN in maintenance phase and on its impact on outcome in an Italian cohort of pediatric patients enrolled in the AIEOP-BFM ALL 2009 protocol. Being an explorative analysis, this study suffers of some limitations. The major drawback is the lack of a complete clinical data collection for the study population, including data on WBC and MP dose intensity at the moment of TGN measurements and on cumulative MP doses and therapy time-off to face drug-induced toxicities during maintenance. Unfortunately, these data were not routinely collected in the central AIEOP database. Toxicities were available only for a subset of the study population. Moreover, there was no objective assessment recommended by the protocol for true patients' compliance to MP therapy. Future dedicated studies in the AIEOP-BFM ALL setting are needed to confirm the lack of impact of TGN levels and variability on relapse, integrating laboratory values with a dedicated clinical data collection by revising patients' medical records at each enrolling center. Further studies are also required to confirm and clarify the rational of the role of *PACSIN2 r*s2413739 on outcome and to establish criteria to use MP pharmacokinetic profiles as predictors of patients' compliance and toxicities.

FIGURE LEGEND

Figure 1: Thiopurine intra-individual variability during maintenance and *PACSIN2* SNP rs2413739. A) CV-TGN distribution according to genotypes; B) Genotype distribution in patients with more stable (CV-TGN_{Q1-Q3}) or greater (CV-TGN_{Q4}) variability among TGN measurements. In brackets; number of patients within each group.

Figure 2: Relapse curves according to A) risk arm; B) *PACSIN2* SNP rs2413739 genotype; C) thiopurine exposure and D) thiopurine variability in maintenance.

Figure 3. Hematological and gastrointestinal severe toxicities according to thiopurine exposure during maintenance. Thiopurine exposure during maintenance was assessed as mean TGN levels measured over time per patient (387 blood samples in 200 children), and graded according to quartiles (Q1, Q2, Q3, Q4), Q1 representing the group with the lower MP systemic exposure).

STUDY HIGHLIGHT

o What is the current knowledge on the topic?

In pediatric ALL therapy, prolonged mild bone marrow suppression of the patient to maintain clinical remission is acknowledged as necessary for sustained event-free survival. Daily mercaptopurine (MP) and weekly methotrexate represent the backbone of maintenance, which lasts up 24 months after diagnosis; protocol-specific WBC target ranges guide drugs dose intensities over time, although this approach has some limitations including, among others, the lack of a direct measurement of drug systemic exposure.

o What question did this study address?

What is the extent and variability of MP active metabolites in the AIEOP-BFM ALL maintenance phase, and what their contribution on outcome? This study has also evaluated the contribution of candidate SNPs in genes involved in MP metabolic pathway on metabolite levels and clinical outcomes.

o What does this study add to our knowledge?

This is the first explorative study conducted on this topic in the AIEOP-BFM ALL therapeutic context; the novelty of the manuscript is corroborated by the scarcity of similar data in the literature. The study returns

objective measurements of MP systemic exposure and variation due to continuous drug adjustments and/or patients adherence, through a therapeutic drug monitoring (TDM) of active MP metabolites in patients' red blood cells.

o How might this change clinical pharmacology or translational science?

MP optimization during ALL maintenance based on TDM has not received great attention yet. TDM can represent a tool for an objective assessment of MP exposure and true patients' compliance to MP therapy, an alternative method for guiding MP dose adjustment to optimize therapeutic response while minimizing toxicity. If replicated, results on the *PACSIN2* rs2413739 on outcome could become a pharmacogenetic marker of relapse.

AUTHOR CONTRIBUTIONS

R.F., G.S., and G.D. wrote the manuscript; R.F., G.S., G.D., and M.R. designed the research; R.F., K.V., A.T., F.F., P.Q., N.B., C.R., A.C., L.R.B., F.L., L.V., and K.G. performed the research; R.F., G.S., D.S,, and M.G.V. analyzed the data.

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SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

Characteristics	Patients
Age at diagnosis (N=290)	
Median (IQR) in years	4.8 (3.0-8.1)
Gender (N=290)	
Male, n (%)	165 (56.9)
Female, n (%)	125 (43.1)
Ethnic group (N=290)	
Caucasians, n (%)	281 (96.9)
Others, n (%)	9 (3.1)
Down Syndrome (N=290)	
Yes, n (%)	5 (1.7)
No, n (%)	285 (98.3)
Immunophenotype (N= 288)	
ALL-B, n (%)	258 (89.6%)
ALL-T, n (%)	30 (10.4%)
Genetic abnormalities (N=202)	
t(1;19), n (%)	5 (2.5)
t(4;11), n (%)	2 (1.0)
t(9;11), n (%)	1 (0.5)
t(12;21), n (%)	50 (24.7)
Other, n (%)	2 (1.0)
None, n (%)	142 (70.3)
Prednisone response (N=288)	
PGR, n (%)	277 (96.2)
PPR, n (%)	11 (3.8)
Risk class (N=290)‡	
Standard, n (%)	97 (33.4)

Medium, n (%)	156 (53.8)
High, n (%)	37 (12.8)
Maintenance	
Treatment length	
Patients, n	259
Median (IQR) in years	1.29 (1.21-1.33)
Grade III-IV toxicities	
Hematological, Ntot, n (%)	47, 31 (65.9)
Gastrointestinal, Ntot,n (%)	52, 3 (5.8)
Follow-up (N=275)	
Median (IQR) in years	4.43 (3.82-5.46)
Outcome at last follow-up (N=290)	
Remission, n (%)	241 (83.1)
Relapse, n (%)	41 (14.1)
Median (IQR) in years^*	1.72 (0.83-2.69)
Death after relapse	
N, Median (IQR) in years	13, 0.97 (0.60-1.58)
Second neoplasm n (%)	2(0.7)
Death without relapse, n (%)	6 (2.1)

Table 1: Demographic and Clinical Characteristics of the study population. Percentage compared to available data; ‡ risk range at the end of the induction phase; ^ since the beginning of maintenance; * measured in 39 patients out of 41 relapsed patients. ALL: acute lymphoblastic leukemia; IQR: interquartile range; n= numbers; PGR "Prednisone good responders", patients with less than 1000 blasts/µl in peripheral blood at day +8 after diagnosis; PPR: "Prednisone poor responders", patients with more than 1000 blasts/µl in peripheral blood at day +8 after diagnosis.

GENE	Position	SNP	N	wt (%)	hz (%)	var (%)	HWE
TPMT	Exon 5	c.238 G>C p.Ala80Pro rs1800462	272	272 (100)	0 (0.0)	0(0.0)	-
TPMT	Exon 7	c.460 G>A p.Ala154Thr rs1800460	269	254 (94.4)	15 (5.6)	0 (0.0)	-
TPMT	Exon 10	c.719 A>G p.Tyr240Cys rs1142345	267	252 (94.4)	15 (5.6)	0 (0.0)	-
NUDT15	Exon 3	c.7379 C>T p.Arg139Cys rs116855232	233	232 (99.6)	1 (0.4)	0 (0.0)	-
ITPA	Exon 2	c.94 C>A p.Pro32Thr rs1127354	261	227 (87.0)	33 (12.6)	1 (0.4)	0.76
ITPA	Intron	IVS2+21 A>C rs7270101	269	208 (77.3)	57 (21.2)	4 (1.5)	0.82
ITPA	Intron	A>C rs6051702	259	177 (68.3)	74 (28.6)	8 (3.1)	0.91
PACSIN2		T>C rs2413739	267	58 (21.7)	117 (43.8)	92 (34.5)	0.09

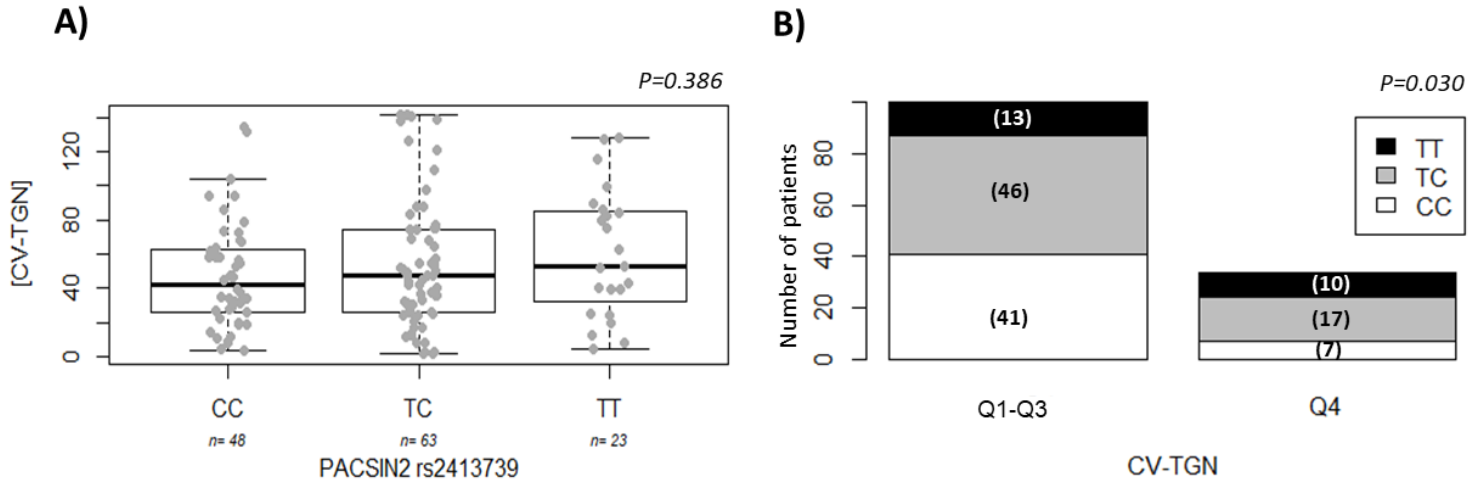
Table 2: SNPs genotype distribution in the study population. HWE: Hardy-Weinberg equilibrium; het: heterozygous; wt: wild type; var: variant; N: total number of patients.

			Maintenance							
			TGN (pmol/8x10^8 RBC)			MMPN (pmol/8x10^8 RBC)			CV-TGN	
	Comparison	**n/N**	**Value**	**Standard error**	**p-value**	**Value**	**Standard error**	**p-value**	**N**	**p-value**
Age	Each year	387/200	10.53	5.30	0.048*	239.97	119.94	0.047*	134	0.435†
Gender	Female *versus* male	387/200	35.49	43.27	0.413*	1543.13	973.44	0.115*	134	0.616‡
TPMT rs1142345	hz *versus* wt	380/195	754.34	71.97	<10^{-4}*	-4870.01	2016.55	0.017*	132	0.507‡
TPMT rs1800460	hz *versus* wt	384/197	756.86	71.97	<10^{-4}*	-4799.22	2013.63	0.018*	134	0.519‡
NUDT15 rs116855232	hz *versus* wt	300/158	-189.37	292.66	0.519	-3026.81	6571.04	0.646	105	
PACSIN2 rs2413739	Each T allele	383/196	-4.55	29.95	0.880*	-521.30	677.09	0.442*	134	0.386‡
ITPA rs1127354	Each A allele	378/194	-126.73	61.60	0.041*	-713.35	1418.01	0.616*	132	0.449‡
ITPA rs7270101	Each C allele	379/194	90.42	46.60	0.054*	499.31	1063.26	0.639*	132	0.728‡
ITPA rs6051702	Each C allele	365/187	42.51	42.27	0.316*	-385.32	963.78	0.690*	127	0.657‡

Table 3: Thiopurine metabolites in patients' erythrocytes and association with demographic and genetic characteristics. hz: heterozygous; IQR: interquartile range; MMPN: methylmercaptopurine nucleotides; n: number of blood samples analyzed; N: number of patients; RBC: red blood cells; TGN: thioguanine nucleotides; wt: wild type. * mean value per patient. P-value according to linear mixed-effect model, for univariate association of TGN and MMPN. †P-value according to Spearman correlation test; ‡ P-value according to Kruskal-Wallis test. Value represents the increase (positive value) or decrease (negative value) in the value of the dependent variable for each independent variable listed.

			Univariate		Multivariate	
		N patients (relapse)	**HR (95% C.I)**	**p-value**	**HR (95% C.I)**	**p-value**
Risk class	SR	93 (7)	1.0			
	MR	151 (28)	2.74 (1.20-6.28)	0.017	3.44 (1.31-9.05)	0.012
	HR	32 (4)	1.96 (0.57-6.73)	0.284	2.77 (0.66-11.66)	0.166
***TPMT**3A**	wt	244 (33)	1.0			
	var	15 (2)	0.94 (0.23-3.94)	0.936		
***NUDT15* rs116855232**	CC	221 (30)				
	CT	1 (0)	undefined	0.998		
***PACSIN2* rs2413739**	CC	89 (6)	1.0			
	TC	113 (17)	2.41 (0.95-6.12)	0.065	2.32 (0.90-5.97)	0.081
	TT	55 (12)	3.58 (1.34-9.56)	0.011	4.14 (1.54-11.11)	0.005
***ITPA* rs1127354**	CC	219 (34)				
	CA	32 (1)	undefined	0.996		
	AA	1 (0)	undefined	0.997		
***ITPA* rs7270101**	AA	201 (28)	1.0			
	AC	52 (5)	0.62 (0.24-1.62)	0.332	0.57 (0.22-1.48)	0.246
	CC	4 (2)	4.35 (1.03-18.30)	0.045	3.67 (0.84-15.99)	0.084
***ITPA* rs6051702**	AA	171 (24)	1.0			
	AC	70 (6)	0.57 (0.23-1.39)	0.212		
	CC	8 (1)	0.94 (0.13-6.99)	0.955		
TGN	Q1	49 (7)	1.38 (0.57-3.31)	0.476		
	Q2-Q4	148 (18)	1.0			
CV-TGN	Q1-Q3	100 (10)	1.0			
	Q4	32 (5)	1.78 (0.61-5.24)	0.294		

Table 4: Influence of clinical and pharmacological variables on relapse. CI: confidence interval; HR: hazard risk; N: number of patients; Q1: first quartile; Q2: second quartile; Q3: third quartile; Q4: fourth quartile; TGN: mean thioguanine nucleotides as measure of thiopurine exposure. CV-TGN: coefficient of variation in thioguanine nucleotides as measure of thiopurine intra-individual variability. P-value according Cox proportional-hazards model.



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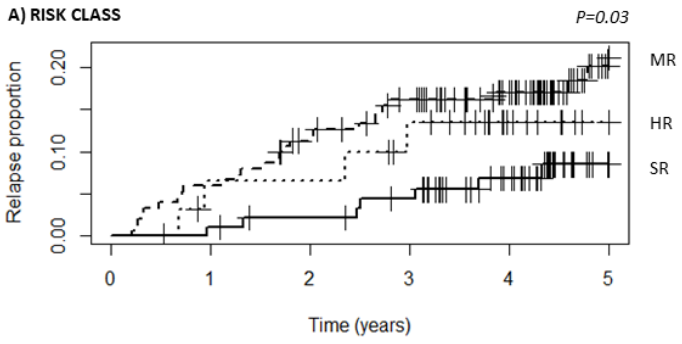
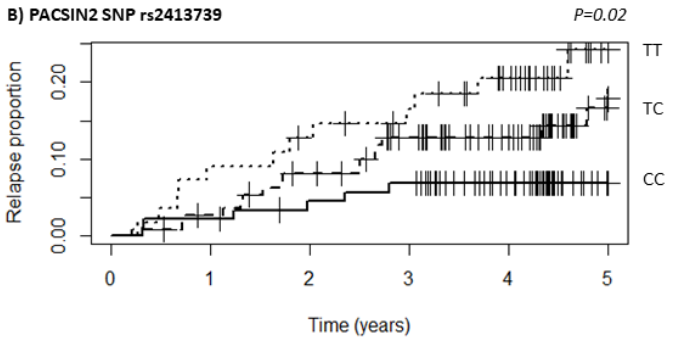
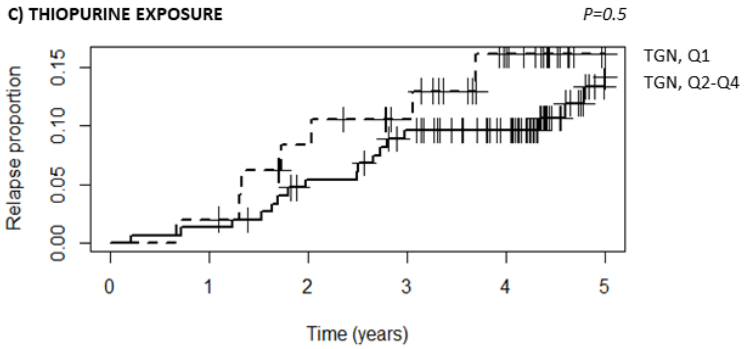


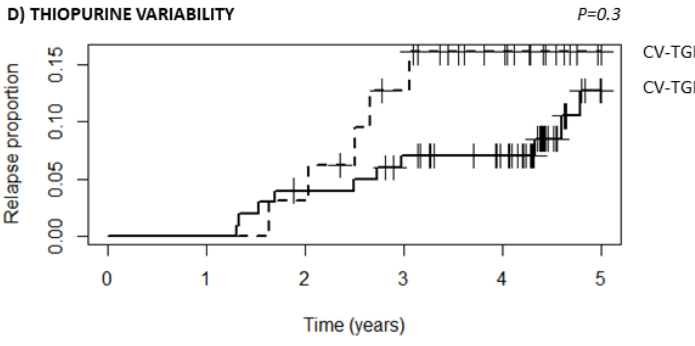
SR	93 (0)	92 (1)	89 (1)	85 (2)	67 (2)	31(1)
MR	151 (0)	142 (9)	129 (9)	118 (6)	94 (1)	42 (3)
HR	32 (0)	28 (1)	28 (0)	24 (2)	14 (0)	7 (0)



CC	89 (0)	87 (2)	83 (2)	81 (0)	62 (0)	26 (0)
TC	113 (0)	108 (3)	100 (6)	88 (5)	68 (0)	34 (3)
TT	55 (0)	50 (5)	47 (2)	43 (2)	35 (2)	15 (1)



Q_1	49 (0)	48 (1)	42 (3)	37 (1)	24(2)	9 (0)
Q_2-Q_4	148 (0)	146 (2)	137 (6)	128 (6)	109 (0)	55 (4)



Q_1Q_3	100 (0)	100 (0)	95 (4)	90 (3)	79 (0)	36 (3)
Q_4	32 (0)	32 (0)	31 (1)	26 (3)	18 (1)	5 (0)

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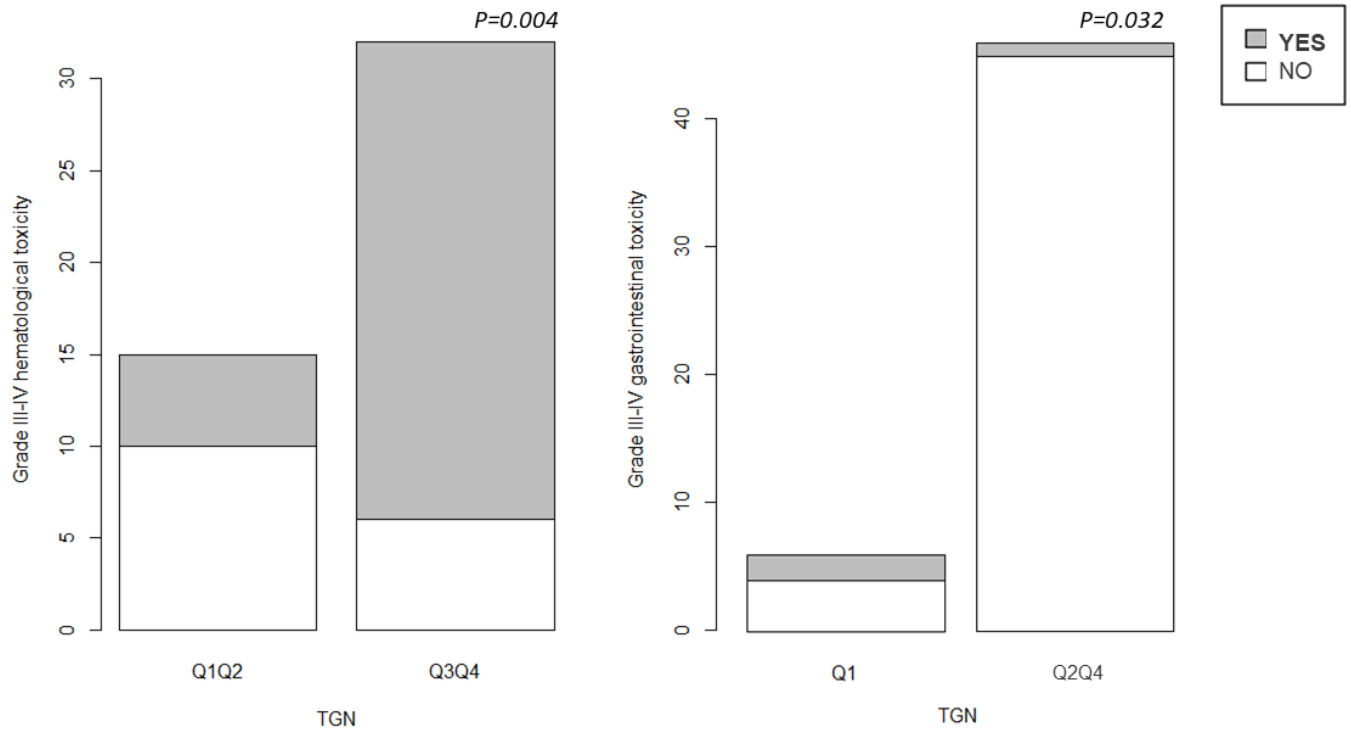

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