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ARTICLE

PACSIN2 rs2413739 influence on thiopurine pharmacokinetics: validation studies in pediatric patients

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Abstract

The aim of the study was to validate the impact of the single-nucleotide polymorphism rs2413739 (T > C) in the *PACSIN2* gene on thiopurines pharmacological parameters and clinical response in an Italian cohort of pediatric patients with acute lymphoblastic leukemia (ALL) and inflammatory bowel disease (IBD). In ALL, *PACSIN2* rs2413739 T allele was associated with a significant reduction of TPMT activity in erythrocytes ($p = 0.0094$, linear mixed-effect model, multivariate analysis considering *TPMT* genotype) and increased severe gastrointestinal toxicity during consolidation therapy ($p = 0.049$). A similar trend was present also for severe hematological toxicity during maintenance. In IBD, no significant effect of rs2413739 could be found on TPMT activity, however azathioprine effectiveness was reduced in patients carrying the T allele (linear mixed effect, $p = 0.0058$). In PBMC from healthy donors, a positive correlation between PACSIN2 and TPMT protein concentration could be detected (linear mixed effect, $p = 0.045$). These results support the role of *PACSIN2* polymorphism on TPMT activity and mercaptopurine adverse effects in patients with ALL. Further evidence on PBMC and pediatric patients with IBD supports an association between PACSIN2 variants, TPMT activity, and thiopurines effects, even if more studies are needed since some of these effects may be tissue specific.

Introduction

Thiopurines, such as 6-thioguanine (TG), mercaptopurine (MP), and its prodrug azathioprine (AZA), are the key drugs used in the treatment of several pediatric diseases, including acute lymphoblastic leukemia (ALL) and inflammatory bowel disease (IBD). These agents exert their therapeutic effects in lymphoid cells by acting as antimetabolites: mimicking the structure of purines, they are converted to thioguanine nucleotide analogs [TGN, meant as the set of all derivatives: thioguanosine mono-, di-, tri-phosphate (tGMP, tGDP, tGTP) and deoxythioguanosine mono-, di-, tri-phosphate (tdGMP, tdGDP, tdGTP)]. TGN are in turn able to interfere with cellular processes: the incorporation of tdGTP into DNA and of tGTP into RNA damages nucleic acids, impairs DNA replication and DNA repair machineries, thus resulting in cell-cycle arrest and apoptosis [1]. The inhibition of the purine de novo synthesis pathway and tGTP-mediated inhibition of the Rho-GTPase Rac1 represents additional mechanisms of action that potentiate the antiproliferative effects of thiopurines in lymphocytes [1]. The thiol moiety of thiopurines, either as free bases or in

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nucleotide form, is substrate of thiopurine methyl transferase (TPMT; EC 2.1.1.67) and the generation of the S-methylated derivatives (MMPN) competes with TGN production. Changes in TPMT activity may affect TGN and MMPN levels, and may therefore account for the inter-individual differences in therapeutic response or toxicities observed after thiopurine administration. Indeed, TGN levels above 235 pmol/8×10^8 red blood cells (RBC) are required for the therapeutic effect in IBD patients [2]; however, higher concentration of TGN (>400 pmol/8×10^8 RBC) can strongly suppress hematopoiesis, leading to severe hematological (HEM) complications (anemia, bleeding, leukopenia, and infections (INF)). In patients with ALL, some clinical protocols suggest MP dose reduction with TGN concentration above 1000 pmol/8×10^8 RBC in order to avoid excessive toxicity [3]. The role of MMPN is controversial: levels above 5700 pmol/8×10^8 RBC have been associated with hepatotoxicity [2, 4]; a contribution to the cytotoxic and immunosuppressive effects of thiopurines, through inhibition of de novo ATP and GTP synthesis, has been postulated for S-methyl-thioinosine monophosphate, a component of MMPN [1].

In Caucasians, ~90–95% of subjects have a normal/high TPMT methylating activity, whereas 5–10% show a reduced and ~0.5% a completely abolished enzymatic activity [5–8]. A strong genotype–phenotype correlation explains the trimodal distribution of TPMT activity: three well-characterized nonsynonymous single-nucleotide polymorphisms (SNPs rs1142345, rs1800460 and rs1800462) in the *TPMT* gene account for more than 95% of TPMT deficiency, with 10% of the population being heterozygous for at least one of them and 0.3% carrying the homozygous-variant genotype responsible for complete protein loss and lack of function [9]. TGN toxic levels are observed in TPMT-deficient patients treated with standard doses of thiopurines [10]. The US Food and Drug Administration and the European Medicines Agency recommend genotyping *TPMT* SNPs prior to thiopurine administration in order to optimize therapy, without affecting treatment efficacy [11]. Guidelines for thiopurine dose adjustment based on TPMT genotype/phenotype have been published by several cooperative groups, including the Clinical Pharmacogenetics Implementation Consortium [10, 12], the Dutch Pharmacogenetic Working Group (https://www.knmp.nl/downloads/pharmacogenetic-recommendations-november-2018.pdf), and others [13].

In 2011, Stocco et al. identified *PACSIN2* (Protein kinase C and casein kinase II interacting protein-2 or Syndapin 2) and its intronic SNP rs2413739 (T > C) as the most important *trans*-acting gene and genetic variant influencing TPMT activity by gene expression and genome-wide analyses on a panel of 30 human HapMap cell lines trios [14]. In ALL patients enrolled in the Total 13B/15 protocols at St. Jude Children's Research Hospital (SJCRH, Memphis, TN), the *PACSIN2* rs2413739 TT genotype was associated with lower TPMT activity during maintenance therapy and with an increased incidence of grade III–IV gastrointestinal (GI) toxicities during the consolidation phase. This latter result was also validated in the independent ALL cohort of children enrolled in the AIEOP-BFM (Associazione Italiana Ematologia Oncologia Pediatrica–Berlin–Frankfurt-Münster) ALL 2000 protocol [14]. Human *PACSIN2* encodes for a ubiquitously expressed protein containing an F-BAR-domain at its N-terminus and an Src homology-3 (SH3)-domain at the C-terminus. F-BAR proteins are the most important regulators of membrane curvature and activate distinct signaling pathways by specific domain-binding partners [15, 16]. In particular, PACSIN2 is involved in vesicle trafficking [16], in regulating the formation and scission of caveolae [17, 18], and in promoting microtubule assembly [16, 19]. Interestingly, PACSIN2 SH3 domain directly interacts with Rac1, a molecular therapeutic target of thiopurines as demonstrated by in vitro studies and in adult IBD patients. [16, 20–22] These data provide the rationale for considering PACSIN2 as a potential biomarker of thiopurine effectiveness and toxicities.

The present study was aimed to characterize more deeply the impact of *PACSIN2* SNP rs2413739 on thiopurine pharmacokinetic and pharmacodynamic parameters in children, either ALL patients treated with the AIEOP-BFM 2009 protocol or IBD patients undergoing AZA therapy. Moreover, the role of this polymorphism on TPMT and PACSIN2 protein concentration was evaluated for the first time to authors' knowledge in an exploratory way in adult healthy donors.

Methods

Study design and populations

MP pharmacological parameters were measured in two independent pediatric patients' cohorts. The first consisted of 280 patients that were newly diagnosed with Philadelphia chromosome-negative ALL at the Hemato-oncological Units of the Pediatric Hospitals IRCCS "Burlo Garofolo" in Trieste ($n = 46$, 16.4%), "Regina Margherita" in Turin ($n = 75$, 26.8%), "Centro Maria Letizia Verga" in Monza ($n = 86$, 31.0%), and "Bambino Gesù" in Rome ($n = 73$, 26.1%). Patients (median (interquartile range, IQ) age: 4.8 (3.0–9.2) years; male: 54.9%) were treated according to the AIEOP-BFM ALL 2009 protocol (ClinicalTrials.gov identifier: NCT01117441, http://clinicaltrials.gov). Biological materials were sent at 4 °C to the Department of Life Sciences of the University of Trieste and processed within 24 h since shipment. Bone marrow aspirates were collected at

diagnosis and processed for pharmacodynamic analysis. For pharmacokinetic analysis, peripheral blood samples were collected in EDTA during the consolidation phase, which consisted of daily MP (25 mg/m^2 *per os)* and biweekly high-dose methotrexate (MTX, 5 g/m^2/dose i.v.), just before the fourth and last biweekly infusion. During the maintenance phase with daily MP (50 mg/m^2 *per os*) and weekly low-dose MTX (20 mg/m^2 *per os*), blood samples were planned to be collected at the scheduled visits on the 3rd, 9th and 15th month.

The second cohort consisted of 119 patients affected by IBD (median age (IQ): 15.1 (12.3–16.9) years; male: 52.1%) enrolled at the Gastroenterology Unit of the IRCCS "Burlo Garofolo" in Trieste, Italy. Sixty-seven of these patients (56.3%) were affected by Crohn's disease (CD), fifty-one (42.9%) by ulcerative colitis (UC), and one (0.8%) by indeterminate colitis. IBD patients had been treated with daily weight-based AZA therapy for at least 3 months before enrollment (median dose (IQ): 2.0 (1.7–2.3) mg/kg/die per os; median treatment length (IQ): 493.5 (231.5–897.5) days). Peripheral blood samples, anticoagulated with EDTA and immediately added with 1 mg of the antioxidant dithiothreitol (DTT, Sigma-Aldrich, Milan, Italy) to preserve the free thiol moiety of thiopurines, were collected at scheduled control visits and used for pharmacogenetic and pharmacokinetic analysis. These blood samples were also sent at 4 °C to the University of Trieste and processed within 24 h. An overview of the study design, highlighting samples collected and pharmacological analyses performed in both cohorts, is given in Supplementary Fig. 1.

Buffy coats of 20 healthy blood donors (median age (IQ): 47 (35.8–57) years; male: 70%) were provided by the Department of Transfusion Medicine, Azienda Ospedaliera Universitaria of Trieste (Italy). Blood was obtained by venepuncture and immediately processed. A total of 4 ml of each buffy coat was used for the isolation of peripheral blood mononuclear cells (PBMC) and for DNA extraction and genotyping as well as for preparing protein lysates for western blot.

The clinical studies were approved by the local ethical committees and appropriate informed consent was obtained from patients/donors and/or their parents or guardians.

DNA extraction and pharmacogenetic analysis

Total genomic DNA was isolated from patient peripheral blood and healthy donor buffy coats using a commercial kit (Sigma-Aldrich, Milan, Italy) according to the manufacturer's protocol. TaqMan® SNP genotyping assays (Applied Biosystems, USA) were used to characterize the SNPs of interest (*TPMT* rs1142345, rs1800460 and rs1800462 and *PACSIN2* rs2413739, Table 1). Samples' genotyping was repeated twice.

Pharmacokinetic analysis

Measurement of thiopurine metabolites in patients' RBC

Thiopurine metabolites were quantified using a previously described method [23] and results were expressed as pmol/8×10^8 RBC. A brief description of the method is reported in Supplementary Material.

TPMT activity in patients' RBC

TPMT activity was measured as previously described [24], and results were expressed as nmoles of methyl-mercaptopurine produced in 1 h per 10^6 RBC (nmol 6-MMP/h/1 × 10^6 RBC). A brief description of the method is reported in Supplementary Material.

Pharmacodynamic analysis: blasts in vitro sensitivity to thiopurines

The in vitro effect of thiopurines on ALL blasts was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based viability assay, as previously described [25]. Nonlinear regression of dose–response data was performed computing IC_{50}, the drug concentration required to reduce viability to 50%. I_{max} was also calculated from the dose–response curve and defined as the percentage of cell death achieved with the highest drug concentration tested (3.0×10^{-4} M for TG and 3.0×10^{-3} M for MP). A brief description of the method is reported in Supplementary Material.

PBMC isolation and western blotting

PBMC were collected by density gradient centrifugation and western blotting analysis was performed as described in the Supplementary Material.

Clinical response

Clinical data were collected by pediatricians blinded to results of pharmacological analysis and according to routine clinical practice. For ALL patients, episodes of INF, GI, pancreatic/hepatic (PAN/HEP), renal (REN), cardiologic (CARDIO), neurological (NEU), and HEM toxicity during consolidation and maintenance phases were graded referring to a simplified version of the National Cancer Institute-Common Terminology Criteria scales (Supplementary Table S1). Clinical data were available only for a small subset of ALL patients (81

Table 1 Candidate SNPs and genotype distribution in pediatric patient cohorts

Gene	SNP	Position	IBD cohort (N° = 119)					ALL cohort (N° = 297)					
			N°	wt (%)	hz (%)	mut (%)	HWE	N°	wt (%)	hz (%)	mut (%)	HWE	*p* value*
TPMT	rs1142345 (*TPMT*3C*)	Exon 10 A719G, Tyr240Cys	112	107 (95.5)	5 (4.5)	0 (0.0)	0.81	194	184 (94.8)	10 (5.2)	0 (0.0)	0.14	1.00
TPMT	rs1800460 (*TPMT*3B*)	Exon 7 G460A, Ala154Thr	112	107 (95.5)	5 (4.5)	0 (0.0)	0.81	210	200 (95.2)	10 (4.8)	0 (0.0)	0.72	1.00
TPMT	rs1800462 (*TPMT*2*)	Exon 5 G238C, Ala80Pro	119	119 (100)	0 (0.0)	0 (0.0)	NA	200	200 (100)	0 (0.0)	0 (0.0)	NA	NA
PACSIN2	rs2413739	Intron (T > C)	89	27 (30.3)	37 (41.6)	25 (28.1)	0.14	212	72 (34)	94 (44.3)	46 (21.7)	0.18	0.50

ALL acute lymphoblastic leukemia, *HWE* Hardy–Weinberg equilibrium, *hz* heterozygote, *IBD* inflammatory bowel disease, *mut* mutated, *N°* number, *NA* not assessed, *wt* wild type

*p value calculated according to Fisher's test comparing genotyping frequencies in the IBD and ALL cohorts

children in consolidation and 47 in maintenance), being still under collection for the others.

Clinical efficacy was assessed in patients with IBD, using Pediatric Crohn's Disease Activity Index (PCDAI) and Pediatric Ulcerative Colitis Activity Index (PUCAI) for CD and UC patients, respectively, at the time of blood sample collection for the metabolites' measurement [26, 27]. Disease was considered inactive if indexes were lower than 10 at the time of sample collection.

Statistical analysis

Statistical analyses were performed using the software R (version 3.4.2).

The association between pharmacological parameters (pharmacokinetic and pharmacodynamic, dependent variable) and pharmacogenomic variables (independent variables) was examined using linear mixed-effect models of the Gaussian family. The effect of gender and age covariates was also examined. In patients with IBD, to study disease activity, linear mixed-effect models of the binomial family were used, considering disease activity as the dependent variable and the pharmacogenomic variables as the independent variables. The mixed effects models were built using the phenotype of interest as the dependent variable, each covariate as the fixed effect and the patients as the random effect in the model. In pharmacokinetic analysis, TGN levels below 50 pmol/8×10^8 RBC were excluded. In patients with ALL, toxicities were separately considered: each was dichotomized as severe (grade III/IV) versus nonsevere (grade I/II or absent), without discriminating among specific adverse episodes. Logistic regression model was used to assess the effect of genotypes on the onset of severe toxicity. All pharmacogenomic analyses evaluated an additive effect of the genotype on the phenotype of interest. For the study on healthy donor blood samples, the association between TPMT, PACSIN2 protein concentration, *PACSIN2* polymorphism was evaluated by mixed-effect linear models. Since our study is a validation study focused on few comparisons, the standard significance threshold of 0.05 was applied to p values and no multiple testing adjustment was performed [28]. Normality of the phenotype was tested by the Shapiro test and appropriate transformation was applied if needed, in order to adjust the normality of the distribution.

Results

PACSIN2 and TPMT genotype distribution in patients' cohorts

Three SNPs in the *TPMT* gene (rs1142345, rs1800460, and rs1800462) and one in *PACSIN2* (rs2413739) were

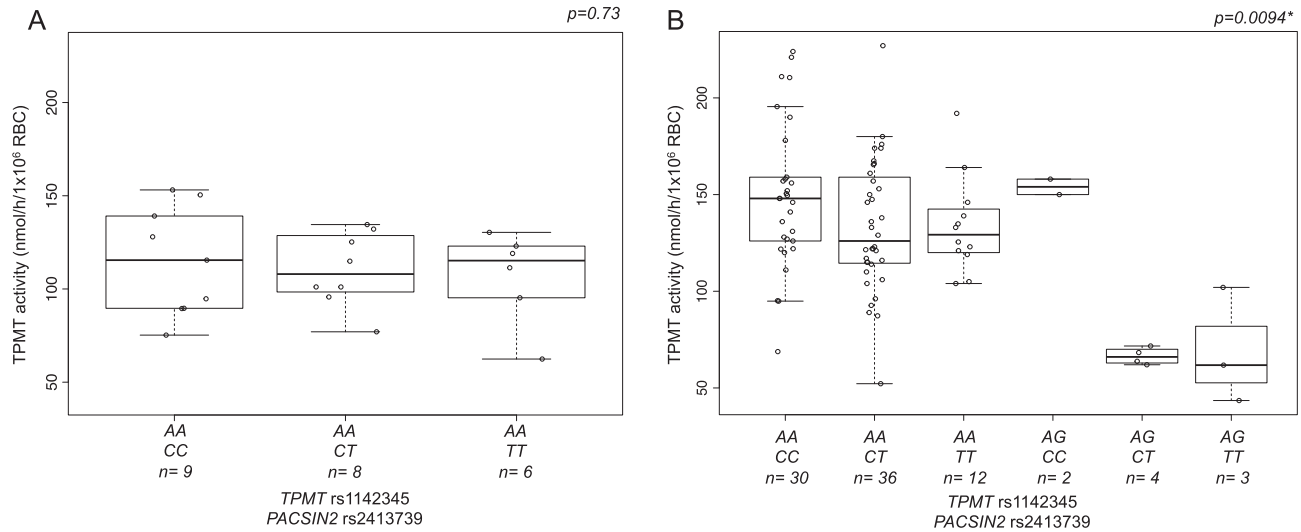

Fig. 1 Correlation between TPMT enzymatic activity and *TPMT* rs1142345 and *PACSIN2* rs2413739 genotypes in a subgroup of (**a**) IBD patients and (**b**) ALL patients. Blood samples of ALL patients were collected during maintenance. Each point represents the median value for the variable considered for each patient. p values were calculated by the linear mixed-effect model, $*p = 0.0094$ for *PACSIN2* rs2413739 according to the multivariate analysis including *TPMT* genotypes

genotyped in ALL and IBD in pediatric patients: results are shown in Table 1. The two cohorts were similar in *TPMT* genotype distribution, with ~5% of patients carrying the low-activity *TPMT* alleles in both populations, as expected from previously published data in Caucasians and in ALL Italian children [8, 12]. Patients with a variant *TPMT* allele were all heterozygous *TPMT*3A* (haplotype rs1142345 and rs1800460), with the exception of one heterozygous *TPMT*3C* (rs1142345) ALL patient: none of them carried the *TPMT*2* (rs1800462) allele and none was homozygote for the variant alleles. Similarly, no significant difference in *PACSIN2* SNP rs2413739 genotype distribution was observed between ALL and IBD children, with ~40% of them carrying the TC genotype and ~30% being TT, as expected for Caucasian [14]. Genotype frequencies were in Hardy–Weinberg equilibrium (HWE, $p > 0.05$) in both cohorts.

PACSIN2 rs2413739 and thiopurine pharmacokinetics

TPMT enzymatic activity

The influence of demographic characteristics on TPMT enzymatic activity was evaluated in pediatric patients undergoing a long-term, thiopurine-based therapy. Interestingly, the enzymatic function was significantly higher in ALL patients compared with IBD patients ($p = 0.032$, linear mixed-effect model). In both cohorts, TPMT activity was not influenced by age and gender (Supplementary Table S2).

For the genetic analysis, *TPMT* genotype was available for all patients with at least one measurement of TPMT enzymatic activity, while *PACSIN2* genotype was available for 23 IBD and 89 ALL patients, respectively (Fig. 1). In IBD, no significant difference in TPMT activity was observed among the *PACSIN2* rs2413739 TT (six patients, 26.1%), TC (eight, 34.8%), and CC (nine, 39.1%) subjects ($p = 0.73$, linear mixed-effect model, Fig. 1a); these children were all *TPMT* wild type. In ALL, univariate analysis demonstrated that TPMT activity was significantly reduced in *TPMT* heterozygotes (rs1142345 and rs1800460, linear mixed-effect model, $p < 10^{-4}$) and in the presence of the *PACSIN2* rs2413739 T allele (linear mixed-effect model, $p = 0.0013$). Genotype effects remained statistically significant after multivariate analysis considering both genes (*TPMT* rs1142345, $p = 10^{-4}$; *PACSIN2* rs2413739, $p = 0.0094$, Fig. 1b, *TPMT* genotype explaining 16,7% of variability in the TPMT activity and *PACSIN2* genotype 9.6%). Interestingly, *PACSIN2* genotype effect was particularly evident within *TPMT* heterozygotes (linear mixed-effect model, $p = 0.040$) in comparison with *TPMT* wild type patients (linear mixed-effect model, $p = 0.044$).

Thiopurine metabolites

Table 2 summarizes the TGN and MMPN levels in the two pediatric cohorts. In ALL patients, a direct correlation was found between age and metabolites: TGN were significantly higher in adolescent in comparison with younger patients in maintenance. Statistical significance was not reached for MMPN; however, a tendency towards higher MMPN concentration in teenagers was observed in maintenance. Metabolites did not differ between male and female patients in either cohort (Table 2).

Table 2 Thiopurine metabolites in patients' RBC and association with demographic characteristics

	IBD cohort		ALL cohort			
			Consolidation		Maintenance*	
N° patients	119		134		144	
N° blood samples	280		134		249	
Metabolites	TGN	MMPN	TGN	MMPN	TGN	MMPN
Median (IQ) pmol/8 × 10^8 RBC	347.5 (231.8–498.5)	1072 (448–2243)	242.9 (156.6–417.4)	1168.2 (540.5–2406.2)	351.44 (243.48–574.55)	4228.58 (1568.26–7957.79)
Age (p value)	0.39	0.17	0.15	0.93	0.03	0.09
Gender (p value)	0.41	0.92	0.21	0.83	0.26	0.38
TPMT variants (p value)	3.9×10^{-3}	1.1×10^{-3}	10^{-4}	0.05	$<10^{-4}$	3×10^{-4}
PACSIN2 rs2413739 (p value)	0.40	0.76	0.37	0.35	0.58	0.61

ALL acute lymphoblastic leukemia, *IBD* inflammatory bowel disease, *IQ* interquartile range, *MMPN* methylmercaptopurine nucleotides, *RBC* red blood cells, *TGN* thioguanine nucleotides

*Multiple measurements over time (3rd, 9th, and 15th months) were reported for 74 patients ($n = 3$, 28 patients; $n = 2$, 46 patients): in these patients, the mean values of TGN and MMPN were considered

Among ALL patients, the presence of a *TPMT* low-activity allele increased the concentration of TGN (linear mixed-effect model, $p < 10^{-4}$) and decreased that of MMPN (linear mixed-effect model, $p = 0.0003$) in patients' RBC during the maintenance phase (thiopurine metabolites measured in 232 blood samples of 131 children). By contrast, *PACSIN2* rs2413739 did not affect metabolites levels (239 blood samples of 137 children analyzed). Similar results were observed in samples obtained during the consolidation phase (increased TGN (linear mixed-effect model, $p = 10^{-4}$) and decreased MMPN concentrations (linear mixed-effect model, $p = 0.047$) for *TPMT* heterozygotes, no genetic effect of *PACSIN2* rs2413739, thiopurine metabolites measured in 113 children).

Among IBD patients, *TPMT* genotype was known for 112 children with thiopurine metabolites measured in 273 blood samples, and *PACSIN2* genotype for 84 children with thiopurine metabolites measured in 209 blood samples. Heterozygous *TPMT*3A* patients showed an increased value of TGN (linear mixed-effect model, $p = 0.0039$) and a significant reduction in MMPN (linear mixed-effect model, $p = 0.0011$) with respect to wild type *TPMT* subjects; *PACSIN2* rs2413739 did not affect TGN and MMPN levels.

PACSIN2 rs2413739 and ALL patients' thiopurine pharmacodynamics

The pharmacodynamic assay with TG and MP was performed on blasts isolated from bone marrow aspirates of 39 and 31 ALL patients, respectively (Supplementary Material). Patients age did not affect blasts in vitro sensitivity, whereas a gender effect was observed for MP: females showed a higher resistance to MP in comparison with males (linear model, $p = 0.033$). All patients were wild type for *TPMT*. The correlation between thiopurine in vitro sensitivity and *PACSIN2* rs2413739 polymorphism was also investigated but no difference according to genotype was found.

PACSIN2 rs2413739 and clinical response

Out of 212 ALL patients with known *PACSIN2* rs2413739 genotype, toxicities data were available for 81 children in consolidation and 47 in maintenance. During consolidation, the incidence of severe (grade ≥ 3) adverse effects were: HEM 48.1%, GI 9.9%, INF 4.9%, PAN/HEP 3.7%, REN 1.2%, CARDIO 0%, NEU 0%; during maintenance, the incidence was: HEM 65.9%, PAN/HEP 19.1%, INF 14.9%, GI 8.5%, CARDIO 2.1 %, REN 0%, NEU 0%. Borderline significance emerged for *PACSIN2* SNP rs2413739 and GI during consolidation, with an increased risk of adverse effect in TT carriers (linear mixed effects $p = 0.04947$, Table 3), and a similar trend was observed also for HEM toxicity during maintenance ($p = 0.0998$).

All IBD patients had been treated continuously with daily weight-based oral AZA therapy for at least 3 months before enrollment (median dose (interquartile range, IQ): 2.0 (1.7–2.3) mg/kg/die; median (IQ) treatment length: 493.5 (231.5–897.5) days). Clinical response to AZA therapy was evaluated in 78 patients (180 samples

Table 3 Grade III–IV toxicities and PACSIN2 rs2413739 genotype in ALL patients during consolidation and maintenance therapy

Consolidation phase ($N = 81$)												
PACSIN2 rs2413739	INF			GI			PAN			REN			HEM		
	No (n)	Yes (n)	p value	No (n)	Yes (n)	p value	No (n)	Yes (n)	p value	No (n)	Yes (n)	p value	No (n)	Yes (n)	p value
CC	26	3	0.325	28	1	0.04947	28	1	0.6290	28	0	0.8816	15	14	0.729
TC	33	0		30	3		31	2		32	1		16	17	
TT	18	1		15	4		19	0		19	0		11	8	
Tot	77	4		73	8		78	3		79	1		42	39	
Maintenance phase ($N = 47$)															
PACSIN2 rs2413739	INF			GI			PAN			CARDIO			HEM		
	No (n)	Yes (n)	p value	No (n)	Yes (n)	p value	No (n)	Yes (n)	p value	No (n)	Yes (n)	p value	No (n)	Yes (n)	p value
CC	17	4	0.443	20	1	0.9222	17	4	0.6712	20	1	0.997	9	10	0.0998
TC	13	2		12	3		13	2		14	0		4	10	
TT	10	1		11	0		8	3		11	0		2	9	
Tot	40	7		43	4		38	9		45	1		15	29	

CARDIO cardiological, *GI* gastrointestinal, *HEM* hematological, *N* number, *PAN/HEP* pancreatic/hepatic, *REN* renal, *tot* total

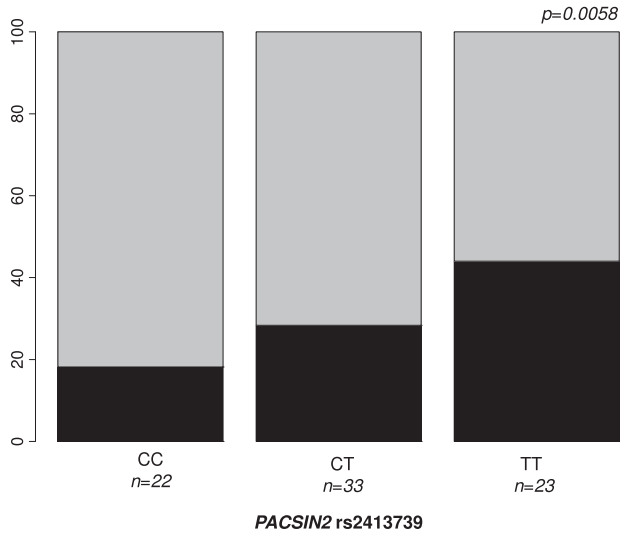

Fig. 2 Disease activity and *PACSIN2* rs2413739 genotypes in IBD patients. Bar plots represent the percentage of patients in remission (gray) and with active disease (black) according to *PACSIN2* rs2413739 genotype. Clinically active disease was assessed as PCDAI and PUCAI values above 10. Disease activity was assessed at the time of the measurement of thiopurine metabolites. One hundred and eighty observations in seventy-eight patients were considered. p value was calculated by the linear mixed-effect model of the binomial family

analyzed): AZA effectiveness was reduced in children carrying the T allele of the SNP rs2413739 (linear mixed effects, $p = 0.0058$; genotyping results: CC in 22, CT in 33, and TT in 23 patients): indeed, a clinically active disease was observed in 43.1% of TT patients, in 28.4% of those with the CT genotype and in 18.2% of the CC subjects (Fig. 2). *TPMT* variant genotype, present in 2 of the 78 patients as *TPMT*3*A heterozygous allele, was not significantly associated with AZA effectiveness.

TPMT and PACSIN2 variants in healthy donors' PBMC

To evaluate the role of *PACSIN2* SNP rs2413739 on TPMT and PACSIN2 protein level, 20 adult healthy donors were genotyped for the SNPs of interest, and western blot analysis of human TPMT and human PACSIN2 was performed on their PBMCs lysates. Only one of the volunteers was heterozygous for *TPMT*3A*; the genotype distribution of *PACSIN2* rs2413739, with six (30%) heterozygous and seven (35%) carrying the TT wild-type genotype, was not in HWE likely because of the small number of individuals considered. In the lysates, TPMT showed a protein concentration that was approximately the double of that measured for PACSIN2 (median (IQ): 77.3 (64.7–93.8)% versus 38.9 (26.9–65.1)%, respectively). (Supplementary Fig. 2). No significant contribution of the polymorphism was found on TPMT and PACSIN2 protein level in this small population. In contrast, a significant

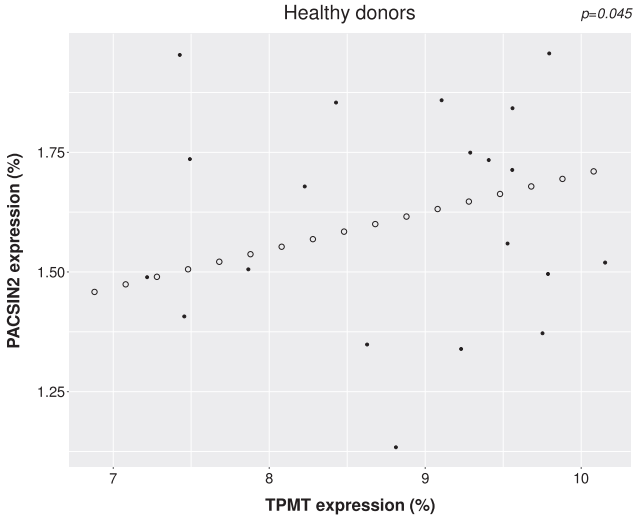

Fig. 3 Association between TPMT and PACSIN2 protein concentrations evaluated by western blotting in PBMC cellular extracts from adult healthy donors. Values were normalized by logarithmic transformation (for PACSIN2 concentration) or square root (for TPMT concentration). Each black point represents the average of the two quantifications performed for each cell extract. White circles represent the predicted PACSIN2 concentration values based on TPMT values, from the mixed-effect linear model built on the basis of these measurements ($p = 0.045$)

direct correlation between the TPMT and PACSIN2 levels was observed (linear mixed effect, $p = 0.045$, conditional $r^2 = 64\%$, Fig. 3), suggesting a putative coordination in their protein levels.

Discussion

Our data document that *PACSIN2* rs2413739 T allele reduces the TPMT enzymatic activity during the MP/MTX-based maintenance therapy of the AIEOP-BFM ALL 2009 protocol but not in the AZA-based IBD therapy, although no SNP effect was observed on metabolite levels. The role of *PACSIN2* rs2413739 on the TPMT enzymatic activity in ALL was first proposed in 2012 in a SJCRH cohort [14], and has been validated in multivariate analysis in the AIEOP-BFM ALL 2009 protocol; the present study confirms also the expected contribution of *TPMT* SNPs rs1142345 and rs1800460 on the TPMT phenotype. Recent genome-wide association studies (GWAS) have not confirmed the *PACSIN2* rs2413739 effect and have provided strong evidence of *TPMT* as the only monogenic trait influencing the TPMT phenotype, finding that only *TPMT* variants are significantly associated with TPMT activity in ALL children ($n = 1026$; $p = 8.6 \times 10^{-61}$) [29] and in a mixed population (two cohorts of adult healthy volunteers and one of pediatric ALL patients, $n = 1212$; $p = 1.2 \times 10^{-72}$) [30]. We hypothesized that the discrepancy on the *PACSIN2* rs2413739 results could be linked to the different times used for RBC sampling, likely due to the greater level of TPMT in newly synthesized erythrocytes [31]. Indeed, in both GWAS, TPMT activity has been measured after induction, a therapeutic phase that causes profound myelosuppression and is followed by bone marrow cellular recovery, whereas in the present study the enzyme activity has been assessed during maintenance, in a therapeutic phase when RBC have already undergone a progressive ageing process and myelosuppression is less severe. Moreover, GWAS studies may detect more easily strong effects, while smaller ones may be more difficult to reproduce [32]. Concomitant drugs represent an alternative hypothesis to explain the discrepant *PACSIN2* rs2413739 results in ALL: MTX, co-administered with MP across all AIEOP-BFM ALL 2009 therapeutic phases, is able to deplete S-adenosyl methionine, a TPMT cofactor particularly important for the stability of the TPMT protein [33]. It is therefore likely that the genetic effect of *PACSIN2* rs2413739 on TPMT activity in ALL children emerges only under more physiological conditions, such as those found during the maintenance phase characterized by low-doses MTX and quite normal production and life cycle of RBC. A recent study has associated the *PACSIN2* rs2413739 genotypes with the incidence of HEM toxicities during maintenance, this finding further supports the effect of this polymorphism in thiopurine biotransformation during this phase [34]. It is possible that the putative weak genetic influence of *PACSIN2* varies with patient age and becomes more evident in younger patients because of higher measurable TPMT activity [35]. Indeed, it has been reported that TPMT activity is higher in infants in comparison with children [36] and in adolescents in comparison with older patients and adults [37], although this age dependence is controversial [14, 38]. This hypothesis could explain the lack of *PACSIN2* rs2413739 influence on TPMT during the AZA-based maintenance immunosuppression in IBD therapy, in contrast to what is observed in the MP/MTX-based ALL treatment. Our IBD patients were all teenagers with a median age of around 15 years, whereas ALL patients were mostly children below 10 years of age. Epigenetic mechanisms, such as the promoter methylation of *TPMT* [39], could stand at the basis of age-related TPMT function. It should be also noted that no direct effect of age on TPMT activity was observed in neither ALL nor IBD cohort, although an effect of age on TGN levels in the ALL cohort (higher in adolescents compared with younger patients) was found: this effect may dependent on the activity of other enzymes and/or transporters involved in the MP metabolizing pathway as well as on environmental and clinical factors. Moreover, the number of TPMT enzymatic measurements in the IBD group was very limited and thus further studies need to be performed to shed light on the contribution of age in thiopurine pharmacokinetics.

For ALL patients, an increased incidence of grade III/IV stomatitis and diarrhea during consolidation therapy in patients with variant T alleles could be confirmed, as previously reported [14]. Moreover, a trend for an association between *PACSIN2* variants and severe HEM toxicities during maintenance therapy was found, confirming previously described data [34]. The results showed in this study are encouraging toward a significant effect of *PACSIN2* variant on chemotherapy-induced adverse events occurrence although they are limited by the small number of individuals with already available clinical data. Pharmacological analyses should be associated to the clinical outcome in the whole ALL cohort before drawing definitive conclusions, and should be interpreted carefully considering also potential underlying confounding variables, such as relevant clinical information (e.g., immunophenotype, variables of treatment response including minimal residual disease, treatment risk groups).

In the IBD cohort, an increased disease activity has been observed for carriers of *PACSIN2* rs2413739 TT genotype. This could be at least partly interpreted as resistance to the thiopurine antimetabolite AZA and could be a TPMT-independent effect, possibly related to phenomena, such as modulation of autophagy or other tissue-specific effects [14]. Due to the low incidence of events, none of which had grade III or IV severity, toxicities were not considered in the analysis for this cohort.

These preliminary clinical results, together with the observation that *PACSIN2* SNP does not influence the MP metabolite levels in any of the two analyzed cohorts, suggest that thiopurine pharmacokinetics is a complex trait, probably influenced by multiple genes and nongenetic factors. However, it can also be hypothesized that *PACSIN2* rs2413739 could have some TPMT-independent tissue-specific effect on thiopurine cytotoxic effects, of particular importance in the gastrointestinal tract. Indeed, rs2413739 polymorphism shows a different tissue-specific gene expression pattern between whole blood and the colon-sigma, as reported for larger cohorts in the public available genotype-tissue expression (GTEx) portal (www.gtexportal.org). GTEx reports a higher PACSIN2 expression in subjects carrying the rs2413739 T allele in blood ($p = 10^{-11}$) but not in colon-sigma ($p = 0.9$). Moreover, TT carriers have also a higher TPMT expression in the former tissue ($p = 0.006$ versus $p = 0.23$). In this study, the polymorphism was not associated with PACSIN2 and TPMT protein concentrations, likely because of the small number of healthy donors analyzed.

This study has some limitations. A small number of samples were considered in some of the subgroups, therefore some of the analyses (e.g: association of *PACSIN2* SNP and TPMT activity in IBD patients or association of *PACSIN2* SNP and chemotherapy cytotoxic effects in ALL patients, as already mentioned) can have low statistical power and requires further investigations in larger cohorts. Being a validation study, no formal power analysis was performed. In this study, no adjustment was done for multiple comparisons; the use of multiple comparison in studying few candidate SNPs in relation to a given phenotype is debated [40], and indeed this study focuses on only few comparisons that comprise two candidate polymorphisms and the standard demographic covariates age and gender. Adjusting significance threshold beyond standard values seemed over conservative to authors also because, being a validation study on the role of *PACSIN2* SNP on pharmacokinetic parameters, our significant results are strengthened by the coherence with previous evidence.

In conclusion, this study addresses an important issue related to the treatment of children with thiopurines either for ALL or IBD and provides new insights on the role of PACSIN2 on TPMT activity and protein that seems to be dependent on the age and clinical condition of the patient. Indeed, the study confirms this effect in children with ALL, but not in teen patients with IBD. Moreover, PACSIN2 seems to modulate thiopurines effect in the gastrointestinal tract also in patients with IBD, since *PACSIN2* polymorphism was associated with AZA efficacy measured as a clinical activity score below 10. Nonetheless, these results have limited validity for clinicians as they are: indeed, they are based on small numbers of individuals and significance in clinical response is partly borderline. Further studies are needed to fully elucidate the molecular mechanism involved in the age and tissue specificity of the association between PACSIN2, TPMT, and thiopurines sensitivity in children needing these drugs.

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Author contributions RF contributed to the study design, genetic analysis, data interpretation, and paper writing; GS contributed to study design, statistical analysis, data interpretation, and paper writing; DF performed HPLC analysis; NG, ID, LV, AC, EB, and SM recruited patients and collected clinical data; MP contributed to western blotting analysis; FL, AB, FF, and AV discussed results and revised the manuscript; GD contributed to study design, results discussion, and paper writing; MR contributed to the study design, coordinated the clinical part, discussed results, and revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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