MULTICENTRIC CASE CONTROL STUDY ON AZATHIOPRINE DOSE AND PHARMACOKINETICS IN EARLY ONSET PEDIATRIC INFLAMMATORY BOWEL DISEASE

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ABSTRACT

BACKGROUND: Early onset inflammatory bowel disease (IBD) is generally aggressive, with a high probability of complications and need of surgery. Despite the introduction of highly effective biological drugs, treatment with azathioprine continues to be important even for early onset IBD; however, in these patients azathioprine response appears to be reduced. This study evaluated azathioprine doses, metabolite concentrations and their associations with patients' age in children with IBD treated at 6 tertiary pediatric referral centers.

METHODS: Azathioprine doses, metabolites and clinical effects were assessed after at least 3 months of therapy in 17 early onset (age<6 years, cases) and 51 non-early onset (age>12 and <18 years, controls) IBD patients. Azathioprine dose was titrated on therapeutic efficacy (response and adverse effects). Azathioprine metabolites and thiopurine methyltransferase activity were determined by HPLC-UV methods.

RESULTS: Frequency of patients in remission was similar among early onset and control group (respectively 82% and 84%, p-value=0.72). Early onset patients required higher doses of azathioprine (median 2.7 vs 2.0 mg/kg/day, p-value=1.1×10^{-4}). Different doses resulted in comparable azathioprine active thioguanine nucleotide (TGN) metabolite concentrations (median 263 vs 366 pmol/8×10^8 erythrocytes, p-value=0.41) and methylmercaptopurine nucleotide concentrations (median 1455 vs 1532 pmol/8×10^8 erythrocytes, p-value=0.60). Lower TGN metabolites/azathioprine doses ratios were found in early onset patients (median 98 vs 184 pmol/8×10^8 erythrocytes/mg/kg/day, p-value=0.017).

Interestingly, early onset patients presented also higher TPMT activity (median 476 vs 350nmol methyl-mercaptopurine/mg hemoglobin/h, p-value=0.046).

CONCLUSIONS: This study demonstrated that early onset IBD patients present increased inactivating azathioprine metabolism, likely because of elevated activity of the enzyme thiopurine methyltransferase.

KEYWORDS: azathioprine; early onset inflammatory bowel disease; thiopurine methyltransferase; pharmacokinetics

INTRODUCTION

Approximately 25% of inflammatory bowel diseases (IBDs), namely Crohn's disease (CD) and ulcerative colitis (UC), has an onset in childhood with an incidence, for patients aged <16 years, between 8 and 11 cases / 100.000 inhabitants / year. The incidence of IBD in children has risen in the last decade, particularly for episodes of illness with onset early in life, defined as early onset (<6 years)(1, 2); incidence of early onset cases is ~10% of all pediatric cases(2). IBD in children is in general more aggressive, both in terms of disease extension and progression, with a high probability of complications and surgery; moreover, the disease proves much more aggressive the earlier the onset is(1). Pharmacological therapy of IBD is primarily aimed at inflammation treatment and control, through the use of drugs able to induce and maintain disease remission(3). Drugs currently available for induction and maintenance therapy of pediatric IBD, like glucocorticoids, azathioprine and biologics are less effective in early onset cases(4). For azathioprine, response to standard doses is reduced in early onset patients in comparison to those with onset at age older than 6 years: open studies showed that a higher dosage of azathioprine compared with conventional regimens should be used in patients with early onset IBD to achieve better response(5, 6). Azathioprine is a pro-drug and requires bioactivation to thioguanine nucleotides (TGN), through a complex pathway of enzymatic reactions, overlapping with the salvage pathway for nucleotides synthesis. Polymorphisms in genes for enzymes involved in the metabolism of azathioprine influence treatment’s efficacy and toxicity(7). Thiopurine methyltransferase

(TPMT) influences substantially azathioprine metabolism at several steps, thereby altering the formation of active TGNs(8). In patients with genetic variants leading to reduced activity of TPMT, the metabolic pathway of azathioprine is shifted in the direction of active TGNs(9). Polymorphisms in TPMT have been associated with an increased risk for azathioprine related adverse events in patients with IBD(10). The determination of azathioprine metabolites concentrations is considered to be an important tool for individualization of thiopurine therapy in order to verify patients' compliance and adequate exposure to active metabolites(11). We therefore hypothesized that early onset IBD patients may present an altered azathioprine pharmacokinetics in comparison to non-early onset patients, leading to decrease sensitivity to azathioprine. The aim of this study was then to evaluate azathioprine doses, metabolite concentrations and their associations with patients' age in early onset pediatric IBD patients in comparison to non-early onset pediatric IBD.

MATERIALS AND METHODS

Ethical considerations

The study was approved by the local ethical committees and appropriate informed consent was obtained from all patients or their parents or tutors.

Patients and eligibility criteria

In this study 68 patients with IBD were enrolled by the participating centers between October 2014 and September 2015. The inclusion criteria were age up to 6 years for cases and more than 12 years and less than 18 years for controls, previous diagnosis of IBD and treatment with azathioprine for at least 3 months and stable dose of azathioprine for at least 1 month. The exclusion criteria were patients with ileostomy or colostomy, disease needing surgery, fulminant ulcerative colitis or toxic megacolon, contemporary presence of other non controlled pathologies, concomitant therapy with anti-TNF biological agents (infliximab or adalimumab). Other co-treatments, such as aminosalycilates, glucocorticoids or enteral therapy were allowed.

The patients enrolled were all the eligible consecutive cases taking azathioprine at the participating centers in the time-frame of the study. For each case, the first three consecutive controls enrolled in each center, matched on IBD diagnosis, were selected.

Therapeutic protocol and sample collection

This case control study was non-interventional and patients were treated according to standard care and enrolled cross-sectionally from patients treated in each participating hospital. Patients were treated with a dose escalating strategy to reduce the risk of adverse events starting however from a relatively high dose (median of 2 mg/kg). At subsequent follow-up visits, the dose was increased or reduced so as to obtain the optimal clinical response; the criteria used to increase or reduce the dose of azathioprine were the level of disease activity and

laboratory parameters used to monitor azathioprine toxicity (in particular leukocytes, erythrocytes and platelets counts, hemoglobin concentration, MCV, liver enzymes ALT, AST and γ-GGT and amylase levels).

Blood samples, collected for measuring azathioprine metabolites, TPMT activity and for genotyping, were taken at the first clinic visit occurring after at least three months of therapy and one month on stable azathioprine dose. Timing of sample collection was independent from clinical response status and results of pharmacokinetic analysis did not affect clinicians in their therapeutic choices.

Definition of clinical response

Clinical response was assessed, using Pediatric Crohn's Disease Activity Index (PCDAI)(12) and Pediatric Ulcerative Colitis Activity Index (PUCAI)(13) respectively for CD and UC patients, at the time of blood sample collection for the metabolites' measurement. Disease was considered inactive if the disease activity index was lower than 10 at the time of sample collection. Successful therapy or inactive disease were not among the inclusion criteria.

Measurement of azathioprine metabolites

Azathioprine metabolites (TGN and methylmercaptopurine nucleotides, MMPN) were measured at the Department of Life Sciences, University of Trieste in patients' erythrocytes using an HPLC assay by Dervieux et al.(14). Blood samples were centrifuged for collection of erythrocytes, as previously described;

erythrocytes were stored at -80°C until analysis, which was performed within a month from collection. Metabolites concentration is expressed as pmol / $8x10^8$ erythrocytes. The ratio between TGN and the dose of azathioprine was calculated considering for each individual measurement the dose the patients was taking the day the blood sample for the metabolites assessment was collected.

Measurement of TPMT activity

TPMT activity was measured for all patients at the Department of Life Sciences, University of Trieste by HPLC assay based on in vitro conversion of mercaptopurine to methyl-mercaptopurine, using S-adenosyl-methionine as the methyl donor(15). TPMT activity is expressed as nmol of methyl-mercaptopurine produced by lysates of patients' erythrocytes, containing 1 mg of hemoglobin, during 1 h of incubation at 37 °C in the presence of mercaptopurine. TPMT activity was measured concurrently to azathioprine metabolites, if the amount of patients' erythrocytes was sufficient.

Genotypes

Genomic DNA was extracted from peripheral blood samples using a commercial kit (SIGMA, Milan, Italy), in order to characterize the most relevant genetic polymorphisms in the candidate gene TPMT (rs1800462, rs1800460, rs1142345), by using TaqMan assays (Thermoscientific, Milan, Italy).

Statistical analysis

Statistical analysis was performed using the software R (version 3.2.4).

The association between pharmacological phenotypes of interest (i.e, therapeutic response, dose of azathioprine, TGN metabolites concentrations, MMPN metabolites concentrations, ratio TGN/dose, TPMT activity, concomitant therapies) and the considered covariates (i.e, demographic variables including age-group classification, IBD type, TPMT genotypes) was evaluated in a univariate analysis by using generalized linear models of appropriate family (gaussian/ANOVA for continuous and binomial/logistic regression for categorical variables). In these univariate analyses, the dependent variable was the pharmacological phenotype of interest and the independent variable the demographic, clinical or pharmacogenetic covariate.

Multivariate analysis was done to test the independence of the significant effects identified in univariate analyses on the phenotypes considered; for this multivariate analysis generalized linear models of the appropriate family were used combining covariates significant in the univariate analysis as the independent variables. For all parametric analysis (i.e, linear models used in the univariate analysis and the multivariate analysis), normality of the phenotype was tested by the Shapiro test and log10 or Box-cox transformation was applied if needed, in order to adjust the normality of the distribution.

RESULTS

Patients enrolled and samples collected

The present study recruited 17 early onset patients with inflammatory bowel disease, matched to 51 later onset pediatric IBD patients, considered as controls. Demographic and clinical characteristics are reported in Table 1. From October 2014 to September 2015 peripheral blood samples have been collected to measure azathioprine metabolites. Among these, 27 were obtained during treatment with azathioprine alone and 41 during treatment with azathioprine and other medications and in particular: 26 with an aminosalicylate, 6 with a glucocorticoid, 6 with an aminosalicylate and a glucocorticoid, 2 with an aminosalicylate and an antibiotic and 1 with enteral nutrition; no significant difference could be observed for co-treatments between cases and controls. In particular, frequency of patients taking aminosalycilates with azathioprine was similar between cases and controls (respectively 41.2% vs 52.9%, p-value logistic regression = 0.40). At the time of the evaluation, none of the patients was on allopurinol, furosemide or any other medication that could interfere with thiopurine metabolism.

Genotyping

TPMT genotyping is available for all cases and 48 controls; in 3 control patients, genotyping could not be performed due to technical reasons. All polymorphisms considered were respecting Hardy-Weinberg equilibrium and their distribution is comparable to what has been reported in the literature for patients of Caucasian ethnicity. Frequency of TPMT variant genotypes was similar among cases and controls: in particular, 1 early onset patient (5.9%) and 2 non-early onset patients

(4.2%) presented the variant heterozygous genotype of rs1142345 (A719G) (p-value logistic regression = 0.77). Among these, the early onset patient and one non-early onset patient presented also the rs1800460 (G460A) variant in heterozygous form. No patient presented the rs1800462 (G238C) variant. Therefore, in terms of variant alleles, 2 patients (1 case and 1 control) were heterozygous for TPMT*3A, while 1 control was heterozygous for TPMT*3C. All other 62 patients with available DNA for genotyping were considered homozygous for the TPMT*1 allele.

Clinical efficacy in patients with early onset IBD in comparison to non-early onset patients

Clinical response was determined: frequency of patients in remission was not different among early onset and control group (respectively 82% and 84%, odds ratio early onset vs non-early onset = 0.76, 95% C.I. = 0.18 – 3.89, p-value logistic regression = 0.72).

Azathioprine doses and metabolites in patients with early onset IBD in comparison to non-early onset patients

Early onset patients required higher doses of azathioprine (median 2.7 vs 2.0 mg/kg/day, p-value ANOVA = 1.1×10^{-4}, Figure 1). This difference was present even considering separately patients with CD and UC (see Figure, Supplementary Digital Content 1). Different doses resulted in comparable azathioprine active TGN metabolites concentrations (median 263 vs 366 pmol /

$8x10^8$ erythrocytes, p-value ANOVA = 0.41, Figure 2) and MMPN concentrations (median 1455 vs 1532 pmol / $8x10^8$ erythrocytes, p-value ANOVA = 0.60, Figure 3). Lower TGN metabolites / azathioprine dose ratios were found in early onset patients (median 98 vs 184 pmol / $8x10^8$ erythrocytes/mg/kg/day, p-value ANOVA = 0.017, Figure 4). Treatment duration was not different between cases and controls (median 553, interquartile range (IQR) 250-670 vs 402, IQR 176-771.5 days, p-value ANOVA = 0.77). For all covariates besides azathioprine dose, considering CD and UC patients separately, the trends were similar to those described in the grouped pediatric IBD patients, even if no significant difference can be calculated, likely because of the reduction in population size in the separated groups (data not shown).

TPMT activity in patients with early onset IBD in comparison to non-early onset IBD patients

TPMT activity could be measured in a subset of patients for which sufficient blood sample was available after the quantification of thiopurine metabolites, in particular 9 early onset and 27 non-early onset IBD patients. This population was representative of the whole group since no demographic variable was different between this subgroup of patients and the whole study population in terms of the demographic, clinical and pharmacological variables. Interestingly, early onset patients presented higher activity of TPMT (Figure 5, median 476 vs 350 nmol of methyl-mercaptopurine/mg hemoglobin/h, p-value ANOVA = 0.046).

Additional univariate analysis on demographic, clinical and pharmacogenetic covariates and azathioprine pharmacokinetics

Type of IBD showed a fully significant effect on the median TGN or TGN/dose ratio in our population (respectively median 284 pmol / $8x10^8$ erythrocytes in Crohn's patients vs 372 pmol / $8x10^8$ erythrocytes in UC patients for TGN, p-value ANOVA = 0.015, and 140 pmol / $8x10^8$ erythrocytes/mg/kg/day in Crohn's patients vs 185 pmol / $8x10^8$ erythrocytes/mg/kg/day in UC patients for TGN/dose ratio, p-value ANOVA = 0.021); therefore an increased concentration of active thiopurine metabolites in UC patients compared to CD was evident, as previously reported(9). Gender did not show any significant association with azathioprine doses, metabolites or TPMT activity in this population. TPMT variant genotype was significantly associated in a univariate analysis with a reduction in MMPN concentration (1610 pmol / $8x10^8$ erythrocytes in TPMT wild-type vs 261 pmol / $8x10^8$ erythrocytes in TPMT variants, p-value ANOVA = 0.017) and an increase in TGN/dose ratio (159 pmol / $8x10^8$ erythrocytes/mg/kg/day in TPMT wild-type vs 291 pmol / $8x10^8$ erythrocytes/mg/kg/day in TPMT variants, p-value ANOVA = 0.0055) as well established.

Multivariate analysis evaluating independence of the effects of the genotypes on azathioprine metabolites concentrations or dose

For azathioprine dose, TGN concentrations, MMPN concentrations and TPMT activity, no multivariate analysis was done, since only one covariate had a fully significant effect in a univariate analysis (see above). For TGN/dose ratio, the

multivariate generalized linear model showed that early onset IBD, UC type of IBD and variant TPMT genotype are independent determinants of increased concentration of TGN metabolites per unit of azathioprine administered. Detailed results of this multivariate analyses are reported in Table 2.

DISCUSSION

In this study, performed on children with IBD treated at 6 Italian tertiary referral centers, we demonstrate for the first time that early onset IBD patients (age <6 years old) have profound differences in azathioprine pharmacokinetics in comparison to non-early onset patients (age between 12 and 18 years old): in particular, the ratio of active TGN metabolites per unit of daily azathioprine dose is significantly reduced in early onset patients. This could explain the much higher doses required by early onset patients, in order to achieve similar efficacy. Indeed, the larger doses administered resulted in similar concentration of active metabolites in early onset and older child with IBD. Interestingly, early onset IBD was associated with increased activity of TPMT, the main enzyme involved in azathioprine biotransformation, further supporting the hypothesis that early onset IBD patients have increased inactivation of thiopurines and therefore may be less sensitive to the drug than non-early onset patients.

Azathioprine is frequently used in IBD and is important to maintain remission; however, a significant proportion of patients does not respond to therapy or develops important adverse events(16, 17). The causes for this inter-individual variability in response to azathioprine are not completely understood; however, a

consistent amount of evidence relates variability in azathioprine response to inter-individual differences in the metabolism of the medication, that are due, at least in part, to genetic polymorphisms of relevant enzymes(9), such as TPMT and NUDT15, which is particularly important in Asian patients (18).

It has been previously described that the activity of one of the most relevant enzymes involved in azathioprine biotransformation, TPMT, is elevated in newborns, in comparison to children(19), and in younger children in comparison to older ones and adults(20). The observation of higher TPMT activity in younger children has not been supported by all published studies(21), however most of the studies not describing a relevant age-effect on TPMT were focused on adult patients and spanning all age groups, with the addition of potential confounders(22). Many studies in children on azathioprine pharmacokinetics, especially those in IBD patients, are still underpowered(23), therefore this study is the first one to clearly present the differences in azathioprine pharmacokinetics of early onset IBD patients. However studies performed in larger populations of patients with acute lymphoblastic leukemia also support lack of efficacy of thiopurines in younger patients, due to reduced concentration of active TGN metabolites and potentially increased TPMT activity(24). Indeed, a recent large study on TPMT activity in children with leukemia confirmed that younger age is associated with increased TPMT activity(25).

Our current study is non interventional, therefore the potential clinical application of its findings are limited; however, the practice of administering high doses of thiopurines in early onset IBD, to improve therapeutic efficacy, is common and

should be explored by properly designed randomized controlled trials in order to define strategies to further improve therapy with thiopurines in this age group. One may assume that an integrated approach including non-genetic (e.g. developmental) and genetic factors may improve our understanding of azathioprine response(26). On these bases, there is a strong clinical recommendation to elucidate the impact of higher azathioprine dosage (3.5 mg/kg/day) in pediatric patients with early onset IBD by a randomized prospective "dose finding" trial to develop an age-specific formulation for a chronic highly debilitating disease, especially for patients' with early onset. Liquid formulations for younger children may also be preferable and could be considered(27).

In this study, we do not provide evidence on the molecular mechanism by which TPMT is increased in early onset IBD. Epigenetic mechanisms, in particular methylation of gene promoters, have been proposed in ontogenesis of enzymes responsible for drug biotransformation(28), such as cytochromes(29). Innovative studies could be performed to test whether similar age-dependent epigenetic mechanisms occur also for TPMT promoter methylation(30). Moreover, besides increased TPMT activity, also other enzymes involved in azathioprine metabolism, such as glutathione transferases, could be reduced in early onset patients and increased during development, affecting azathioprine pharmacokinetics and efficacy, and could be evaluated in further studies(9, 17). In addition to differences in biotransformation, other pharmacokinetic factors, influenced by age, may contribute to the requirement for higher azathioprine

doses described by our study, such as changes in absorption and bioavailability(5, 31). TPMT activity might have been affected by concomitant mesalazine treatment (32); however, frequency of patients in therapy with mesalazine was not significantly different between cases and controls. Azathioprine therapy itself could have influenced TPMT activity: since cases were on higher doses of azathioprine, TPMT activity might be raised because of that (33); however, induction of TPMT activity by thiopurine treatment in patients with IBD is controversial (34). Finally, our early onset population did not include patients with very-early onset disease (less than 1 year old): studies focusing in this important although rare patients' population could be considered (2). One strength of this study is that all laboratory tests for azathioprine metabolites, TPMT activity, and TPMT genotype were done at the same centralized laboratory (35).

In conclusion, this study demonstrates that early onset IBD patients present an increased inactivating azathioprine metabolism, possibly because of elevated activity of the enzyme TPMT.

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TABLES

Table 1: demographic and clinical characteristic of patients enrolled					
		All patients (n = 68)	Age group		
			early onset (age < 6 years) (n = 17)	non-early onset (age >12 - <18 years) (n = 51)	p-value*
Age (years) at time of sample collection**		14.7, 2.7 – 18.0	5.1, 2.7-5.8	15.7, 12.0-18.0	-
Gender	Female (%)	28 (43)	8 (47)	21 (41)	0.67
	Male (%)	39 (57)	9 (53)	30 (59)	
Type of IBD	Crohn's disease (%)	16 (23)	4 (23)	12 (23)	1
	Ulcerative colitis (%)	52 (76)	13 (76)	39 (76)	

*: p-values are from generalized linear models; **: for continuous variables, median and ranges are reported.

Table 2: multivariate analysis for covariates with a significant effect in the univariate analysis				
Azathioprine related pharmacological phenotype (dependent variable)	Independent variable in multivariate generalized linear model	Comparison	Effect*	p-value**
Ratio TGN/dose	Age of IBD onset	Early vs Later	1.1	0.013
	TPMT Variant genotype	Heterozygous vs wild-type	2.5	0.0074
	IBD type	ulcerative colitis vs Crohn's disease	0.89	0.047

*: The effect size represents the increase (positive value) or decrease (negative value) in the value of the dependent variable for each independent variable listed; **: p-values are from a generalized linear model.

FIGURE LEGENDS

Figure 1: the boxplots show stable azathioprine doses (mg/kg/day) in patients with early onset IBD (age < 6 years old) or non-early onset IBD (age > 12 years and < 18 years old) obtained after at least three months of therapy; empty and solid points display azathioprine dose values for patients with wild-type and variant TPMT respectively; p-value is from ANOVA.

Figure 2: the boxplots show TGN concentrations (pmol/8x10^8 erythrocytes) in patients with early onset IBD (age < 6 years old) or non-early onset IBD (age > 12 years and < 18 years old); empty and solid points display TGN concentration values for patients with wild-type and variant TPMT respectively; p-value is from ANOVA. TGN concentrations values were log-transformed to adjust normality.

Figure 3: the boxplots show MMPN concentrations (pmol/8x10^8 erythrocytes) in patients with early onset IBD (age < 6 years old) or non-early onset IBD (age > 12 years and < 18 years old); empty and solid points display MMPN concentration values for patients with wild-type and variant TPMT respectively; p-value is from ANOVA. MMPN concentration values were transformed according to Box-Cox method (final exponent 0.1) to adjust normality.

Figure 4: the boxplots show the ratio between TGN concentrations and azathioprine doses (pmol/8x10^8 erythrocytes mg/kg/day) in patients with early onset IBD (age < 6 years old) or non-early onset IBD (age > 12 years and < 18

years old); empty and solid points display MMPN concentration values for patients with wild-type and variant TPMT respectively; p-value is from ANOVA. TGN/azathioprine dose ratios were transformed according to Box-Cox method (final exponent 0.4) to adjust normality.

Figure 5: the boxplots show TPMT activity values (nmol of methylmercaptopurine produced by patients' erythrocytes lysates containing 1 mg of hemoglobin during 1 h of incubation at 37 °C in the presence of mercaptopurine) in patients with early onset IBD (age < 6 years old) or non-early onset IBD (age > 12 years and < 18 years old); empty and solid points display TPMT activity values for patients with wild-type and variant TPMT respectively; p-value is from ANOVA.

Supplementary Digital Content 1.docx

Figure 1

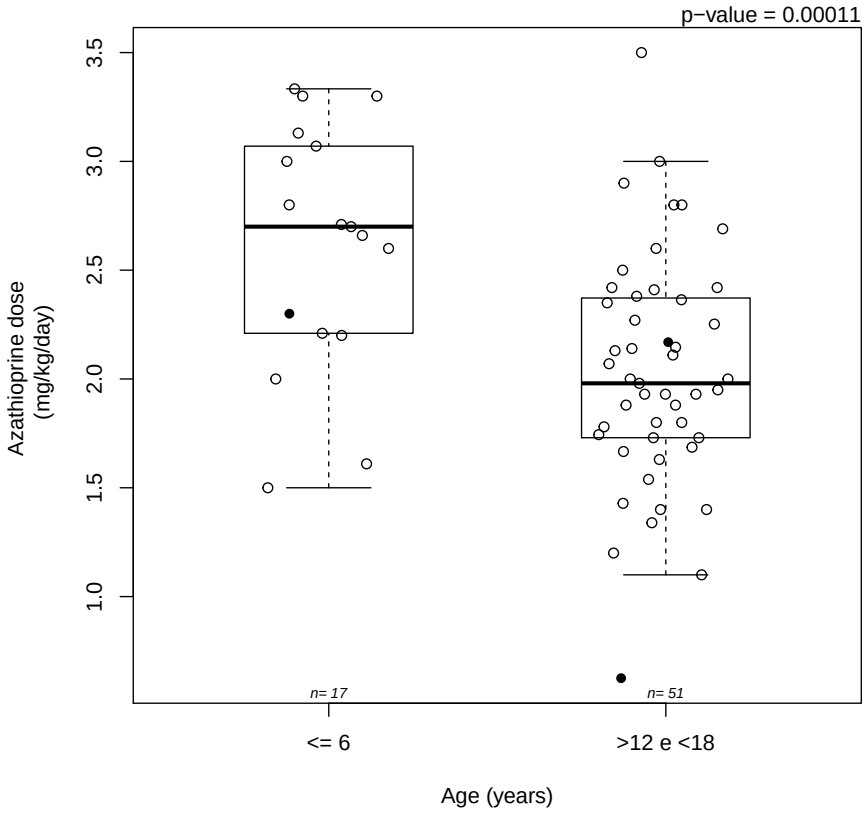

p−value = 0.00011
Azathioprine dose
(mg/kg/day)
1.0
1.5
2.0
2.5
3.0
3.5
n= 17
n= 51
<= 6
>12 e <18
Age (years)

Figure 2

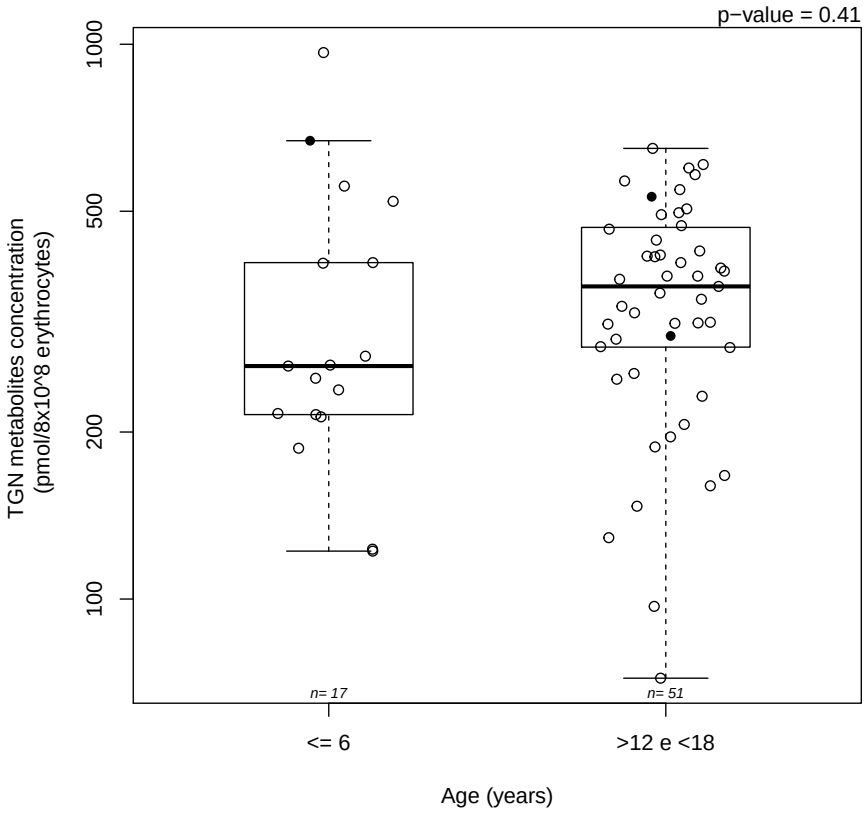

p−value = 0.41
TGN metabolites concentration
(pmol/8x10^8 erythrocytes)
100
200
500
1000
n= 17
n= 51
<= 6
>12 e <18
Age (years)

Figure 3

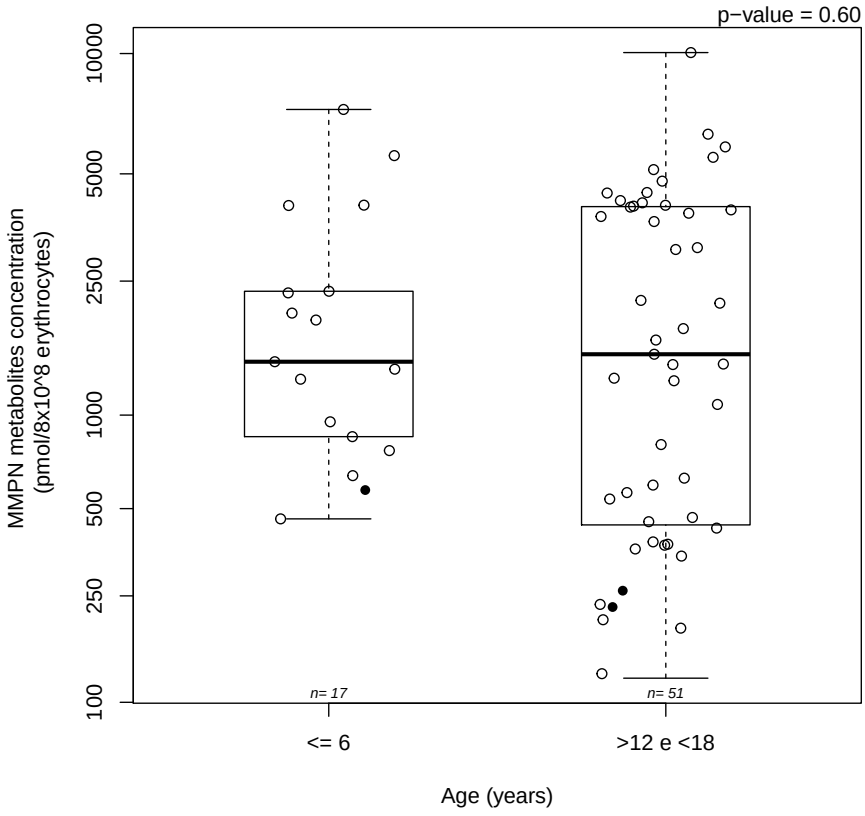

p−value = 0.60
MMPN metabolites concentration
(pmol/8x10^8 erythrocytes)
100
250
500
1000
2500
5000
10000
n= 17
n= 51
<= 6
>12 e <18
Age (years)

Figure 4

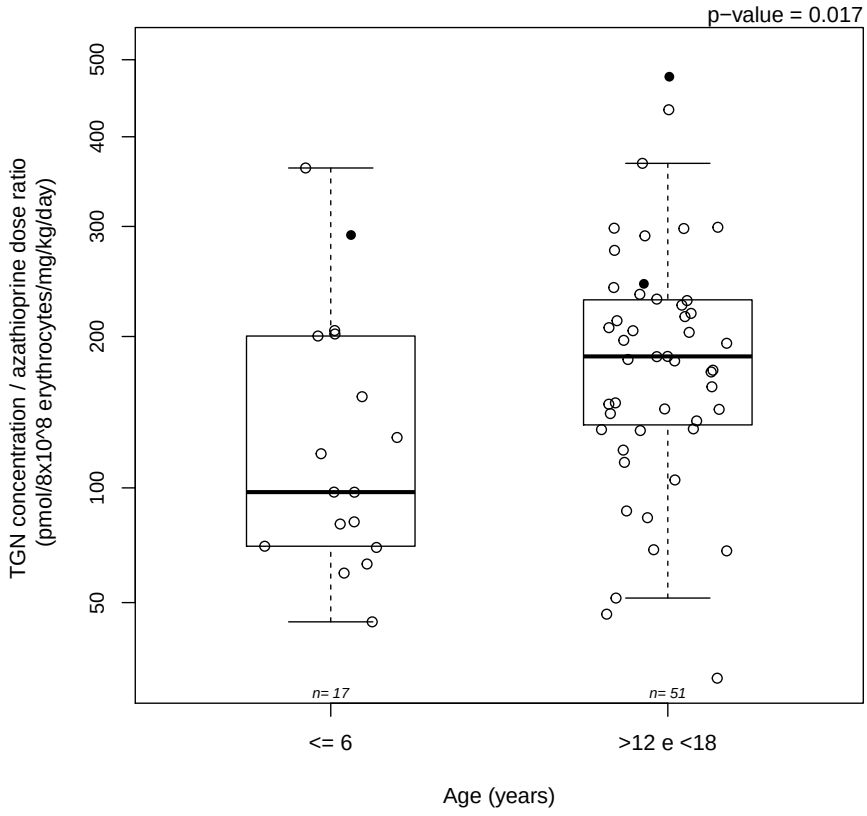
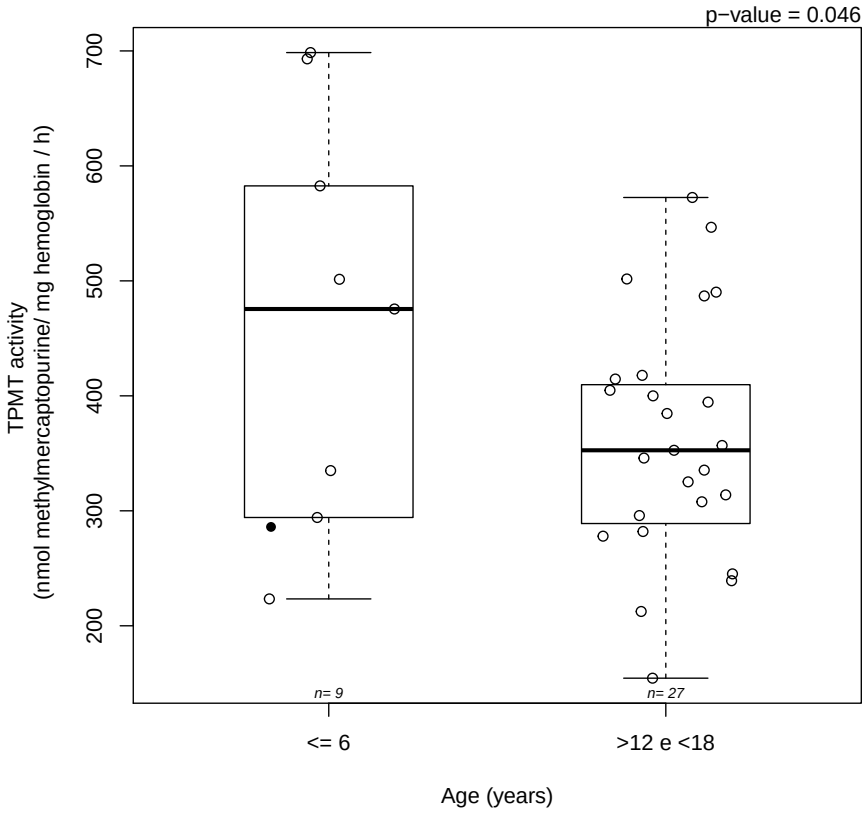

p−value = 0.017
TGN concentration / azathioprine dose ratio
(pmol/8x10^8 erythrocytes/mg/kg/day)
50
100
200
300
400
500
n= 17
n= 51
<= 6
>12 e <18
Age (years)

Figure 5


p−value = 0.046
TPMT activity
(nmol methylmercaptopurine/ mg hemoglobin / h)
200
300
400
500
600
700
n= 9
n= 27
<= 6
>12 e <18
Age (years)