Biomedicine & Pharmacotherapy 157 (2023) 113901

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

DNA methylation of the TPMT gene and azathioprine pharmacokinetics in children with very early onset inflammatory bowel disease

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ARTICLE INFO

Keywords:
Azathioprine
DNA methylation
TPMT
Very early onset inflammatory bowel disease

ABSTRACT

Background: Thiopurine methyltransferase (TPMT) is a crucial enzyme for azathioprine biotransformation and its activity is higher in very early onset inflammatory bowel disease (VEO-IBD) patients than in adolescents with IBD (aIBD).

Aims: The aims of this pharmacoepigenetic study were to evaluate differences in peripheral blood DNA methylation of the TPMT gene and in azathioprine pharmacokinetics in patients with VEO-IBD compared to aIBD.

Methods: The association of age with whole genome DNA methylation profile was evaluated in a pilot group of patients and confirmed by a meta-analysis on 3 cohorts of patients available on the public functional genomics data repository. Effects of candidate CpG sites in the TPMT gene were validated in a larger cohort using pyro-sequencing. TPMT activity and azathioprine metabolites (TGN) were measured in patients' erythrocytes by HPLC and associated with patients' age group and TPMT DNA methylation.

Results: Whole genome DNA methylation pilot analysis, combined with the meta-analysis revealed cg22736354, located on TPMT downstream neighboring region, as the only statistically significant CpG whose methylation

Abbreviations: ANOVA, analisys of variance; CD, Crohn's disease; CpG, cytosine-phosphate-guanine; FDR, false discovery rate; HPLC, high performance liquid chromathography; IBD, inflammatory bowel disease; IQR, interquartile range; MMPN, methylmercaptopurine nucleotide; SNP, single nucleotide polymorphism; TGN, thioguanine nucleotide; TPMT, thiopurine methyltransferase; UC, ulcerative colitis; VEO-IBD, very early onset inflammatory bowel disease; aIBD, adolescents with inflammatory bowel disease.

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https://doi.org/10.1016/j.biopha.2022.113901
Received 20 June 2022; Received in revised form 12 October 2022; Accepted 17 October 2022
Available online 30 November 2022

increases with age, resulting lower in VEO-IBD patients compared to aIBD (median 9.6% vs 12%, p = 0.029). Pyrosequencing confirmed lower cg22736354 methylation in VEO-IBD patients (median 4.0% vs 6.0%, p = 4.6 $\times 10^{-5}$). No differences in TPMT promoter methylation were found. Reduced cg22736354 methylation was associated with lower TGN concentrations (rho = 0.31, p = 0.01) in patients with VEO-IBD and aIBD.
Conclusion: Methylation of cg22736354 in TPMT gene neighborhood is lower in patients with VEO-IBD and is associated with reduced azathioprine inactivation and increased TGN concentrations.

1. Introduction

Inflammatory bowel diseases (IBDs), encompassing Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the gastrointestinal tract [1]. IBD can occur at any age, and in about 20% of cases have an onset during childhood, with recent evidence suggesting that the incidence of pediatric IBD is increasing [2]. Patients diagnosed before the age of 6 years are defined as very early onset IBD (VEO-IBD) and have some specific features such as a strong genetic predisposition, a predominant colonic involvement, and a higher risk for a more aggressive course with an adverse effect on growth, development, pubertal maturation and bone health [3–5]. Moreover, it has been shown that children with VEO-IBD may have therapeutic peculiarities with different responses to drugs compared to older children [3].

Azathioprine is an established treatment for IBD and commonly used to maintain remission of IBD [6]. Azathioprine is a pro-drug and needs to be activated through a complex pathway of enzymatic reactions into thioguanine nucleotides (TGNs). Thiopurine-methyltransferase (TPMT) plays an important role in the biotransformation of azathioprine because it catalyzes the S-methylation of mercaptopurine, converting it into an inactive form. The TPMT gene is highly polymorphic, and the most relevant variant alleles are coding nonsynonymous single-nucleotide polymorphisms (SNPs) [7]. Recently, age has been demonstrated to affect TPMT activity. Indeed, patients with VEO-IBD had increased TPMT activity and needed a higher pro-kg dose of azathioprine to reach a therapeutic level of TGNs compared to adolescents with IBD [8]. It has been reported that epigenetic modifications, mostly DNA-methylation, may determine the interindividual variability in drug response [9]. Moreover, chronological age induces remarkable changes in genome-wide DNA methylation levels [10–13] and sets of cytosine-phosphate-guanine (CpG) dinucleotide statistically associated with chronological age, called "epigenetic clock", have been identified [14].

Considering this evidence, the hypothesis of an age-dependent epigenetic regulation of the TPMT gene is highly possible.

The primary aim of the study was to evaluate the differences in the peripheral blood DNA methylation of the TPMT gene and its neighboring regions in a cohort of VEO-IBD patients compared to adolescent IBD patients. The secondary aim was to validate the differences in azathioprine pharmacokinetics and TPMT gene expression in VEO-IBD patients compared to adolescent IBD patients and evaluate the association between DNA methylation and pharmacokinetic variables.

2. Methods

2.1. Patients

A multicentric case-control observational study was performed, with patients enrolled between January 2018 and June 2021. Written and signed informed consent was obtained from the parents or guardians of the patients to join the study. The study protocol was approved by the Ethics Commitee ("CEUR-2018-Os-002-Burlo"). Patients diagnosed with IBD, according to clinical, radiological, endoscopic findings as suggested by the Porto criteria [15] and with ongoing treatment with azathioprine, were considered eligible. IBD was diagnosed according to the Porto criteria and classified according to the Paris Classification [16]. Patients with VEO-IBD younger than 6 years at enrollment were considered cases, while adolescent patients with IBD aged 12–18 years were considered as controls. Immunogenic or genetic testing were performed in order to exclude monogenic causes of VEO-IBD. Adolescent patients with IBD were paired by the type of IBD in the ratio of 1:3. Azathioprine dose was at the discretion of the treating physician according to the international guidelines and to the clinical status of the patient. Other cotreatments, such as aminosalicylates, steroids, or enteral nutrition, were allowed. The exclusion criteria included: concomitant therapy with anti-tumor necrosis factor biological agents, colostomy, fulminant colitis, and the presence of any of the following conditions: infections (e.g., HIV), tumors, organ transplant, kidney, liver, hematological, endocrine, cardiac, neurological or cerebral diseases.

Samples with TGN concentration under 50 pmol/8 $\times$ 10^8 erythrocytes were excluded as a signal of inadequate compliance to therapy. Samples with azathioprine duration under 90 days were excluded in order to have azathioprine active nucleotides at the steady state concentration in patients' blood. Blood samples, collected for measuring azathioprine metabolites, TPMT activity, TPMT gene expression, TPMT DNA methylation, and genotyping, were taken at the first visit after at least three months of therapy.

2.2. DNA extraction

Genomic DNA was extracted from IBD patients' peripheral blood using a commercial kit (SIGMA, Milan, Italy) and was used for the genotyping and the epigenetic DNA methylation analysis, representing DNA from peripheral blood mononuclear cells/leukocytes [17].

2.3. Genotypes

Genotype of the most relevant SNPs in the candidate gene TPMT (rs1800462, rs1800460, and rs1142345) was assessed using TaqMan assays (Thermoscientific, Milan, Italy). Samples' genotyping was repeated twice to confirm the results.

2.4. DNA bisulfite conversion for methylation analysis

Genomic DNA obtained from whole blood of patients was treated with sodium bisulfite using the EZ 96-DNA methylation kit (Zymo Research, Irvine, CA, USA), following the manufacturer's standard protocol to determine the pattern of methylation. In particular, bisulfite-treated DNA not-methylated cytosine residues are converted to uracil, while 5-methylcytosine residues remain unchanged.

2.5. Illumina methylation EPIC bead-chip array

Genome-wide DNA methylation profiles were obtained using an Illumina Methylation EPIC BeadChip array kit. All experimental methods were performed according to the manufacturer's protocols (Illumina). The IDAT file, a raw data file containing the intensities of the probes in the array, was analyzed using the ChAMP package of R/Bioconductor [18]. The probes for which detection p-values were < 0.01 across all samples were used. Average beta values were used for comparisons. Statistical analysis of the methylation profiles was then focused on CpGs located in the TPMT gene, promoter and neighborhood ($\pm$ 50,000 bp upstream/downstream TPMT gene), assuming that a regulatory element may influence expression of a target gene within 50,

000 bp [19,20], for a total of 32 CpGs (Supplementary Table 1).

2.6. GEO cohorts for meta-analysis

In order to obtain further evidence on DNA methylation differences in the TPMT gene promoter and neighborhood according to patients' age, already available datasets were identified on the public functional genomics data repository Gene Expression Omnibus (GEO) and analyzed. All the available studies on DNA methylation in pediatric IBD (measured before the initiation of any therapy) and on healthy children were identified and selected for analysis. In particular, the GSE112611 DNA methylation microarray dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112611) includes quantile-normalized beta values for DNA methylation data from peripheral blood mononuclear cells of 12 VEO-IBD patients and 113 adolescent IBD patients, together with 52 age-matched healthy controls. All DNA samples of the CD patients' cohort (GSE112611) were taken before starting azathioprine therapy. On the other hand, GSE62219 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62219) includes quantile-normalized DNA methylation beta values, from peripheral blood mononuclear cells, of 10 healthy control children, all younger than five years. The GSE62219 dataset was built on Illumina HumanMethylation450 BeadChip while the GSE112611 dataset on Illumina Infinium Methylation EPIC Bead Chip. Patients' characteristics are summarized in Supplementary Table 2.

2.7. Pyrosequencing analysis for DNA methylation

To analyze the methylation of candidate CpG sites in the TPMT promoter (human genome assembly [hg] chr6:18,154,513 and chr6:18154820) and in those emerging as significant from the pilot analysis on CpG sites (cg22736354) located on TPMT downstream neighboring region, pyrosequencing assay was performed, using the PyroMark Q96 MD (Qiagen, Inc.; Germantown, MD). Methylation was then quantified with Pyro Q-CpG (version 1.0.9; Biotage, Inc.). Regarding the cg22736354 assay, due to the assay design that allows to sequence fragments up to 150 bases, other 3 CpG sites have been analyzed, 2 upstream CpGs (chr6:18,122,713 and chr6:18,122,715) and one downstream CpG (hg19 chr6:18,122,722) of cg22736354. Supplementary Table 3 provides the list of the primers and sequencing probes, specific for the bisulfite converted DNA sequence, used to analyze these sites. The percent methylation at each CpG site (beta) was used to measure DNA methylation.

2.8. Measurement of azathioprine metabolites

Azathioprine metabolites (TGN and methylmercaptopurine nucleotides, MMPN) were measured at the Advanced Translational Diagnostics Laboratory, IRCCS Burlo Garofolo Hospital, Trieste in IBD patients' erythrocytes using a high-performance liquid chromatography (HPLC) assay by Dervieux et al. [21] on an Agilent Technologies 1260 HPLC instrument. Blood samples were centrifuged to collect erythrocytes whom were stored at − 20 °C until analysis. Metabolites' concentration is expressed as pmol/ 8×10^8 erythrocytes. The ratio between TGN and the dose of azathioprine was calculated considering for each measurement the dose the patients were taking the day the blood sample for the metabolite's assessment was collected.

2.9. Measurement of TPMT activity

TPMT activity was measured at the Advanced Translational Diagnostics Laboratory, IRCCS Burlo Garofolo Hospital, Trieste in IBD patients' erythrocytes using an HPLC assay based on in vitro conversion of mercaptopurine to methylmercaptopurine, using S-adenosyl-methionine as the methyl donor [22] on an Agilent Technologies 1260 HPLC instrument. TPMT activity is expressed as pmol of methylmercaptopurine produced by 10^9 patients' erythrocytes during 1 h of incubation at 37 °C in the presence of mercaptopurine.

2.10. TPMT RNA expression in total blood

Whole blood of IBD patients was collected into PAXgene blood RNA tubes for immediate stabilization of intracellular RNA and stored at − 80 °C until the RNA extraction. PreAnalytix RNA isolation kit was used to extract total RNA according to the manufacture's protocol. Then, the RNA concentration and purity were evaluated spectrophotometrically (NanoDrop 2000, EuroClone®). The reverse transcription was performed using the High-Capacity RNA to-cDNA Kit (Applied Biosystem) with 200–1000 ng of total RNA per 20 μL of reaction containing 10 μL of 2 x RT Buffer, 1 μL of 20x RT Enzyme Mix as described above.

Expression levels of the TPMT gene (TaqMan® assay ID: Hs02786624_g1) were normalized using the GAPDH housekeeping gene (TaqMan® assay ID: Hs00909010_g1) and were reported as $2^{-\Delta Ct}$ [23]. The thermal cycler used was the CFX96 real-time system-C1000 (Bio--Rad Laboratories).

2.11. Statistical analysis

For all analyses, normality of the variables was tested by the Shapiro test, and log10, square root, or BoxCox transformation were applied if needed to adjust the normality of the distribution.

The association between pharmacological phenotypes of interest for normally distributed variables (i.e., azathioprine dose, TGN metabolites concentrations, MMPN metabolites concentrations, TGN/dose ratio, TPMT activity, and TPMT expression) and the considered covariates (i.e., demographic variables including age-group classification, IBD type, and TPMT genotypes) was evaluated in a univariate analysis using generalized linear models of appropriate family (Gaussian/analysis of variance [ANOVA] for continuous and logistic regression for categorical variables). The dependent variable was the pharmacological phenotype of interest, and the independent variables were the demographic, clinical, or pharmacogenetic covariates. For DNA methylation and azathioprine therapy duration, due to their non-normal distribution and the impossibility to normalize them, Wilcoxon test and Spearman correlation were used to test the associations.

Data available on GEO of 3 different cohorts (GSE112611, GSE112611, GSE62219) have been analyzed independently using Wilcoxon test or Spearman correlation, and then data have been pooled together for a meta-analysis using the weighted inverse Z method accounting for direction of effect but without weighing for sample size [24]. False discovery rate (FDR) was used to adjust for multiple comparisons.

Multivariate analysis was performed 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 significant covariates in the univariate analysis as the independent variables.

When sub-cohorts were used for analyses, a comparison with the whole cohort was made to check for differences in demographic and pharmacological variables.

According to data distribution, continuous data are presented as mean with standard deviation or medians with interquartile ranges (IQR); categorical data are presented as absolute numbers and percentages.

All p-values < 0.05 were considered statistically significant. All statistical analyses were conducted using the software R, version 4.1.0.

3. Results

3.1. Patients

Thirty VEO-IBD patients and 90 adolescent IBD patients were

enrolled (flowchart of patients selection, supplementary figure 1). Demographic and clinical characteristics are reported in Table 1. None of the patients with VEO-IBD had monogenic disease. No differences in gender and IBD type between VEO-IBD and adolescents were found. All sub-cohorts used for the different analyses were considered representative of the whole cohort because no relevant statistically significant differences emerged.

3.2. Azathioprine doses and metabolites

Azathioprine treatment duration was not different between VEO-IBD patients and adolescent IBD patients (median 388.5 days, IQR 238.8 – 661.2 versus 413.0 days, IQR 149.5 – 1045.5, p-value Wilcoxon test = 0.51, data not shown). VEO-IBD patients were treated with higher doses of azathioprine (median dose of 2.5 mg/kg/day, IQR 2.0–2.8) compared to adolescent IBD patients (median dose of 1.9 mg/kg/day IQR range 1.7–2.3) (p-value ANOVA: 0.0003, Fig. 1 A). TGN concentration was lower in VEO-IBD patients (median 256.5 pmol/8 $\times$ 10^8 erythrocytes, IQR 153.3–345.4) than in adolescent IBD patients (median 357.5 pmol/8 $\times$ 10^8 erythrocytes, IQR 268.6–459.0) (p-value ANOVA = 0.01, Fig. 1B). The multivariate generalized linear model showed that VEO-IBD and variant TPMT genotype were independent determinants of increased TGN plasma concentrations (p-value ANOVA: 0.008, estimate: 0.19; p-value ANOVA: 0.0005, estimate: −0.51, respectively). Furthermore, MMPN level was comparable between the two groups (median 1385.1 pmol/8 $\times$ 10^8 erythrocytes, IQR 657.5–2078.0, versus 1429.5 pmol/8 $\times$ 10^8 erythrocytes, IQR 522.2–4096.0, p-value ANOVA = 0.71, Fig. 1 C). Finally, lower TGN metabolites/azathioprine dose ratio was found in VEO-IBD patients (median 96.7 pmol/8 $\times$ 10^8 erythrocytes/(mg/kg/d), IQR 66.3–154.2) compared to adolescent IBD patients (median 179.6 pmol/8 $\times$ 10^8 erythrocytes/(mg/kg/d), IQR 134.6–234.4) (p-value ANOVA = 0.0005, Fig. 1D). The multivariate generalized linear model showed that VEO-IBD (p-value: 0.0003, estimate: 0.08) and variant TPMT genotype (p-value: 0.0006, estimate: −0.14) were independent determinants of TGN metabolites concentration per unit of azathioprine administered.

3.3. TPMT activity

TPMT activity was measured in a subset of 17 VEO-IBD and 55 adolescent IBD patients (patients' demographic, clinical, and pharmacological variables are reported in Supplementary Table 4). VEO-IBD patients presented higher TPMT activity than adolescent IBD patients (median 253.0 pmol of methylmercaptopurine/10^9 erythrocytes/h, IQR 178.2–309.9 versus 207.66 pmol of methylmercaptopurine/10^9 erythrocytes/h, IQR 175.71–244.56, p-value ANOVA = 0.046, Fig. 2). The multivariate generalized linear model showed that VEO-IBD (p-value: 0.035, estimate: −37.9) and variant TPMT genotype (p-value: 0.003, estimate: 109.5) were independent determinants of TPMT activity.

Table 1
Demographic and clinical characteristics of patients enrolled. Age at the time of enrolment and disease duration are expressed as mean ± standard deviation. p-values are from generalized linear models and logistic regression. Abbreviations: CD, Crohn disease; UC, ulcerative colitis; VEO-IBD, very early onset IBD patients.

	All patients	VEO-IBD	Adolescent IBD	p-value
	n = 120	n = 30	n = 90	
Age (years)	12.0 ± 4.8	4.4 ± 1.4	14.6 ± 1.9	< 0.01
Sex				
Female (%)	54 (45.0%)	14 (46.7%)	40 (44.4%)	0.19
Male (%)	66 (55.0%)	16 (53.3%)	50 (55.6%)	
IBD type				
CD (%)	35 (29.1%)	6 (20.0%)	29 (32.2%)	0.78
UC (%)	85 (70.9%)	24 (80.0%)	61 (67.8%)	
Disease duration (days)	1070.8 ± 900.0	674.8 ± 385.1	1202.8 ± 982.2	0.073

3.4. Illumina methylation EPIC BeadChip array pilot analysis

TPMT gene DNA methylation was initially analyzed in whole blood of a subgroup composed of 10 VEO-IBD and 8 adolescent IBD patients (patients' demographic, clinical, and pharmacological variables are reported in Supplementary Table 4). In this cohort, significant differences were found in azathioprine duration and median TGN levels (p-value Wilcoxon: 0.01 and 0.01, respectively).

Thirty-two CpG sites, available on the EPIC array, on TPMT gene (hg19 chr6:18128545–18155374) and its neighboring regions (± 50000 base pair of the TPMT gene), have been analyzed. Azathioprine duration was different between VEO-IBD patients and adolescent IBD patients (p-value = 0.003, Wilcoxon test), with VEO-IBD presenting the lower values. At the same time, TGN levels, azathioprine dose and TPMT activity showed no difference (data not shown). As reported in Table 2, five CpG sites resulted differentially methylated between VEO-IBD patients and adolescent IBD patients (p-value < 0.05, Wilcoxon test). One CpG was located on the TPMT gene body (cg0670276), while 3 were located upstream and 1 on the downstream neighboring regions.

3.5. Meta-analysis between our cohort and 3 other cohorts available on GEO

To reduce the potential bias of the different azathioprine duration observed in the sub-cohort for the analysis of the TPMT gene methylation by EPIC array, a metanalysis to compare DNA methylation and patients' age between our cohort and 3 other cohorts available on the GEO platform, was performed. The metanalysis showed 4 significant CpGs after false discovery rate (FDR) adjustment (Table 3). Two of the identified CpGs were significant also in our IBD cohort (cg22736354 and cg04231636). The direction of the methylation in relation to age was concordant in each cohort for all the CpGs identified. Interestingly, cg22736354 was the only CpG whose methylation increases with age and was selected for further validation in a larger cohort of pediatric IBD patients, while for cg04231636, DNA methylation decreases with age.

3.6. Validation of the DNA methylation of cg22736354 using pyrosequencing

Methylation analysis with pyrosequencing was performed in a subset of patients composed of 20 VEO-IBD patients and 48 adolescent IBD patients (patients' demographic, clinical, and pharmacological variables are reported in Supplementary Table 4). DNA extracted from peripheral blood was analyzed by pyrosequencing to quantify the methylation of cg22736354 (hg19 chr6:18,122,719). The methylation of cg22736354 was lower in VEO-IBD patients (median methylation 4.0%, IQR 3.8–4.2) compared to adolescent IBD patients (median methylation 6.0%, IQR 5.0–8.0) (p-value Wilcoxon: 4.6 $\times 10^{-5}$, Fig. 3). Other 3 CpG sites adjacent to cg22736354 have been analyzed in the same assay due to its technical design (i.e., pyrosequencing that allows the sequencing of 10–20 bp in a targeted region). In particular the methylation of 2 CpG sites upstream (hg19 chr6:18,122,713 and chr6:18,122,715) and 1 CpG site downstream (hg19 chr6:18,122,722) of cg22736354 have been analyzed. The methylation of all the 3 CpG resulted significantly lower in VEO-IBD patients compared to adolescent IBD patients, as reported in supplementary table 5 and Fig. 3.

3.7. Association between pharmacological variables and cg22736354 DNA methylation

The association between all the pharmacological variables (TGN, azathioprine dose, azathioprine duration and MMPN) and the methylation of cg22736354 was assessed in the same 20 VEO-IBD patients and 48 adolescent IBD patients using Spearman correlation, showing a statistically significant association between cg22736354 methylation and both TGN concentration and TGN metabolites/azathioprine dose ratio

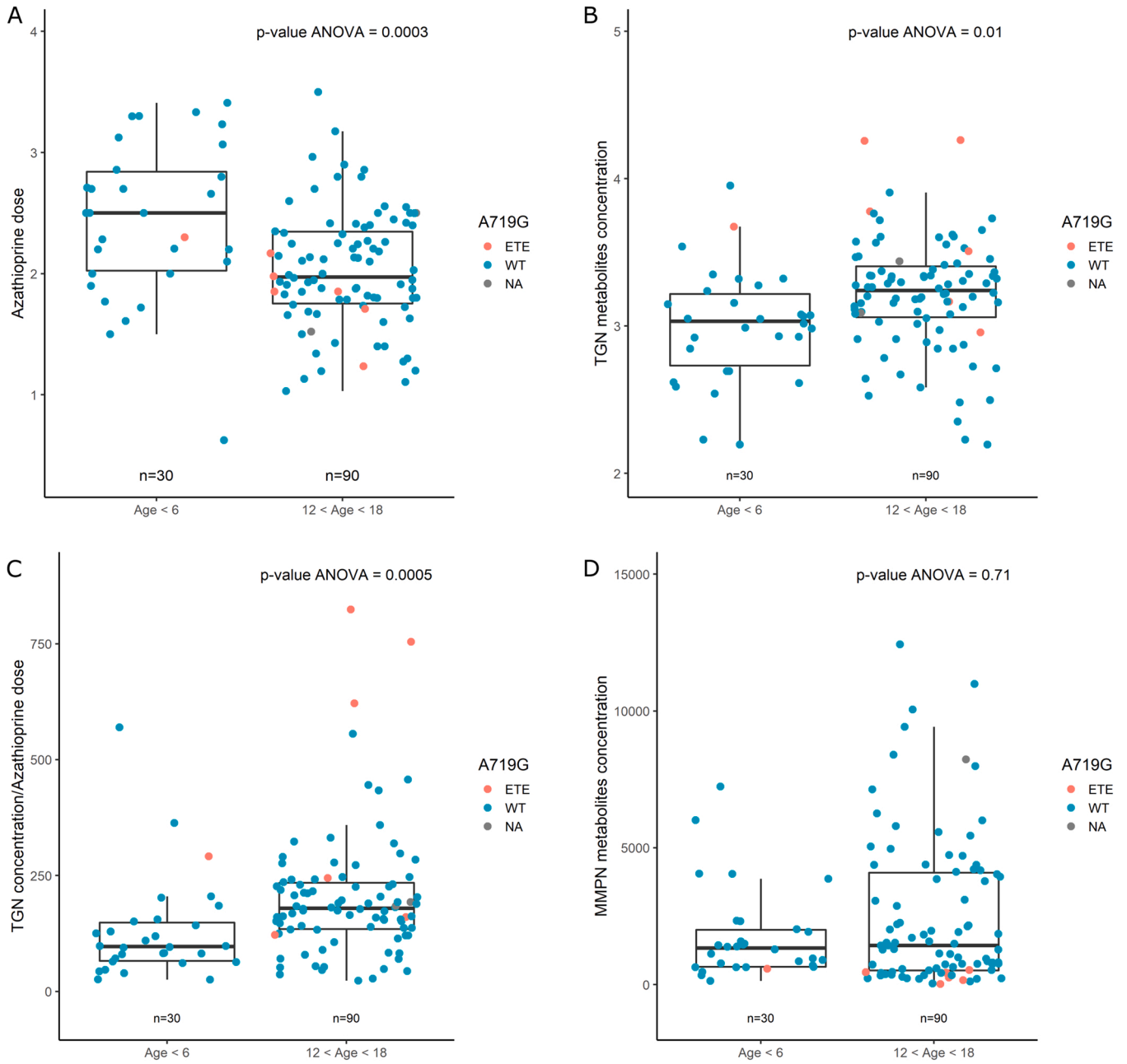

Fig. 1. (A) azathioprine doses (mg·kg^{-1}·d^{-1}), (B) TGN concentration (pmol/8 × 10^8 erythrocytes), (C) MMPN concentration and (pmol/8 × 10^8 erythrocytes) (D) TGN/Dose of azathioprine (pmol/8 × 10^8 erythrocytes mg^{-1}·kg·d^{-1}) in VEO-IBD patients (age < 6 years) compared to adolescent IBD patients (aged > 12 and <18 years), obtained after at least 3 months of therapy; blue and red points display values for patients with wild-type and variant TPMT for A719G respectively while black points refer to two patients for whom genotyping was not possible due to technical problems.

(p-value: 0.01 and 0.01 respectively) (Fig. 4) while no associations were found for azathioprine dose, azathioprine duration, MMPN levels (p-value: 0.47, 0.33 and 0.93 respectively, data not shown). For the correlation between TPMT activity and methylation of cg22736354 data was available for only 11 VEO patients and 31 adolescent IBD patients, showing no statistically significant association (p-value ANOVA: 0.67, data not shown).

3.8. DNA methylation of two CpG sites on TPMT promoter

Methylation analysis of 2 candidate CpG sites located on TPMT promoter was performed on the 20 VEO-IBD patients and 48 adolescent IBD patients previously described. No difference in the methylation of both chr6:18154813 and chr6:18154820 between VEO-IBD patients and adolescent IBD patients was found (supplementary table 6).

3.9. TPMT gene expression

TPMT gene expression was measured in a subset of 14 VEO-IBD patients and 24 adolescent IBD patients (patients' demographic, clinical, and pharmacological variables are reported in Supplementary Table 4). Median TPMT expression was not statistically significant between the two groups (p-value ANOVA = 0.47, Supplementary figure 2). Moreover, no association between TPMT expression and TPMT genotype was present (p-value: 0.84) and no correlation between TPMT expression and cg22736354 emerged in our analysis (p-value: 0.37).

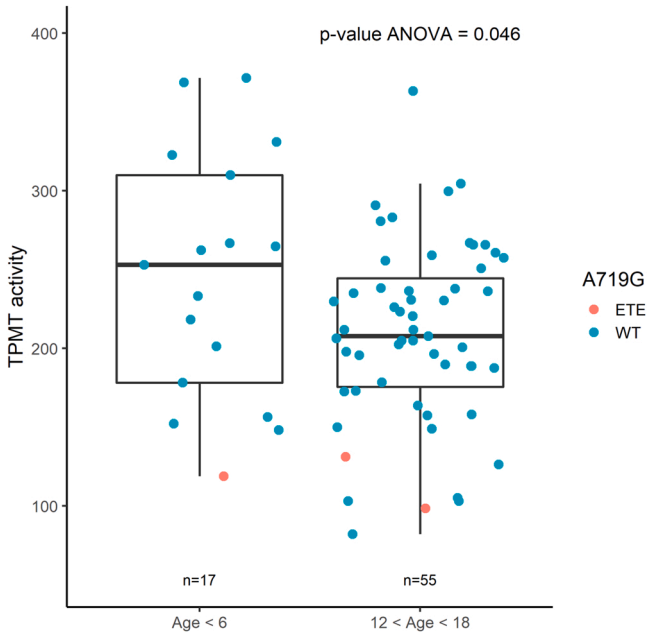

Fig. 2. TPMT activity (pmol/10^9 erythrocytes/h) in patients with VEO-IBD (aged < 6 years) and adolescent IBD patients (aged > 12 and <18 years); blue and red points display TPMT activity values for patients with wild-type and variant TPMT for A719G respectively.

4. Discussion

This is the first pharmacogenomic study demonstrating that epigenetic age-associated DNA methylation differences influence the pharmacokinetics of azathioprine in pediatric IBD patients.

It has been previously shown that VEO-IBD patients have peculiar azathioprine pharmacokinetics compared to adolescents. In particular, they present lower TGN plasma concentration normalized to azathioprine dose, likely because of an age-dependent increase in TPMT activity [8]. Indeed, the standard dose of thiopurines may not be adequate for IBD patients younger than 6 years and higher azathioprine dose is often required to achieve comparable TGN levels [25]. Moreover, elevated TPMT activity has been shown in newborns in comparison with children [26] and younger children in comparison with older ones and adults [27].

The molecular mechanism underlying the age-related progressive reduction in TPMT activity in IBD patients has not yet been identified.

The pilot analysis of the peripheral blood DNA methylation of candidate CpG positions in the TPMT gene showed that the methylation of cg22736354 was the only one that increased significantly with age in all the cohorts evaluated. This site, including 3 adjacent CpG sites, was used for subsequent validation in a larger cohort of pediatric IBD patients.

All the 4 CpGs resulted markedly demethylated in VEO-IBD patients, indicating an age-dependent regulation of all the regions including cg22736354. Moreover, the methylation of cg22736354 was significantly associated with TGN, and TGN/dose ratio in the subgroup analyzed.

Several studies have previously reported the age-dependent changes in DNA methylation of cg22736354. In particular, this specific CpG site was found to overlap both in Hannum [28] and Horvath [14] epigenetic clocks, which use predictable changes in the DNA methylation of several CpG sites across the genome, to estimate chronological age with elevated accuracy [29]. Hannum et al. used DNA methylation profiles from whole blood to identify 71 CpG sites that correlate with chronological age. In comparison, Horvath et al. used data from 51 different tissues from multiple studies to identify 353 CpG sites whose methylation levels can be combined to form an age predictor. In both clocks, the methylation of cg22736354 positively correlated with chronological age. Moreover, other studies identified the methylation of cg22736354 as an accurate age predictor, with methylation that increases with age [30,31] even though no studies are available to our knowledge about the biological role of cg22736354 methylation changes.

Interestingly, the cytosine cg22736354 emerged as the only CpG site in cis-acting methylation quantitative trait loci with rs76244256 (located on TPMT body) and rs7744541 (located on TPMT upstream neighboring region) that intersect with both the Hannum and Horvath clocks [32], indicating a possible influence of these 2 SNPs in regulating cg22736354 methylation.

Besides, the TPMT promoter structure has been very well characterized in the past [33], and several studies reported a tight regulation of TPMT expression and activity according to variable number tandem repeats located on its promoter [34,35], indicating the importance of TPMT promoter in regulating the gene expression. To our knowledge, no studies are available about the role of TPMT promoter methylation in regulating its expression or activity. In this study, the methylation of 2 CpG sites located on TPMT promoter resulted very low and almost identical between VEO-IBD and older pediatric patients with IBD, supporting the hypothesis of a more substantial impact of cg22736354 methylation in regulating azathioprine pharmacokinetics in young

Table 2
Statistically significant CpG sites in the pilot study cohort. Abbreviations: Chr6, chromosome 6; VEO-IBD, very early onset IBD patients.

Probe ID	Gene name	Position on Chr6	% of methylation in VEO-IBD	% of methylation in adolescent IBD	p-value Wilcoxon
cg22736354	NHLRC1	18122719	0.088 ± 0.02	0.124 ± 0.03	0.0266
cg18068140	NHLRC1	18123164	0.69 ± 0.04	0.65 ± 0.04	0.0434
cg01869138	NHLRC1	18123241	0.83 ± 0.03	0.78 ± 0.05	0.0205
cg06702726	TPMT	18152154	0.98 ± 0.00	0.96 ± 0.01	0.0342
cg04231636	KDM1B	18186411	0.36 ± 0.02	0.33 ± 0.03	0.0342

Table 3
Metanalysis results. Only statistically significant CpG sites are reported. Abbreviations: Chr6, chromosome 6; VEO-IBD, very early onset IBD patients.

ProbeID	Gene Name	Position on Chr6	% of methylation in VEO-IBD	% of methylation in adolescent IBD	Meta p-value	FDR
cg22736354	NHLRC1	18122719	0.096 ± 0.01	0.12 ± 0.02	0.0037	0.029
cg16879574	NHLRC1	18123047	0.17 ± 0.03	0.13 ± 0.01	1.01×10^{-6}	3.13×10^{-5}
cg0072000	NHLRC1	18123049	0.10 ± 0.02	0.07 ± 0.02	0.0019	0.0196
cg18068140	NHLRC1	18123164	0.68 ± 0.06	0.63 ± 0.06	0.0110	0.056
cg01869138	NHLRC1	18123241	0.83 ± 0.03	0.78 ± 0.04	0.0340	0.153
cg08448780	TPMT	18148790	0.83 ± 0.05	0.80 ± 0.05	0.0105	0.056
cg04231636	KDM1B	18186411	0.38 ± 0.05	0.33 ± 0.06	0.0001	0.002

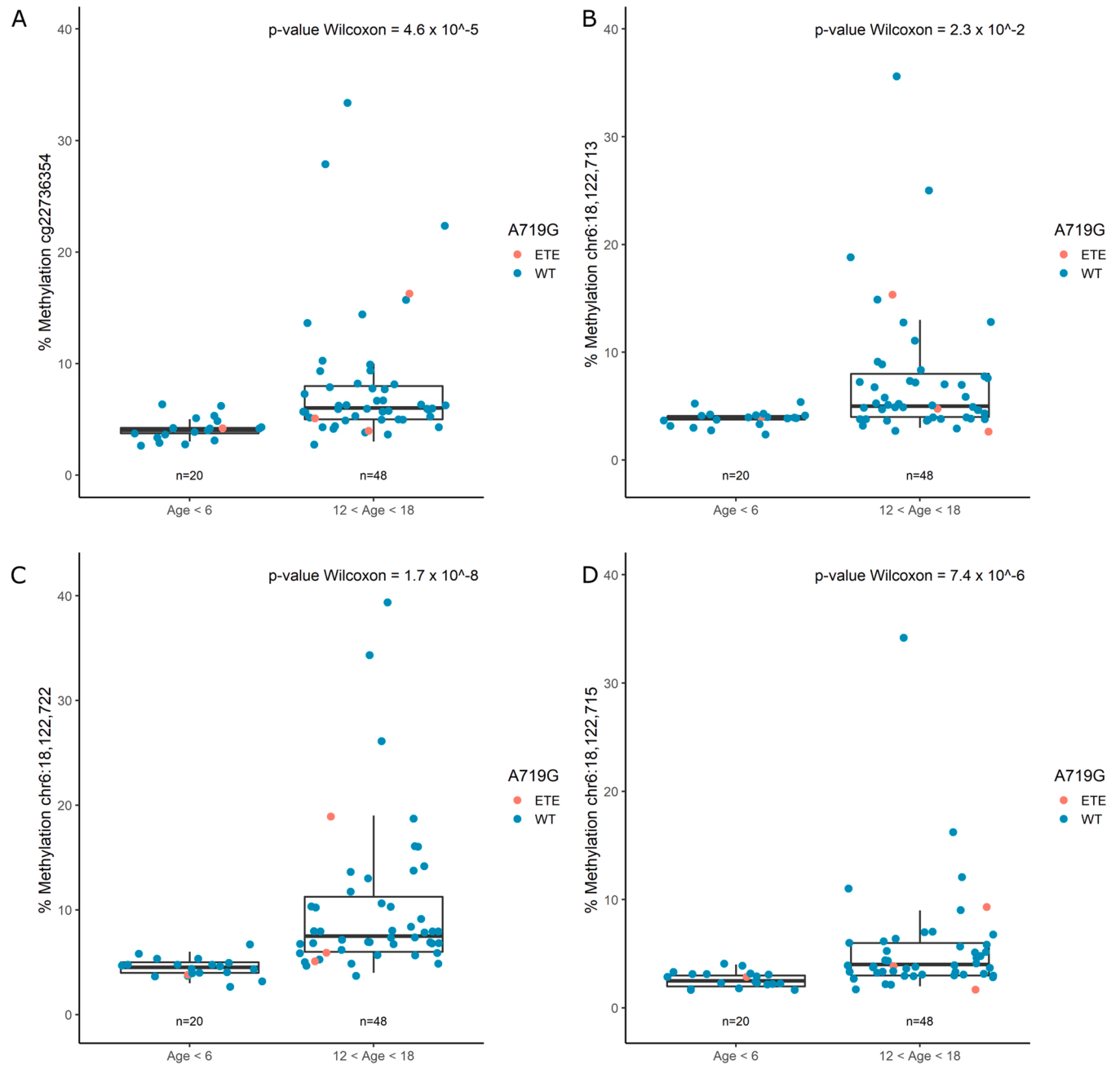

Fig. 3. cg22736354 (A), chr6:18,122,713 (B), chr6:18,122,715 (C) and chr6:18,122,722 (D) methylation (%) in patients with VEO-IBD (aged < 6 years) and in adolescent IBD patients (aged > 12 and <18 years); blue and red points display methylation values for patients with wild-type and variant TPMT for A719G respectively.

patients with IBD.

It has previously been reported that the methylation of the neighboring regions of a gene can alter the expression of the gene itself [36] due to altered recruitment and binding capacity of methylation sensible transcription factors such as sp1 [37], known to be an essential transcription factor also for TPMT [38]. Another example of a positive correlation between DNA methylation in the far upstream neighboring region and gene expression has been described by Rauscher et al. for EN1 for PITX2, APC and LHX2 breast cancer-associated genes [39].

In this study, we identified also a statistically significant position (cg04231636) which methylation was higher in VEO-IBD compared to adolescent IBD patients. The functional consequences of this difference are unclear: it is possible that this is a site of hydroxymethylation, an epigenetic modification known to increase gene expression, but further studies are needed to validate this hypothesis [40].

The main limit of this study is the lack of an in vitro model able to recapitulate the molecular mechanisms by which small differences in cg22736354 methylation could alter TPMT expression and activity. It has been already reported that the association of DNA methylation with an observed phenotype can occur also through methylation levels in the range of 1–5%, at single CpGs or over short genomic regions [41,42]. Moreover, DNA methylation differences in the range between 0.1% and 10% have been associated also with differences in drug response [43,44]. Another limit is that some pharmacological variables could not be measured in all the patients enrolled because of the reduced availability of blood samples especially in VEO-IBD patients. However, the subcohorts used for the different analyses were representative of the whole cohort, not influencing the generalizability of the results. Moreover, the

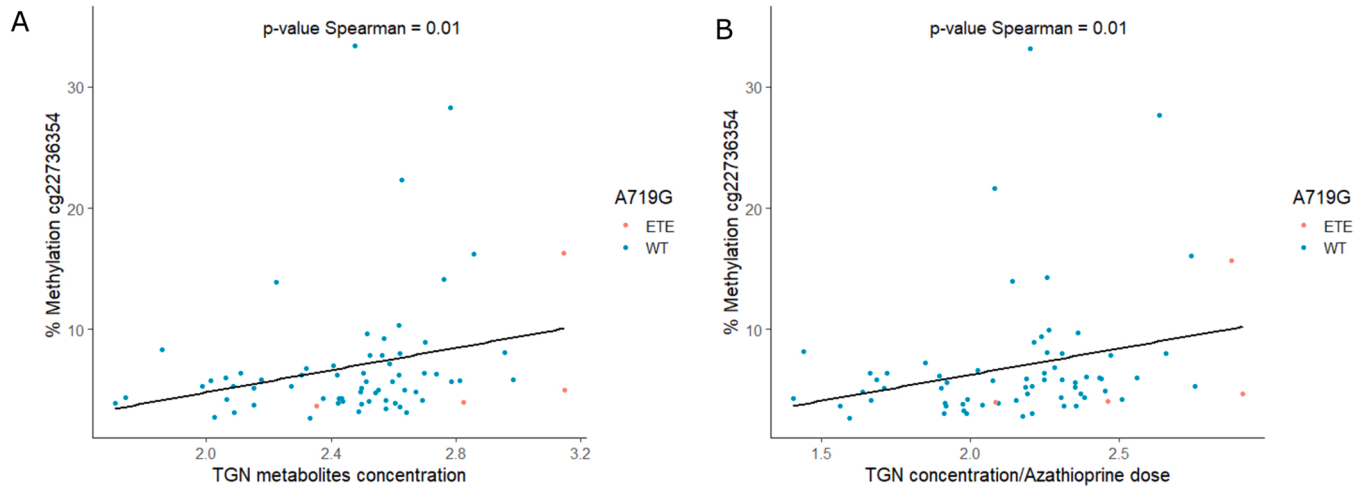

Fig. 4. Correlation between cg22736354 methylation (%) and Log TGN concentration (pmol/8 × 10^8 erythrocytes) (A) and Log TGN/Dose ratio (pmol/8 × 10^8 erythrocytes $mg^{-1}{\cdot}kg{\cdot}d^{-1}$) (B) respectively in patients with VEO-IBD (aged < 6 years) and in adolescent IBD patients (aged > 12 and <18 years); blue and red points display methylation values for patients with wild-type and variant TPMT for A719G respectively.

small number of patients in the gene expression cohort was insufficient to underline both a statistically significant difference in TPMT expression between VEO-IBD and adolescent IBD patients. Furthermore, the small number of patients in common both in the TPMT activity cohort and DNA methylation validation cohort was not sufficient to obtain a statistically significant correlation between TPMT activity and cg22736354 methylation. Finally, other enzymatic explanation for the pharmacokinetic differences in azathioprine pharmacokinetics were not investigated in this work [45].

Further studies are needed to understand whether these findings are applicable to other diseases in which azathioprine is used and to investigate the molecular mechanism by which the methylation of the neighboring region of TPMT, which includes cg22736354, influences TPMT expression. In particular, modulating the methylation of cg22736354 in vitro, with a CRISPR-Cas9 system for targeted DNA methylation, as reported by Vojta and collaborators [46], could be considered. Other pharmacokinetic factors influenced by age, such as changes in absorption and biotransformation [47], may also contribute to defining azathioprine pharmacokinetics differences and should be evaluated.

In conclusion, this study provided proof that age-related epigenetic changes should be considered for therapy personalization in IBD. In particular, an epigenetics-based pharmacological tool may be further developed to help patients with IBD immediately receive the optimal dose of azathioprine, avoiding lack of efficacy or side effects related to an initial inadequate dose of the drug.

Funding

This work was supported by the Italian Ministry of Health, through the contribution given to the Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy, grant RC 21/17.

CRediT authorship contribution statement

Matteo Bramuzzo and Gabriele Stocco: study conception and design. Davide Selvestrel, Marina Aloi, Serena Arrigo, Sabrina Cardile, Erika Cecchin, Mauro Congia, Simona Gatti, Francesco Graziano, Marianna Lucafò, Stefano Martelossi, Massimo Martinelli, Luca Scarallo, Elisabetta Francesca Stacul, Caterina Strisciuglio, Giovanna Zuin, Debora Curci, Sofia Pagarin, Susan Thompson, Carl D. Langefeld, Erika Cecchin and Gabriele Stocco: acquisition, analysis, and interpretation of the data. Davide Selvestrel, Gabriele Stocco, Giuliana Decorti, Matteo Bramuzzo drafted the initial manuscript. Davide Selvestrel, Gabriele Stocco and Matteo Bramuzzo: critical discussion. Matteo Bramuzzo and Giuliana Decorti: study supervision. All authors contributed to the article and approved the submitted version.

Conflict of interest statement

All authors read and approved the final manuscript. All authors have no conflict of interest.

Data Availability

Data will be made available on request.

Acknowledgments

The authors wish to thank Dr. Rossano Rossino, from the Laboratorio di Genetica Genomica Ospedale Pediatrico Microcitemico A Cao, Dipartimento di Scienze mediche e sanità pubblica, Università di Cagliari, for technical assistance.

Data Transparency Statement

Data, analytic methods, and study materials will be made available to other researchers upon motivated request to the corresponding author.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113901.

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