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Azathioprine dose tailoring based on pharmacogenetic information: Insights of clinical implementation

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ABSTRACT

Azathioprine is commonly used as an immunosuppressive antimetabolite in the treatment of acute lymphoblastic leukemia, autoimmune disorders (such as Crohn's disease and rheumatoid arthritis), and in patients receiving organ transplants. Thiopurine-S-methyltransferase (TPMT) is a cytoplasmic trans-methylase catalyzing the S-methylation of thiopurines. The active metabolites obtained from thiopurines are hydrolyzed into inactive forms by the Nudix hydrolase 15 (NUDT15). The *TPMT**2 (defined by rs1800462), *3A (defined by rs1800460 and rs1142345), *3B (defined by rs1800460), *3C (defined by rs1142345), *6 (defined by rs75543815), and *NUDT15* rs116855232 genetic variant have been associated, with the highest level of evidence, with the response to azathioprine, and, the approved drug label for azathioprine and main pharmacogenetic dosing guidelines recommend starting with reduced initial doses in TPMT intermediate metabolizer (IM) patients and considering an alternative treatment in TPMT poor metabolizer (PM) patients. This study aims to assess the clinical impact of azathioprine dose tailoring based on *TPMT* genotyping studying the azathioprine toxicity and efficacy, treatment starts, and dose adjustments during follow-up, comparing TPMT IM/PM and normal metabolizer (NM) patients. It also studied the association of *NUDT15* rs116855232 with response to azathioprine in patients receiving a tailored treatment based on TPMT and characterized the *TMPT* and *NUDT15* studied variants in our population. Results show that azathioprine dose reduction in TPMT IM patients (*TPMT**1/*2, *1/*3A, or *1/*3C genotypes) is related to lower toxicity events compared to TPMT NM (*TPMT* *1/*1 genotype), and lower azathioprine dose adjustments during follow-up without showing differences in the efficacy. The results support the hypothesis of existing other genetic variants affecting azathioprine toxicity.

1. Introduction

Thiopurines are purine analogs, first synthesized in the early 1950s by Elion and Hitchings [1] including 6-thioguanine, 6-mercaptopurine (6-MP), and its prodrug azathioprine, the 1-methyl-4-nitro imidazolyl derivative of guanine [2].

Azathioprine is commonly used as an immunosuppressive antimetabolite, alone or combined with other drugs, usually corticosteroids, in the treatment of acute lymphoblastic leukemia, autoimmune disorders (such as Crohn's disease and rheumatoid arthritis), and in patients receiving organ transplants [3].

It has a narrow therapeutic index, and the dose varies depending on the situation from 0.5 to 5 mg/Kg/day as initial doses to 1–4 mg/Kg/day maintenance doses. But, in most cases, starting with 0.5–1.5 mg/kg/day and increasing maintenance doses up to 2–2.5 mg/Kg/day is recommended [4].

In any case, the dose should be adjusted according to clinical needs and hematologic tolerance, and the dose should be reduced by 25% when co-administered with xanthine oxidase inhibitors, such as allopurinol, oxipurinol, or thiopurinol. Additionally, whenever an adverse

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drug event (ADE) occurs, the dose should be reduced, or treatment discontinued.

The most prevalent ADE is leukopenia (5–25% of patients receiving treatment) [5]. Other common ADEs are hepatotoxicity, pancreatitis, and gastrointestinal intolerance [6], in addition to depression of bone marrow function, myelotoxicity, other blood and lymphatic system disorders [7,8], and an increased risk of certain types of cancer in long-term treatment [9,10]. In any case, the benefit outweighs the risk of malignancy or other ADEs.

1.1. Azathioprine pathway and action

Azathioprine is a prodrug that is metabolized to 6-MP by glutathione transferase or by non-enzymatic conversion (only 1% of all biotransformation), which takes place in red blood cells [11].

Fig. 1 shows the azathioprine pathway and mechanism of action described below.

The 6-MP is transported into the cell by the nucleoside transporters SLC28A2, SLC28A3, SLC29A1, and SLC29A2 [3], and metabolized by three competitive metabolic pathways. The main metabolic pathway involves the intracellular transformation of 6-MP into pharmacologically active thioguanine nucleotides (TGNs) through a multi-step enzymatic pathway resulting in thioinosine monophosphate (TIMP), thioguanosine monophosphate (TGMP), and finally thioguanine triphosphate (TGTP) nucleotides [2,5]. The TPMT removes TGMP from the medium, transforming it into methyl thioguanosine monophosphate (meTGMP), it also deviates TIMP from the formation of TGTP resulting in 6-mercaptopurine ribose (MPR) [12] and finally, methyl mercaptopurine ribose (meMPR), causing hepatotoxicity [3].

The second competing metabolic pathway for 6-MP involves the extracellular oxidation of 6-MP to thiouric acid by xanthine dehydrogenase (XDH). The other intracellular metabolic pathway involves the methylation of 6-MP by TPMT to methyl mercaptopurine (meMP) [2,5].

Azathioprine achieves its immunosuppressive effect through the insertion of TGNs into DNA and RNA, thus distorting nucleic acid structures, inhibiting enzymes involved in DNA replication and repair, altering DNA structure, causing apoptosis of lymphocytes, and preventing bone marrow proliferation and hematopoiesis [13]. Furthermore, TGTP inhibits the activity of the guanosine triphosphatase "GTPase Ras-related C3 botulinum toxin substrate" (Rac1), dysregulating the proliferation of T lymphocytes and the repression of immune responses [14], and methyl thioinosine monophosphate (meTIMP) inhibits the de novo synthesis of purines, adenosine triphosphate (ATP) and guanosine triphosphate (GTP) in unstimulated cells [15].

Therefore, azathioprine contributes to immunosuppressive effects, based on a balance between pro-apoptotic and anti-metabolic pathways [15].

1.2. Thiopurine-S-methyltransferase (TPMT)

TPMT is a cytoplasmic trans-methylase found in almost all human tissues with 245 amino acids, and 28 kDa [16]. It catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds, as well as thiopurines such as azathioprine, 6-MP, and 6-thioguanine [3].

Different studies have shown that there is a relationship between TGN levels, linked to TPMT activity, and the therapeutic or toxic effect of azathioprine. If TPMT has deficient activity, a greater amount of substrate will be transformed thus resulting in higher TGN levels and toxicity [17]. Conversely, high TPMT activity is associated with lower TGN levels and a loss of drug effect, and increased meMPR and meTIMP resulting in liver damage and inhibition of purine biosynthesis, respectively [2].

In humans, the TPMT enzyme is encoded by the *TPMT* gene, located on the short arm of chromosome 6 (6p22.3 region). The *TPMT* gene is highly polymorphic, with more than 30 single nucleotide polymorphisms (SNPs) described, most of them non-synonymous [18], inherited in a codominant manner [19], affecting the enzyme activity, but showing variable frequencies in the general population [20,21]. The four most frequent variants are the *TPMT**2 (defined by rs1800462), *3A (defined by rs1800460 and rs1142345), *3B (defined by rs1800460) and *3C (defined by rs1142345) alleles, causing the enzyme loss of function through different mechanisms [22]. Some *TPMT* variants as those defining the *TPMT**2, *3A, *3B, *3C, *3D, *5, and *6 alleles, lead to amino acid substitutions, while others, such as the *TPMT**4

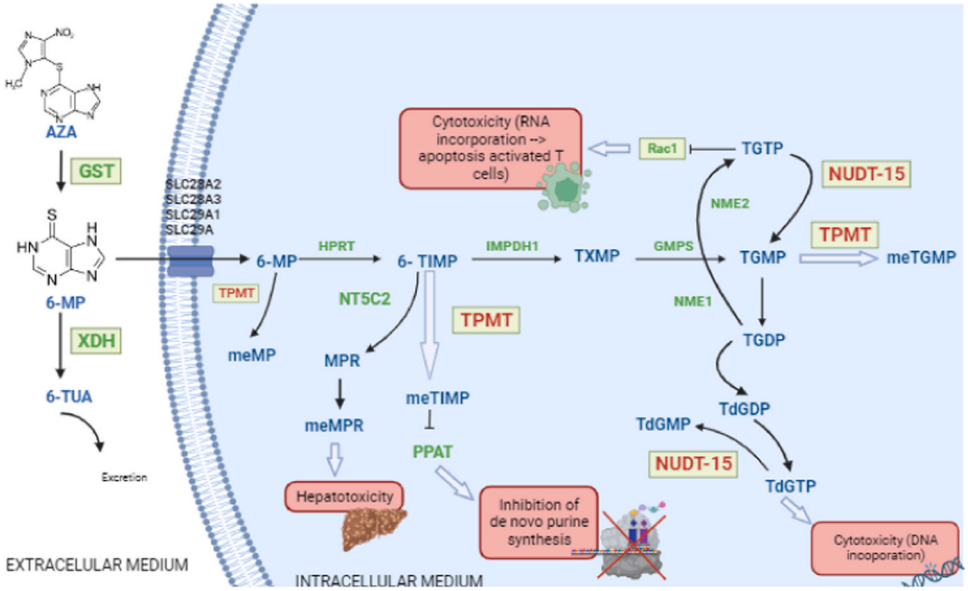

Fig. 1. Azathioprine pathway and mechanism of action. AZA: azathioprine; GST: Glutation S-transferase; 6-MP: 6-mercaptopurine; XDH: xanthine deshydrogenase; TUA: thiouric acid; meMP: methyl mercaptopurine; HPRT: hypoxanthine phosphoribosyl transferase; TIMP: thioinosine monophosphate; IMDPH: inosine monophosphatase dehydrogenase; TXMP: thioxantosine monophosphate; GMPS: guanosine monophosphatase synthase; TGMP: thioguanosine monophosphate; me: methyl; TGDP: thioguanine diphosphate; TGTP: thioguanine triphosphate; NME: nucleoside diphosphate kinase; Rac1: GTPase Ras-related C3 botulinum toxin substrate; NT5C2: 5′-nucleotidase, cytosolic II; MPR: mercaptopurine ribose; PPAT: phosphoribosyl pyrophosphate amidotransferase; TPMT: Thiopurine-S-methyltransferase; NUDT15: Nudix hydrolase 15.

(defined by rs1800584) leads to the destruction of a splice site [20].

1.3. Nudix hydrolase 15 (NUDT15)

NUDT15 is encoded by a gene in the Nudix (nucleoside diphosphates linked to another moiety X) family, targeting the Hydrolase 15. NUDT15 is an enzyme that belongs to the Nudix hydrolase superfamily.

TGNs as TGTP are active metabolites obtained from mercaptopurine. Both are substrates of the NUDT15 and may be hydrolyzed into inactive forms. This impeds their insertion into DNA and RNA, the consequent apoptosis of lymphocytes by their effect on enzymes involved in DNA replication and repair, and the negative impact on bone marrow proliferation and hematopoiesis [23,24].

As we can see, NUDT15 is a negative regulator of thiopurine activation and toxicity. Genetic variants in *NUDT15,* especially the rs116855232, were related to decreased metabolism of thiopurines, resulting in early leukopenia in Crohn's and inflammatory bowel disease (IBD) [24], azathioprine [25] and thiopurine [26–28] toxicity, and decreased doses of mercaptopurine [24] and thiopurines [29].

1.4. Pharmacogenetics of azathioprine

Different genetic variants have been associated with the response to azathioprine with different levels of evidence. The *TPMT* and *NUDT15* genes are the most determinant ones modifying the patients' response to this drug. Specifically, the *NUDT15* rs116855232 variant, *TPMT**2 (defined by rs1800462), *3A (defined by rs1800460 and rs1142345), *3B (defined by rs1800460), *3C (defined by rs1142345), and *6 (defined by rs75543815) alleles have been associated, with a level of evidence 1A [30], with the response to azathioprine.

As we can see (Table 1), the *TPMT* rs1800460 and rs1142345 can be located in the same chromosome, defining the *TPMT**3A allele, or in a different one, defining the *TPMT**3B and *3C, respectively. The *TPMT**1/*3A genotype is usually assigned to an individual carrying both genetic variants, instead of *TPMT**3B/*3C, considering the high linkage disequilibrium (LD) between them and the frequency of these genotypes in the European population (*TPMT**1/*3A: 6.45%; *TPMT**3B/*3C: 0.0028%).

Regarding the *NUDT15* rs116855232, it defines the *NUDT15**3 when inherited alone or *NUDT15**2 if located in the same chromosome within the *NUDT15* rs746071566 (Table 1). Both *NUDT15**2 and *3 are no function alleles.

Dosing guidelines based on pharmacogenetic (PGx) information from the Dutch Pharmacogenomics Working Group (DPWG) [31] and the Clinical Pharmacogenetics Implementation Consortium (CPIC) [32] categorize individuals non carrying a *TPMT* or *NUDT15* mutated allele (*TPMT**1/*1 and *NUDT15**1/*1 genotypes) as NMs, and those carrying one only mutated allele as IMs. On the other hand, CPIC classifies those carriers of two alleles different from *1 as PMs (such as *TPMT**3A/*3C or *NUDT15**2/*3), possible IMs (such as *TPMT**3A/*7 or *NUDT15**3/*4) or indeterminate (such as *TPMT**17/*19 or *NUDT15**5/*6), whereas DPWG classifies them all as PMs.

The *Food and Drug Administration* (FDA)-approved drug label of azathioprine recommends considering genotyping or phenotyping patients for *TPMT* deficiency and genotyping for *NUDT15* deficiency in patients with severe myelosuppression, alternative therapy in patients with homozygous *TPMT* or *NUDT15* deficiency, and lower doses in patients with heterozygous deficiency.

Also, the DPWG guidelines (31) recommend in TPMT intermediate metabolizers (IM) or NUDT15 IM patients to start with 50% of the standard dose and adjust the initial dose based on toxicity (monitoring of blood counts) and effectiveness, considering that dose adjustment is not required for doses lower than 1.5 mg/kg per day for azathioprine and that more restrictive dose reductions are necessary if the patient also presents a IM or PM phenotype for NUDT15 or TPMT, respectively. In TPMT PM or NUDT15 PM patients, it recommends choosing an alternative or starting with 10% of the standard dose, considering that any adjustment of the initial dose should be guided by toxicity (monitoring of blood counts) and effectiveness, and, if the dose is decreased, it recommends advising patients to seek medical attention when symptoms of myelosuppression (such as severe sore throat in combination with fever, regular nosebleeds, and tendency to bruising) occur.

Regarding the CPIC guidelines (32), in a similar way, it recommends in TPMT IM and/or NUDT15 IM patients, start with reduced starting doses (30%−80% of a normal dose) if the normal starting dose is 2–3 mg/kg/day (e.g., 0.6 – 2.4 mg/kg/day) and adjust doses of azathioprine based on the degree of myelosuppression and disease-specific guidelines. In TPMT PM and/or NUDT15 PM patients, it recommends for non-malignant conditions to consider alternative non-thiopurine immunosuppressant therapy, and for malignant conditions, to start with drastically reduced normal daily doses (reduce daily dose by 10-fold) and adjust doses of azathioprine based on the degree of myelosuppression and disease-specific guidelines.

2. Hypothesis and objectives

The drug label for azathioprine, DPWG (31), and CPIC (32) guidelines recommend starting with reduced initial doses in TPMT IM patients

Table 1
Genetic variants with related dosing recommendation in CPIC or DPWG guidelines.

GENE	*allele	Major Nucleotide Variation	rs	MAF^	CPIC (32)	DPWG (31)	Evidence
TPMT	*2	c.238 G>C	rs1800462	0.00244	Yes	Yes	1 A
	*3A	c.460 G>A	rs1800460	0.035413	Yes	Yes	1 A
		c.719 A>G, C	rs1142345	0.040659	Yes	Yes	1 A
	*3B	c.460 G>A	rs1800460	0.035413	Yes	Yes	1 A
	*3C	c.719 A>G, C	rs1142345	0.040659	Yes	Yes	1 A
	*4	c.626–1 G>A	rs1800584	0.00016	Yes	Yes	> 3
	*6	c.539 A>T	rs75543815	< 0.00001	Yes	Yes	1 A
	*7	c.681 T > G	rs72552736	0.00004	Yes	Yes	> 3
	*8	c.644 G>A	rs56161402	0.000769	Yes	Yes	> 3
	*5, *9-*18	-	-	-	No	Yes	> 3
NUDT15	*2	c.7973 C>T	rs116855232	0.002889	Yes	Yes	1 A
		50delGAGTCG; 55_56insGAGTCG	rs746071566	0.00235	Yes	Yes	3
	*3	c.7973 C>T	rs116855232	0.002889	Yes	Yes	1 A
	*4	c.7974 G>A	rs147390019	0.000061	Yes	Yes	> 3
	*5	c.52 G>A	rs186364861	0.000031	Yes	Yes	> 3
	*6	50delGAGTCG; 55_56insGAGTCG	rs746071566	0.00235	Yes	Yes	3

Rs: Reference single nucleotide polymorphism (SNP); MAF: Minor Allele Frequency; CPIC: Clinical pharmacogenetics Implementation Consortium; DPWG: Dutch Pharmacogenomics Working group; *TPMT*: Thiopurine-S-methyltransferase; *NUDT15*: Nudix hydrolase 15; ^Reported MAFs refer to European population in the Allele Frequency Aggregator (ALFA) and obtained from dbSNP

and considering an alternative treatment in TPMT PM patients. In 2018 and 2019, respectively, the DPWG and CPIC guidelines extended these recommendations to NUDT15 IM/PM patients.

This study aims to assess the clinical impact of azathioprine dose tailoring based on *TPMT** 2 (defined by rs1800462), *3A (defined by rs1800460 and rs1142345), *3B (defined by rs1800460), and *3C (defined by rs1142345) alleles studying the distribution of ADEs (toxicity), azathioprine efficacy, treatment starts, and dose adjustments during follow-up, between TPMT IM/PM and NM patients.

Despite of azathioprine dose tailoring based on *TPMT* genotype, which had been implemented in the clinical practice before 2018, a large number of patients still showed ADEs. In this study it is also stated the association of *NUDT15* rs116855232 variant with response to azathioprine in patients receiving a tailored treatment based on *TPMT*, and *TMPT* and *NUDT15* studied variants were characterized in our population.

3. Materials and methods

Observational study including patients prescribed azathioprine regardless of the indication of use in *Hospital Universitario Clínico San Cecilio* (Granada, Spain), between Jan/01/2019, and Feb/30/2023, after *TPMT**2 (defined by rs1800462), *3A (defined by rs1800460 and rs1142345), *3B (defined by rs1800460), and *3C (defined by rs1142345) genotyping, and dose tailoring based on DPWG and CPIC guidelines recommendations.

The Research Ethics Committee of Granada approved the study (Code: 0610-N-19; Date of approval: Apr/24/2019). All the participants signed the written informed consent, and the principles of the Declaration of Helsinki were followed.

The following inclusion criteria were regarded: Spanish patients treated with azathioprine after prescription by a physician at our hospital, with requested *TPMT* PGx test before azathioprine prescription, available 3-month follow-up period based on medical records, and non-azathioprine or mercaptopurine treatment during three years before recruitment. Not signing the informed consent or asking for the withdrawal of the study resulted in the exclusion of the study.

The main endpoints were the toxicity and efficacy of azathioprine. The toxicity endpoint was ADEs to azathioprine. The efficacy endpoint was achieved if patients met two criteria. First, the positive clinical assessment regarding the progression of the illness treated with azathioprine by the physician recorded in the clinical records. Second, the non-discontinuation of azathioprine treatment during follow-up. ADEs causality and severity were assessed based on the Liverpool Causality Assessment Tool (LCAT) [33], and Common Terminology Criteria for Adverse Events (CTCAE) [34], respectively. Only ADEs categorized as probable or definite based on the LCAT, and severity grade 2 or higher were considered for the study. Included ADEs were categorized as "leukopenia and blood disorders", "gastrointestinal and hepatic disorders", and "others". The efficacy endpoint was reviewed by recruiter physicians to avoid considering a lacking efficacy in patients going into remission of their illness.

First, the clinical impact of azathioprine dose tailoring was assessed based on *TPMT* genotyping studying the association of included *TPMT* variants with toxicity and efficacy events, treatment starts/discontinuations, and dose adjustments during follow-up.

In May 2023, to explain part of the ADEs occurred during the follow-up period, *NUDT15* rs116855232 was tested, which was the only genetic polymorphism associated with azathioprine response with the highest level of evidence, MAF higher than 0.001 in European population according to the Allele Frequency Aggregator (ALFA), and not being tested in daily clinical routine in our hospital. We studied the association of *NUDT15* (rs116855232) with ADEs during a 3-months follow-up period since the recruitment date.

For *TPMT* and *NUDT15* characterization in our population, the Hardy-Weinberg (H-W) equilibrium, genotypes distribution, and LD of included variants for *TPMT* were tested.

The clinical impact of the PGx testing was studied collecting data about prescriptions of azathioprine in our hospital, hospital units requesting the PGx test, number of PGx tested patients, time of PGx test request (before/after azathioprine prescription), grade of acceptance of dosing recommendations based on the PGx test, and ADEs to azathioprine.

3.1. Patients and data management

Whenever a doctor considers prescribing azathioprine, they request genotype *TPMT**2 (defined by rs1800462), *3A (defined by rs1800460 and rs1142345), *3B (defined by rs1800460), and *3C (defined by rs1142345) to the hospital pharmacy unit. At this point, a nurse takes the saliva samples from the patient using sterile cotton swabs which are genotyped in less than 48 h. The laboratory communicates the results to the pharmacy within 72 h of sample collection, and the hospital pharmacists translate genotypes into phenotypes and dosing recommendations.

Patients with a *TPMT**1/*1 genotype were categorized as TPMT NM, and those with a *TPMT**1/* 2, *1/*3C and *1/*3A genotypes were categorized as IMs as stated by CPIC (32) and DPWG (31).

According to DPWG guidelines (31), TPMT IM patients intended to treat with azathioprine were recommended to "Start with 50% of the standard dose. The adjustment of the initial dose should be guided by toxicity (monitoring of blood counts) and effectiveness. Dose adjustment is not necessary for doses lower than 1.5 mg/kg/day". Also, according to these guidelines, TPMT PM patients would be recommended to "Choose an alternative or start with 10% of the standard dose, any adjustment of the initial dose should be guided by toxicity (monitoring of blood counts) and effectiveness".

The PGx dosing recommendation is communicated to the doctor, uploading a clinical report in the patient's medical record, who makes the final decision on the prescription and treatment regimen.

The remaining DNA and saliva samples are stored as a private biosample collection registered with the Carlos III Health Institute (C.0007322). These samples were used for retrospectively testing the *NUDT15* rs116855232 in May/2023.

3.2. Tested genetic variants in the study and PGx tests

The genetic variants included in the study were those in *TPMT* and *NUDT15* reported in PharmGKB [35,36] with a level of evidence 1 A for their association with azathioprine response and MAF higher than 0.001. These were the *TPMT* rs1800462, rs1800460, rs1142345, and *NUDT15* rs116855232. With these variants the *TPMT**2 (defined by rs1800462), *3A (defined by rs1800460 and rs1142345), *3B (defined by rs1800460), *3C (defined by rs1142345) can be inferred.

*TPMT**2, *3A, *3B, and *3C were used for PGx dose tailoring of azathioprine in the daily clinical routine before the recruitment, and *NUDT15* rs116855232 was tested after the recruitment end date to know its influence on azathioprine response, especially about its influence on unexplained toxicity events among *TPMT* wildtype patients.

DNA was isolated using standard procedures, and DNA extraction was carried out following the method by Freeman et al. [37] and Gomez-Martín A. et al. [38].

For genotyping, the TaqMan® allelic discrimination assay (Life Technologies, Foster City, CA, USA) was used. The call rate for all tested SNPs was $> 98\%$. Quality control for the genotyping results was achieved with negative controls and randomly selected samples included as duplicates.

3.3. Statistical analysis

First, a descriptive analysis of the collected data (Table 2) was performed, calculated the distribution (number, *n*; and percentage, %) of

Table 2
Baseline characteristics of azathioprine treated patients.

Parameter	N = 102 n (%)
Diagnosis	
Vogt Koyanagi Harada disease	6 (5.88)
Vasculitis	11 (10.78)
Behçet's disease	12 (11.76)
Systemic lupus erythematosus	13 (12.74)
Others	60 (58.82)
Initial doses (mg/24 h)	
50	44 (43.13)
75	7 (6.93)
100	43 (42.57)
> 100	7 (6.86)
Doses changed	33 (32.4)
Clinical parameters	
Age	48.94 ± 19.20
Women	63 (61.76)
Weight n = 70	75.50 ± 23.08
Smoker n = 101	15 (14.85)
Diabetes	8 (7.77)
Hyperlipemia	24 (23.30)
Hypertension	28 (27.18)
Concomitant Treatment	
Allopurinol	4 (3.92)
Methotrexate	5 (4.9)
Salicylate	10 (9.8)
ACE	21 (20.59)
Corticoids	84 (82.35)
Genetic characteristics	
TPMT Genotype	
*1/*1 (*wildtype*)	94 (92.15)
*1/*2	2 (1.96)
*1/*3A	5 (4.90)
*1/*3C	1 (0.98)
NUDT15 genotype	
*1/*1	101 (99.02)
*1/rs116855232	1 (0.98)
TPMT/NUDT15 phenotype	
NM/NM	93 (91.18)
IM/NM	8 (7.84)
NM/IM	1 (0.98)
Endpoint	
Toxicity (ADEs) n = 39	
Leukopenia and BD	10 (25.64)
GI and hepatic disorders	22 (56.41)
Others	7 (17.95)
Efficacy	
Yes	87 (85.29)

ACE: Angiotensin-converting enzyme inhibitors; *TPMT*: Thiopurine Methyl Transferase; *NUDT15*: Nudix Hydrolase 15; NM: Normal metabolizer; IM: Intermediate metabolizer;ADE: Adverse drug event; BD: Blood disorders; GI: Gastrointestinal

genotypes, phenotypes, and MAF of the included *TPMT* and *NUDT15* variants. After that, an LD analysis of the genetic polymorphisms in *TPMT* was carried out and the H-W equilibrium was tested.

The association of each SNP with toxicity and efficacy was studied by doing a genotype comparison analysis, and a phenotype comparison association study with the response. First, to study if the actual PGx test is useful in clinical practice, it was carried out the association study with the response of *TPMT* genotypes, used for the dose tailoring of azathioprine in clinical routine. After that, the association with the response of *NUDT15* rs116855232 was studied to explain part of the toxicity events in azathioprine-treated patients receiving standard doses.

The descriptive analysis (Table 2), MAFs, and genotype/phenotype distribution were conducted using R commander. For the association studies of genetic variants with response, LD analysis, and H–W equilibrium analyses we used the SNPstats online tool [39].

The Chi-square test or Fisher exact test was used, odds ratios (OR) calculated, and p-values considering $p < 0.05$ statistically significant.

4. Results

In *Hospital Universitario Clínico San Cecilio* (Granada, Spain), between Jan/01/2019 and Mar/01/2023 n = 877 patients were prescribed azathioprine. Most of them (n = 553; 63.06%) by the Digestive Medicine Department, n = 110 (12.54%) by the Internal Medicine Department, n = 46 (5.25%) by the Neurology Department, n = 45 (5.13%) by the Rheumatology Department, and n = 123 (14.03%) by other hospital departments.

In total, N = 149 PGx tests were requested in our hospital, but only n = 102 patients were finally treated with azathioprine. All of them are from the Internal Medicine Department, meaning 92.73% of azathioprine-prescribed patients by this Hospital Department between Jan/01/2019, and Mar/01/2023 (Fig. 2).

Among N = 149 patients tested for *TPMT**2, *3A, *3B and *3C we found n = 134 *TPMT**1/* 1 (wildtype) patients, n = 2 *1/*2, n = 4 *1/*3C, and n = 9 *1/*3A. No patients were recessive homozygous for the tested rs. Regarding the n = 47 patients non treated with azathioprine we found n = 40 *TPMT**1/*1 (wildtype) patients, no *TPMT**1/*2, n = 3 *1/*3C, and n = 4 *1/*3A, translated into n = 40 TPMT NM and n = 7 IM patients. Regarding just the n = 102 TPMT-tested patients and finally treated with azathioprine, we found n = 2 *TPMT**1/*2, n = 1 *1/*3C, and n = 5 *1/*3A. These genotypes were translated into n = 94 NM patients, n = 8 IM patients, and no PM patients. All of them were dose-adjusted based on our recommendation. TPMT IM patients were recommended to receive the 50% of the standard dose, this was followed by physicians in all the cases.

Among treated patients (Table 2), the mean age was 48.94 ± 19,19 years old, with different diagnoses, 61.76% women, and around 50% of patients treated with at least 100 mg/24 h.

4.1. Association study of TPMT and NUDT15 variants with the response

In the association study of *TPMT* SNPs with the toxicity endpoint, there were no significant differences in ADEs distribution regarding *TPMT* variants used for the tailoring of azathioprine doses. Even among n = 8 TPMT IM patients and dose adjusted based on PGx result, only n = 1 (12.5%) suffered an ADE during follow-up but n = 38 NM patients (40.43%). It shows that carrying at least one allele among *TPMT**2, *3A, *3B, or *3C and receiving reduced doses of azathioprine is related to a decreased number of ADEs during a 3-month follow-up period (OR= 0.21; 95% CI= 0.02 – 1.78; p = 0.09) without differences in the efficacy (OR= 0.48; 95% CI= 0.09 – 2.65; p = 0.42) (Table 3).

Extended results about the association of *TPMT* genotypes with the toxicity and efficacy endpoints are shown in supplementary tables 3A and 3B, including a comparison between wildtype (*TPMT**1/*1) and mutated genotypes. First, in a single genotype analysis excluding patients carrying any other mutated genotype (supplementary table 3A), and second a grouped genotype analysis including those n = 8 mutated patients (supplementary table 3B).

Regarding the *NUDT15* rs116855232, just one patient carried this variant and was treated with azathioprine. This patient had a *TPMT**1/*1 genotype, suffered no ADEs during follow-up, had a positive medical evaluation during follow-up, and had no treatment discontinuation.

4.2. Usefulness of TPMT and NUDT15 genotyping in daily clinical practice

Among azathioprine-treated patients (n = 102), n = 94 were categorized as NM patients not carrying a *TPMT* variant, and n = 33 (32.4%) received adjusted doses after the treatment started (n = 25 dose increasements; n = 8 dose reductions). Regarding those patients carrying at least one *TPMT* variant and receiving a dosing recommendation, n = 8 (100%) were prescribed azathioprine following the PGx recommendations, and doses were not adjusted after treatment started in any case.

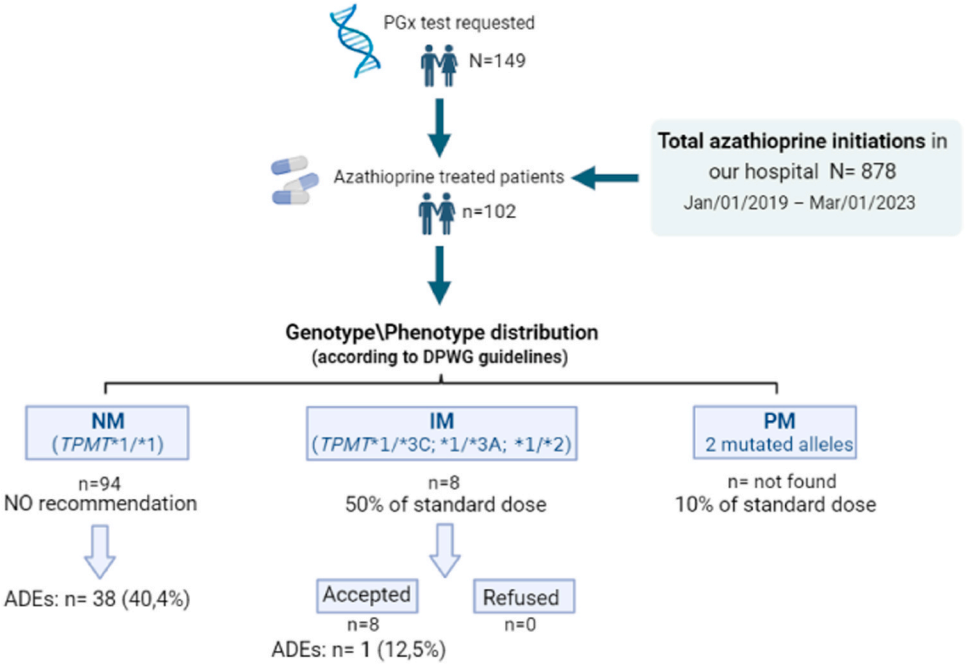

Fig. 2. Management of patients. PGx: Pharmacogenetic; DPWG: Dutch Pharmacogenomics Working Group; NM: Normal metabolizer; IM: Intermediate metabolizer; PM: Poor Metabolizer; ADE: Adverse drug event.

Table 3
Association study of genetic variants used for azathioprine dose tailoring with the toxicity (ADEs) and efficacy endpoints.

		ADE n (%)		OR (95% CI)	p-value
		Yes	No		
Phenotype analysis					
TPMT	IM	1 (2.6)	7 (11.1)	0.21	0.09
	NM	38 (97.4)	56 (88.9)	(0.02–1.78)	
Genotype analysis					
*TPMT**2	*1/*2	0 (0)	2 (3.2)	0.00	0.16
	Non-*1/*2	39 (100)	61 (96.8)	(0.00 - NA)	
*TPMT**3B	*1/*3B	1 (2.6)	4 (6.3)	0.39	0.37
	Non-*1/*3B	38 (97.4)	59 (93.7)	(0.04–3.61)	
*TPMT**3C	*1/*3C	1 (2.6)	5 (7.9)	0.31	0.24
	Non-*1/*3C	38 (97.4)	58 (92.1)	(0.03–2.72)	
*TPMT**3A	*1/*3A	1 (2.6)	4 (6.3)	0.39	0.37
	Non-*1/*3A	38 (97.4)	59 (93.7)	(0.04–3.61)	
		Efficacy n (%)		**OR (95% CI)**	**p-value**
		Yes	**No**		
Phenotype analysis					
TPMT	IM	6 (6.9)	2 (13.3)	0.48	0.42
	NM	81 (93.1)	13 (86.7)	(0.09 – 2.65)	
Genotype analysis					
*TPMT**2	*1/*2	1 (1.1)	1 (6.7)	0.16	0.23
	Non-*1/*2	86 (98.8)	14 (93.3)	(0.01 – 2.76)	
*TPMT**3B	*1/*3B	4 (4.6)	1 (6.7)	0.67	0.74
	Non-*1/*3B	83 (95.4)	14 (93.3)	(0.07 – 6.49)	
*TPMT**3C	*1/*3C	5 (5.8)	1 (6.7)	0.85	0.89
	Non-*1/* 3C	82 (94.2)	14 (9.3)	(0.09 – 7.86)	
*TPMT**3A	*1/*3A	4 (4.6)	1 (6.7)	0.67	0.74
	Non-* 1/* 3A	83 (95.4)	14 (93.3)	(0.07 – 6.49)	

SNP: Single nucleotide polymorphism; OR: Odds ratio; CI: Confidence interval; IM: Intermediate metabolizer; PM: Poor metabolizer; NM: Normal metabolizer; NA: not applicable; *TPMT**2 is defined by rs1800462; *TPMT**3A is defined by rs1800460 and rs1142345; *TPMT**3B is defined by rs1800460; *TPMT**3C is defined by rs1142345.

There were n = 47 non-treated patients. It was aimed to explain if the PGx test influenced the prescription of azathioprine comparing the distribution of IM patients among treated and non-treated patients.

The results showed that carrying at least one *TPMT* variant is associated with not adjusting the azathioprine doses since the treatment start (p = 0.042) (Table 4) compared to not carrying *TPMT* variants. Furthermore, it is shown a bigger proportion of patients carrying a *TPMT* mutated allele among non-azathioprine treated patients.

4.3. *TPMT and NUDT15 characterization*

N = 149 patients were tested for *TPMT**2, *3B, *3C, and *3A, and *NUDT15* rs116855232, so including patients finally treated with azathioprine (n = 102) and n = 47 patients receiving an alternative treatment. Among the N = 149 patients tested for *TPMT* and *NUDT15* variants, including treated and non-treated patients, n = 15 patients carry at least a genetic variant among included SNPs. All the included SNPs were in the H-W equilibrium in our population and showed no differences with the Iberian Peninsula population of the 1000 Genomes Project (36) (Table 5).

The results showed an LD between *TPMT**3B (defined by rs1800460)

Table 4
Association study of TPMT phenotype with treatment starts, and dose adjustments during follow-up.

		Treatment start (N = 149) n (%)		OR (95% CI)	p-value
		YES	NO		
TPMT	**IM**	8 (7.84)	7 (14.89)	0.49 (0.14 – 1.7)	0.184
	NM	94 (92.16)	40 (85.11)		
		Dose adjustment during follow-up (N = 102) n (%)		**OR (95% CI)**	**p-value**
		YES	**NO**		
TPMT	**IM**	0 (0)	8 (11.59)	0 (0 – 1.17)	0.042
	NM	33 (100)	61 (88.41)		

OR: Odds ratio; CI: Confidence interval; IM: Intermediate metabolizer; PM: Poor metabolizer; NM: Normal metabolizer

Table 5
Distribution of TPMT and NUDT15 genotypes.

	Genotypes N = 149			MAF	H-W	Comparison with 1000 Genomes*
	Wt	Het	Hom			
***TPMT**2 (rs1800462)**	147	2	0	0.007	1	0.700
***TPMT**3B (rs1800460)**	140	9	0	0.030	1	0.406
***TPMT**3C (rs1142345)**	136	13	0	0.044	1	0.918
***NUDT15* (rs116855232)**	146	3	0	0.010	1	0.070

Wt: Wildtype; Het: Heterozygous; Hom: recessive homozygous; MAF: Minor Allele Frequency; H-W: p-value of the Hardy-Weinberg equilibrium analysis; *MAF comparison between our population and Iberian Peninsula (Ibs) population in the 1000 Genomes Project [40]

Table 6
Linkage disequilibrium analysis of TPMT variants.

	***TPMT**2**	***TPMT**3B**	***TPMT**3C**
***TPMT**2**	-	p-value= 0848 D= <0001 D´= 0,7652 r = 0,0111	p-value= 0,7997 D= <0001 D´= 0,8375 r = −0,0147
***TPMT**3B**	-	-	**p-value= 0 < 0.001** **D= 0,0288** **D´= 0,9982** **r = 0,8248**
***TPMT**3C**	-	-	-

and *3C (defined by rs1142345) ($p < 0.001$) (Table 6). Provided data in Tables 5 and 6 show that if a patient carries the *TPMT**3B (defined by rs1800460), the *TPMT**3C (defined by rs1142345) is also inherited, but a patient may carry the *TPMT**3C (defined by rs1142345) alone.

5. Discussion

This study aims to assess the clinical impact of *TPMT* genotyping for azathioprine dose tailoring in our hospital, considering the distribution of requested PGx tests among the total number of azathioprine prescriptions, grade of acceptance of PGx results, and the association of genotypes with treatments starts, dose adjustments, and the efficacy and toxicity of azathioprine among patients. This study also aims to help as a guideline for the clinical implementation of the *TPMT* PGx test for azathioprine dose tailoring. The *NUDT15* rs116855232 variant was retrospectively tested to study its influence on unexplained toxicity events among these patients, and the *TPMT**2 (defined by rs1800462), *3B (defined by rs1800460), *3C (defined by rs1142345), *3A (defined by rs1800460 and rs1142345) alleles, and *NUDT15* rs116855232 variant were characterized in our population.

These results show that among non-azathioprine treated patients there is a bigger proportion of patients carrying at least one *TPMT* mutated allele defined by genetic variants recommended to be tested in the azathioprine drug label. This means that the *TPMT* genotyping is used not only to dose tailoring azathioprine prescribed patients but to decide the final treatment when there is an available alternative.

Results suggest that carrying at least one *TPMT* mutated allele and receiving reduced initial doses of azathioprine is related (but not significant) to fewer ADEs during a 3-month follow-up (Table 3; $p = 0.09$) and no azathioprine dose adjustment since the treatment start (Table 4; $p = 0.042$) without differences in the efficacy (Table 3; $p = 0.42$) compared to carrying no *TPMT* variants. This means that *TPMT* genotyping conditions drug choice and dose adjustment, and dose adjustment is not harmful for IM patients, in fact, it is beneficial, as they reach efficacy with lower ADEs. On the other hand, *NUDT15* impact could not be assessed and it did not explain the remaining toxicity events since only one patient was found carrying this variant among treated patients and no association was found with the toxicity endpoint.

This might be explained based on two different hypotheses. First, azathioprine is being overdosed, *TPMT*-based dose tailoring is useless and receiving reduced doses should be recommended regardless of the genotype. Second, many other genetic variants not tested in our clinical practice affect azathioprine response, which we consider a more plausible hypothesis based on the following evidence.

5.1. Limitations

This study has limitations. It is not a study comparing intervention and control cohorts with/without an azathioprine dose-tailored treatment based on *TPMT/NUDT15* genotype, and we neither categorized patients on their pathology. On the other hand, this was not the aim of the study since we tried to see in our daily clinical practice, based on real world data, how the *TPMT* test is being used and its clinical impact, helping as a guide for the clinical implementation of *TPMT* genotyping for azathioprine dose tailoring. It is not possible to analyze the impact of *NUDT15* rs116855232 in the response to azathioprine since we found only one patient carrying this variant, and we did not study all the genetic variants affecting azathioprine response but those included are those we are testing in clinical routine, and the only ones associated with azathioprine response with a level of evidence 1A and translated into dosing recommendations based on DPWG guidelines (31). Furthermore, we did not retrospectively genotype the azathioprine-treated patients who were not genotyped when azathioprine was prescribed. As we used data from our daily clinical practice, recruited patients had different diagnoses showing different toxicity events. Because of this, we could not show categorized results depending on different toxicity events. This might explain if *TPMT* genotyping for azathioprine dose tailoring and reduced doses prevented dose-dependent side effects. As we could know what to expect regarding number of requested PGx test, pathologies or toxicity events, we neither could calculate a sample size.

Further studies should be performed considering larger populations, other variants in *TPMT*, *NUDT15*, or other genes, rare haplotypes including these variants, and other clinical parameters affecting the follow-up of recruited patients.

5.2. Previous results about TPMT-azathioprine interaction

The azathioprine-*TPMT* drug-gene interaction has been widely studied, and the influence of certain *TPMT* variants on azathioprine response has been confirmed. In patients with inflammation and treated with azathioprine the TPMT IM or PM was associated with an increased risk of discontinuation and myelosuppression compared to *TPMT**1/*1 (TPMT NM) [41]. In patients treated with azathioprine carrying at least one *TPMT**2, *3C, or *3A allele compared to the *TPMT**1/*1 genotype, results showed that genotypes including these variants are associated with increased risk of transplant rejection in heart transplantation [42]; decreased hematological indices [43], increased likelihood of hematopoietic toxicity [44], and myelosuppression [45], in people with kidney transplantation; increased likelihood of nausea and vomiting in Rheumatoid Arthritis [46]; and increased likelihood of hematological toxicity or hepatotoxicity in patients with neurological problems [47].

The association of *TPMT* variants with azathioprine response was particularly corroborated among IBD patients. In this regard, the *TPMT**1/*2, *1/*3A, and *1/*3C genotypes are associated with increased likelihood of overall ADEs, bone marrow toxicity [48], and myelosuppression [49] in IBD, and decreased dose of azathioprine, mercaptopurine or purine analog in children with IBD [50]. Furthermore, the TPMT IM phenotype was associated with an increased likelihood of ADEs in IBD patients when treated with azathioprine, mercaptopurine, or purine analogs compared to *TPMT**1/*1 [51]. TPMT IM was also associated with decreased risk of leukopenia when

compared to TPMT PM [52].

Regarding the Spanish population, a previous study by Casajús A. et al. [53] investigated the influence of the same *TPMT* variants, in addition to concomitant treatments, and demographic characteristics on the incidence of ADEs in patients who start the treatment with azathioprine. That study concluded that *TPMT* genotyping before azathioprine prescription reduces ADEs incidence in patients carrying a *TPMT* variant to a similar level as non-carriers, but suggests differences in drug tolerability according to phenotype, without assessing the efficacy of azathioprine among recruited patients. Our results support those by Casajús A. et al. about the association of ADE reduction with the *TPMT* genotype.

As commented above, this background supports the hypothesis of existing genetic variants affecting azathioprine response not being tested in clinical routine.

5.3. Insights related to DPWG and CPIC dosing guidelines

Both the DPWG and CPIC azathioprine dosing guidelines recommend in TPMT IM and/or NUDT15 IM patients to start with reduced doses and adjust the initial dose based on toxicity and effectiveness, and choosing an alternative or starting with 10% of the standard dose in TPMT PM and/or NUDT15 PM. However, neither DPWG nor CPIC guidelines provide different recommendations to patients with TPMT IM/PM and NUDT15 IM/PM compared to patients with TPMT NM and NUDT15 IM/PM phenotypes (31,32).

On the other hand, there are discrepancies regarding which variants translate into different TPMT phenotypes, thus into dosing recommendations. The DPWG guideline for azathioprine and *TPMT* just mentions TPMT phenotypes (NM, IM or PM) to provide a dosing recommendation (31) and we have to take a look to the "General background text Pharmacogenetics – Thiopurine-S-methyltransferase (TPMT)" by the KNMP to see how phenotypes are assigned depending on genotypes, and just considering *TPMT**1 to *18.

On the other hand, the DPWG risk analysis document for azathioprine and *TPMT* [54] refers to CPIC guidelines to consider as TPMT IM patients those carrying a mutated allele among *TPMT**2, *3A, *3B, *3C, *4, *11, *14, *15, *23 or *29, and TPMT PM if carrying two mutated alleles among them. Furthermore, regarding the CPIC guidelines, the 2018 update for thiopurine dosing based on *TPMT* and *NUDT15* genotypes (32) states that just the *TPMT**2, *3A, *3B, *3C, *4, *6, and *8 alleles are responsible for TPMT IM or PM phenotypes and refers to "possible IM" and "indeterminate" phenotypes. For example, *TPMT**3A/*7 and *NUDT15**3/*4 genotypes are categorized as "possible IM", and *TPMT**17/*19 or *NUDT15**5/*6 as indeterminates.

Besides DPWG and CPIC guidelines, if we look at the FDA-approved label for azathioprine, it also recommends considering an alternative therapy in patients with homozygous *TPMT* or *NUDT15* deficiency and reduced dosages in patients with heterozygous deficiency, but only mentions the *TPMT**2, *3C and *NUDT15**2, and *3 alleles.

As we can see, there is evidence supporting the influence of TPMT and NUDT15 metabolizing status on azathioprine response but a lack of homogeneity about the genetic variants that should be tested to make a PGx dosing recommendation, even when dosing guidelines include some genetic variants not showing a great level of evidence about their association with differences in drugs responses.

In this study, we tested the SNPs characterizing the *TPMT**2, *3A, *3B, *3C, which were being used in our daily clinical routine, and the *NUDT15* rs116855232 characterizing the *NUDT15**2, or *3 alleles to explain those toxicity events shown by patients receiving a TPMT-based tailored treatment. We chose these variants since these were the only ones with a level of evidence 1A for their association with azathioprine response and MAF higher than 0.001. This might be considered unbiased criteria to choose which variants may be implemented in the clinical practice.

Furthermore, in this study it was found that carrying at least one *TPMT* mutated allele and receiving reduced initial doses of azathioprine is related (although not significant) to a decreased number of ADEs during a 3-month follow-up period ($p = 0.09$) and no azathioprine dose adjustment since the treatment start ($p = 0.042$) without differences in the efficacy ($p = 0.42$) compared to carrying no *TPMT* variants. Thus, based on these results, lower initial doses might be recommended regardless of the genotype, and, *TPMT* and *NUDT15* PGx tests performed to warn about possible ADEs during follow-up recommending avoiding higher doses if possible in patients carrying a *TPMT* variant.

Apart from this, we also found some inconsistencies regarding the *TPMT* genotype assignment process. We know that the *TPMT* rs1800460 and rs1142345, characterizing the *3B and *3C respectively, both together in the same allele, are the *TPMT**3A (rs1800460 + rs1142345). So those patients carrying the *TPMT* rs1800460 and rs1142345 cannot be assigned to *TPMT**1/*3A or *TPMT**3B/*3C genotypes. This is especially important since the *TPMT**1/*3 A translates into TPMT IM phenotype and 50% dose lowering, and *TPMT**3B/*3C into TPMT PM and a reduced daily dose by 10-fold or consider an alternative treatment.

5.4. Impact of azathioprine PGx test on daily clinical practice in our hospital

The usefulness of pharmacogenetics has been widely studied. It was even proved that the genotype-guided treatment for several drugs reduces the incidence of adverse drug events, its implementation can help to make drug therapy increasingly safe [55], and its cost-effective in some cases [56]. Nowadays, an increasing number of drug labels for different drugs include PGx information. In most cases, the PGx information is limited to a recommendation for testing before the treatment starts, but, in some cases, the PGx test is mandatory before the prescription [57]. Azathioprine is used by different hospital departments in different pathologies, and the PGx test is still a recommendation [58].

One engaging thing shown in this study is that only the internal medicine department requests the PGx test. As we can see above, $n = 877$ patients were prescribed azathioprine in our hospital during the recruitment period, but only $n = 102$ among them were tested, all of them from the internal medicine department, representing 92.73% of azathioprine prescriptions made by this hospital department. It proves the lack of knowledge about PGx depending on healthcare professionals.

This point is interesting regarding the use of azathioprine for digestive pathologies. In our hospital, the digestive unit prescribed $n = 553$ (63.06%) azathioprine treatments without PGx test requests, but, as commented above, the *TPMT*-azathioprine drug-gene interaction is particularly interesting in patients with IBD, with the highest level of evidence about the *TPMT* and *NUDT15* association with azathioprine response.

6. Conclusions

Based on our results we conclude that azathioprine dose reduction in TPMT IM patients (*TPMT**1/*2, *1/*3A, or *1/*3C genotypes) is related to lower toxicity events compared to TPMT NM (*TPMT* *1/*1 genotype), and less azathioprine dose adjustments during follow-up without showing differences in the azathioprine efficacy.

Our results support the hypothesis of existing other genetic variants significantly affecting azathioprine toxicity.

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Varón: Conceptualization, Methodology, Visualization, Formal analysis, Writing – original draft. **Michelle Carolina Rojas Romero:** Software, Investigation, Resources. **José Luis Callejas-Rubio:** Validation, Data curation, Writing – review & editing. **José Cabeza-Barrera:** Supervision, Project administration. **Alba Rodríguez-Nogales:** Validation, Data curation, Writing – review & editing. **Julio Gálvez:** Validation, Data curation, Writing – review & editing. **Rocío Morón-Romero:** Conceptualization, Methodology, Visualization, Formal analysis, Writing – original draft.. *All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115706.

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