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RESEARCH ARTICLE

Klebsiella pneumoniae contributes to altered cytotoxicity of thiopurines in vitro: Possible implications of biotransformation and bacterial metabolism

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Abstract

Background and purpose: Thiopurines are used in paediatric inflammatory bowel disease (IBD), but some patients do not respond. Because the gut microbiota influences drug efficacy and IBD-patient microbiota presents increased bacterial abundance, we investigated the impact of candidate Enterobacteriaceae on drug cytotoxicity, metabolism and efficacy.

Experimental approach: Thiopurines were exposed in vitro to bacteria for 4 h at 37°C and drug concentrations measured by UV spectrophotometry. Cytotoxic effects and drug metabolite concentrations on NALM6 and JURKAT cells were determined after treatment with thiopurines exposed or not to bacteria. Drugs were measured in *Klebsiella pneumoniae* lysates and bacterial conditioned media were used for metabolomic analyses. Shotgun metagenomic sequencing was performed on eight IBD-patient faecal stools.

Key results: Incubation of thiopurines with *K. pneumoniae*, but not *Escherichia coli* and *Salmonella enterica*, reduced thiopurine concentrations and cytotoxicity on NALM6 and JURKAT cells. Thiopurine metabolites were lower in cells treated with drugs previously exposed to *K. pneumoniae*. Internalisation of drugs was demonstrated by their detection in lysates after bacterial incubation. Untargeted metabolomics revealed biotransformation of thiopurines by *K. pneumoniae*, as reactions of deconjugation, reduction, glycosylation, acetylation or conjugation with propionic

Abbreviations: AZA, azathioprine; CD, Crohn' disease; CPS, capsular polysaccharide; dTGTP, deoxythioguanosine triphosphate; dTGUA, deoxythioguanosine; HPLC-UV, high performance liquid chromatography coupled with a UV detector; IBD, Inflammatory bowel disease; IQR, interquartile range; LC–MS/MS, liquid chromatography tandem mass spectrometry; LC-QTOF-MS, liquid chromatography time-of-flight mass spectrometer; MIC, minimum inhibitory concentration; MeTG, methylthioguanine; MeTGDP, methylthioguanosine diphosphate; MeTGMP, methylthioguanosine monophosphate; MeTGTP, methylthioguanosine triphosphate; MeTIDP, methylthioinosine diphosphate; MeTIMP, methylthioinosine monophosphate; MeTITP, methylthioinosine triphosphate; MMPN, methylated derivatives; MP, mercaptopurine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TG, thioguanine; TGDP, thioguanosine diphosphate; TGMP, thioguanosine monophosphate; TGN, thioguanine nucleotides; TGTP, thioguanosine triphosphate; TIDP, thioinosine diphosphate; MeTGDP, methylthioguanosine diphosphate; TIMP, thioinosine monophosphate; TITP, thioinosine triphosphate; TPMT, thiopurine methyl transferase.

Matthias Schwab and Gabriele Stocco equally contributed to this work.

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acid. Incubation with thiopurines led to changes in the secretion of endogenous bacterial metabolites. *K. pneumoniae* faecal abundance was associated with lower thiopurine metabolite concentrations in erythrocytes of paediatric IBD-patients.
Conclusions and Implications: *K. pneumoniae* decreases the cytotoxicity of thiopurines through internalisation of MP and TG. We revealed potential bacterial drug biotransformation, as well as negative correlations between bacterial abundance and drug metabolites.

KEYWORDS
paediatric, IBD, *K. pneumoniae*, microbiota, thiopurines

1 | INTRODUCTION

Thiopurines, (such as **mercaptopurine** [MP], its prodrug **azathioprine** [AZA], and **thioguanine** [TG]), are commonly used to maintain remission in inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC) (Konidari & Matary, 2014). Noteworthy, paediatric IBD patients generally have a more aggressive course of the disease than adults with a higher probability of complications and surgery (Konidari & Matary, 2014; Stocco et al., 2017). Thiopurines are purine analogues and act as antimetabolites to exert their cytotoxic activity on lymphocytes. In particular, they are activated to thioguanine nucleotides (TGN) via several enzymes of the salvage pathway of purine nucleotide biosynthesis, starting with hypoxanthine phosphoribosyl transferase (HPRT). Thioguanine nucleotides insert on nucleic acids causing cell damage, cell arrest and apoptosis (Franca et al., 2019). Mechanistically, the metabolites deoxythioguanosine triphosphate (dTGTP) and thioguanosine triphosphate (TGTP) substitute the corresponding guanosine triphosphates in DNA and RNA, respectively, causing the abovementioned effects (Moon & Loftus, 2016). Their antiproliferative activity towards lymphocytes occurs because of additional mechanisms, such as the inhibition of de novo purine synthesis and suppression of the function of the **Rho-GTPase Rac1** (Franca et al., 2019). Thiopurines, both as free bases and nucleotide forms, are substrates of the enzyme thiopurine methyl transferase (TPMT), which is responsible for catalysing the conversion into methylated derivatives (MMPN), such as the inactive methylmercaptopurine and cytotoxic methylthioinosinic metabolites (Franca et al., 2019).

Despite the proven efficacy of these drugs, some patients do not achieve satisfactory results or develop adverse effects. To date, interindividual variability has been principally related to genetic polymorphisms of TPMT and nudix hydrolase 15 (NUDT15) with functional consequences. In detail, genetic polymorphisms such as *TPMT*2*, *TPMT*3A* and *TPMT*3C* are associated with lower activity of this enzyme, leading to higher levels of TGN and myelotoxicity (Franca et al., 2019). Furthermore, *NUDT15* variants are associated with a lower activity of this enzyme, which is responsible for the dephosphorylation of TGTP to its monophosphate, leading to higher incorporation of this triphosphate into nucleic acids and thus to myelosuppression (Tanaka & Saito, 2021). Because of treatment failure and toxicity, up to 30% of IBD patients must discontinue thiopurine therapy. Interindividual variability can only be explained in part by genetic polymorphisms (de Boer et al., 2007; González-Lama & Gisbert, 2016).

There is growing evidence of the impact of intestinal microbiota on the efficacy of therapies (Franzin et al., 2021; Geller et al., 2017; Zimmermann et al., 2019a). It has been shown that the efficacy and cytotoxicity of nucleoside analogues such as **gemcitabine** (Geller et al., 2017), **famciclovir** (Zimmermann et al., 2019a) and brivudine (Zimmermann et al., 2019b) are influenced by gut microbes. Among other nucleoside analogues, thiopurines also are susceptible to metabolism by microorganisms (Oancea et al., 2017). In particular, Oancea and colleagues investigated the bacterial metabolism of TG in vitro (Oancea et al., 2017). TGNs were detected in *Escherichia coli*, *Bacteroides thetaiotaomicron* and *Enterococcus faecalis* after in vitro exposure of logarithmically growing cultures to TG. In addition, the metabolites were measured in faecal samples taken from *Hprt*$^{-/-}$ mice after

What is already known?

- Gut bacteria are known to influence the efficacy of therapies.

What does this study add?

- *Klebsiella pneumoniae* reduces thiopurines cytotoxicity by internalising and probably metabolising these drugs.

What is the clinical significance?

- *K. pneumoniae* abundance could be linked to lower efficacy to thiopurine in IBD patients.

incubation with TG, demonstrating a bacterial biotransformation (Oancea et al., 2017).

Dysbiosis, which refers to a reduced diversity of gut bacterial species, is associated with several diseases (Horta-Baas et al., 2017; Lucafo et al., 2020; Rajagopala et al., 2016; Sartor & Wu, 2017). In particular, the proliferation of Enterobacteriaceae, belonging to Proteobacteria phylum, at the expense of other bacteria, is a recurring condition in patients with IBD (Franzin et al., 2021; Lucafo et al., 2020; Sartor & Wu, 2017; Schippa et al., 2009). Furthermore, among Enterobacteriaceae, several species, such as *E. coli*, *Salmonella enterica* and *K. pneumoniae*, have the potential to become harmful and promote the development of IBD (Aleandri et al., 2015; Hold et al., 2014; Nagao-Kitamoto & Kamada, 2017).

Given that some patients treated with thiopurines experience therapeutic failure and that gut bacteria may influence drug efficacy and toxicity, this study aimed to investigate in vitro the role of *E. coli*, *S. enterica* and *K. pneumoniae* in mediating the cytotoxic effects of thiopurines. These bacterial species belong to the Enterobacteriaceae family and are known to be enriched and potentially harmful in the intestinal microbiota of IBD patients.

2 | METHODS

2.1 | Bacterial strains and culture conditions

Escherichia coli ATCC 25922, *K. pneumoniae* ATCC 13883 (LGC standards, Sesto San Giovanni, Italy) and a clinical isolate of *S. enterica* were grown in Luria Broth at 37°C under aerobic conditions, according to microbiology laboratory standard procedures.

2.2 | Immortalised cell lines and cell culture

NALM6 B (RRID:CVCL_UJ05) and JURKAT T (RRID:CVCL_0065) cell lines were cultured in RPMI 1640 medium (Euroclone, Milan, Italy) containing 10% foetal bovine serum (FBS) (Sigma-Aldrich, Milan, Italy), 1% L-glutamine 200 mM (Euroclone, Milan, Italy), 1% penicillin 10,000 UI ml^{-1} (Euroclone, Milan, Italy) and 1% of streptomycin 10 mg ml^{-1} (Euroclone, Milan, Italy) and incubated in a humidified atmosphere at 37°C with 5% CO_2; cell passage was performed twice a week.

2.3 | Paediatric patients

Eight paediatric CD patients, undergoing thiopurine therapy for at least 3 months, were included. Three (37.5%) were males; the median age was 13.55 (interquartile range [IQR]: 3.72); the median dose normalised by weight (mg kg^{-1}) was 2.29 (IQR:0.76); and the median duration of therapy was 15.8 months (IQR:11.02). Remission was assessed based on the disease activity score, the Paediatric Crohn's Disease Activity Index (PCDAI). All patients were in remission with the median PCDAI corresponding to 1.25 (IQR:3.75). A cutoff of faecal calprotectin <250 mg kg^{-1} was considered indicative of mucosal remission in the absence of endoscopic evaluation. No other treatment, except for the thiopurines, was administered to the patients.

2.4 | Materials

All chemicals and reagents used were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy). Pure water was obtained from a Milli-Q system (Millipore, Milan, Italy) and used for the preparation of aqueous solvents. Stock solutions of AZA, MP and TG (Sigma-Aldrich, Milan, Italy) were prepared in 0.1 M sodium hydroxide (Sigma-Aldrich, Milan, Italy).

2.5 | Antimicrobial activity test

Susceptibility of microorganisms to the thiopurines AZA, MP, TG and methylprednisolone (Sigma-Aldrich, Milan, Italy), was preliminary evaluated by the 'Broth Microdilution Susceptibility Test'. Methylprednisolone, commonly used for the treatment of IBD, was added to confirm the specific effect of thiopurines on *K. pneumoniae*. Following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Weinstein, 2018), the test was performed in Mueller–Hinton broth in 96-well round-bottom microtitre plates on a final inoculum of 1–5 $\times$ 10^5 CFU ml^{-1} (CFU: colony forming units). Each plate included positive controls (bacterial strain without drugs), negative controls (medium only) and serial fourfold dilutions of each drug, ranging from 50 to 400 μM. The minimum inhibitory concentration (MIC) values were taken as the lowest concentration of drugs resulting in the complete inhibition of visible growth after 24 h of incubation at 37°C.

2.6 | Capsular polysaccharide (CPS) extraction

CPS was extracted at the Laboratory of Molecular Biomedicine of the University of Trieste, using a previously described method (Bellich et al., 2020). In brief, 50 μl of a 1:100 dilution of the overnight culture of *K. pneumoniae* was grown on cystine-lactose-electrolyte-deficient agar (CLED agar) for 3 days at 30°C. Upon the formation of a thick patina, about 3 ml of 0.9% NaCl were added to each Petri dish and the bacteria were scraped from the surface. The collected bacteria were left under gentle stirring for 2 h at 10°C and then centrifuged at 48000 $\times$ *g* at 4°C for 30 min. The supernatant was recovered, checked by UV spectroscopy to confirm the absence of proteins and nucleic acids, dialysed first against 0.1 M NaCl, then water and finally precipitated with four volumes of cold ethanol. The polysaccharide was recovered by centrifugation at 1900 $\times$ *g* at 4°C for 30 min, resuspended in water, dialysed against water, filtered (Millipore membranes 0.8 μm), taken to pH = 7 and lyophilised.

2.7 | In vitro exposure of drugs to bacterial strains and to *K. pneumoniae* CPS

Bacterial strains grown overnight in Luria Broth were diluted 1:50 in fresh Broth, incubated at 37°C with shaking for about 2 h until they reached a concentration of about 7×10^8 CFU ml^{-1} (optical density at 600 nm of 0.70 mA) and diluted in M9 Minimal Salts Medium (Sambrook et al., 1989) at a final concentration of 10^7 CFU ml^{-1}. AZA, MP, TG and methylprednisolone (400 μM) were added to each bacterial suspension and to M9 medium only and incubated for 4 hat 37°C. Additionally, thiopurines were incubated with three serial dilutions (3.75–15–60 μg ml^{-1}) of *K. pneumoniae* CPS, extracted and purified as previously described (Bellich et al., 2020). Concentrations of CPS for the in vitro exposure were chosen based on estimates of CPS levels produced by microorganisms during incubation. At the end of the in vitro exposure, all samples were filtered and stored at −80°C until their use for the tests indicated in the succeeding discussions.

2.8 | MTT cytotoxicity test

To compare the effects of the tested drugs incubated or not with bacteria, the cytotoxicity on cell lines, exposed to the treatments, was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

NALM6 and JURKAT cells were seeded at a density of 2×10^4 or 1×10^4 cells per well, respectively, in 96-well plates. Seven serial dilutions in RPMI 1640 complete medium of thiopurines (ranging from 0.2 to 15 μM of AZA, from 0.3 to 20 μM of MP and from 0.08 to 5 μM of TG), previously exposed or not to bacteria, were added to each well and incubated for 72 h. The cytotoxicity of thiopurines, exposed to increasing concentrations of *K. pneumoniae* CPS, on NALM6 and JURKAT cells was evaluated with the same procedure and conditions described earlier.

In order to evaluate if the variation of cytotoxic effects were specific for thiopurines, NALM6 cells were treated with six serial dilutions of methylprednisolone (ranging from 2 x 10^{-4} to 4 μM) exposed or not to bacteria and incubated for 72 h.

Cytotoxicity was determined by adding 20 μl of MTT solution (5 mg ml^{-1} in phosphate buffered saline) (Sigma-Aldrich, Milan, Italy). Living cells reduce the yellow MTT to a blue formazan product. After 4 h of incubation at 37°C, the formazan product was dissolved in 100 μl dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Milan, Italy), and the plates were read at 540 and 630 nm using the Automated Microplate Reader EL311 (BioTek Instruments, Vermont, USA). The percentage of cell viability was expressed as a ratio to controls consisting of cells not treated with drugs but exposed to M9 or bacterial-conditioned media.

2.9 | Spectrophotometric analysis

To determine the concentration of thiopurines exposed or not to the bacterial strains or to *K. pneumoniae* CPS, absorbance peaks were analysed by UV spectrophotometry (280 nm for AZA, 320 nm for MP and 340 nm for TG) using UV-6300PC Double Beam Spectrophotometer (VWR, Milan, Italy). Calibration curves of each compound were made to quantify the amount of thiopurines in the samples.

2.10 | Sample preparation for the measurements of thiopurine metabolites in lysates and of thiodeoxyguanosine (dTGUA) in DNA of NALM6 and JURKAT cells

Three million NALM6 cells and 1.5×10^6 JURKAT cells were seeded in 50 ml tubes and treated with the most significant concentrations derived from the MTT tests of AZA (15 μM), MP (2.5 μM) and TG (1.25 μM) exposed or not to *K. pneumoniae*. Timing of treatment depended on the type of experiment: for the measurements of thiopurine metabolites, cells were treated for 48 and 72 h, whereas quantification of dTGUA in DNA required a 72-h treatment. At the end of the incubation, cells were counted by Trypan blue dye exclusion assay. After centrifugation at 600 × *g* for 5 min at room temperature, the supernatant was removed and dried pellets were collected and stored at −80°C until the subsequent analyses.

2.11 | Quantification of thiopurine metabolites by LC–MS/MS

A mixture of 250 μl of EDTA 50 mM, 15 μl of dithiothreitol solution 30 mg ml^{-1} and 10 μl of internal standard working solution (20 pmol μl^{-1} [$^2H_3$]MeTGMP, 60 pmol μl^{-1} [$^2H_3$]MeTGDP/[$^2H_3$]MeTGTP, 100 pmol μl^{-1} [$^2H_3$]MeTIMP, 160 pmol μl^{-1} [$^2H_3$]MeTIDP/[$^2H_3$]MeTITP, 40 pmol μl^{-1} [$^2H_4$]TGMP, 80 pmol μl^{-1} [$^2H_4$]TGTP/[$^2H_4$]TGDP) (obtained by chemical synthesis as previously reported; Hofmann et al., 2012) was added to the dried cell pellets and vortex mixed. Proteins were denatured by heating for 5 min at 95°C. All samples were subsequently extracted by the addition of 50 μl of methanol followed by the addition of 250 μl of dichloromethane with thorough mixing after each step. After centrifugation at 16100 × *g* for 20 min, 10 μl of the supernatant was used for liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis as previously described (Hofmann et al., 2012) on an Agilent 6495B triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany) coupled to an Agilent 1290 Infinity II HPLC system with electrospray ionisation mode in positive polarity. Chromatographic separation was carried out on a Biobasic AX column (2.1 × 50 mm, 5 μm particle size, Thermo Electron, Egelsbach, Germany) using water: acetonitrile 7:3 (*v*/*v*), with 10 mM ammonium acetate at pH 6 as mobile phase A and with 1 mM ammonium acetate at pH 10.5 as mobile phase B. The metabolites detected were TGMP (thioguanosine monophosphate), TGDP (thioguanosine diphosphate), TGTP (thioguanosine triphosphate), MeTGMP (methylthioguanosine monophosphate), MeTGDP (methylthioguanosine diphosphate), MeTGTP (methylthioguanosine triphosphate), TIMP (thioinosine monophosphate), TIDP (thioinosine

diphosphate), TITP (thioinosine triphosphate), MeTIMP (methylthioinosine monophosphate), MeTIDP (methylthioinosine diphosphate) and MeTITP (methylthioinosine triphosphate). Metabolite concentrations were expressed as pmol per million cells (pmol/Mio).

2.12 | Quantification of dTGUA in DNA by LC–MS/MS

DNA was extracted from cellular pellets of NALM6 and JURKAT cells treated with thiopurines previously exposed or not to *K. pneumoniae* using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), and subsequently 1 μg of genomic DNA was digested with 15 U Degradase PlusTM (Zymo Research) for 16 h in a total volume of 30 μl. Samples were spiked with 6 μl of internal standard solution (4.2 pmol μl^{-1} [$^2H_4$]6-thioguanosine in water: acetonitrile 1:1 *v*/*v*) (obtained by chemical synthesis as previously reported; Hofmann et al., 2012), mixed and centrifuged. Ten microlitres of the supernatant was used for LC–MS–MS analysis with a previously described method (Atreya et al., 2016) adapted on an Agilent 6495B triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany) coupled to an Agilent 1290 Infinity II HPLC system with electrospray ionisation mode in positive polarity. HPLC separation was achieved on a Poroshell 120 SB-C8 column (150 × 2.1 mm, 3 μm particle size, Agilent) using (A) 0.0075% formic acid in water and (B) 0.0075% formic acid in acetonitrile as mobile phases at a flow rate of 0.4 ml min^{-1}. Gradient runs started at 3.5% B, followed by linear increase to 5% B from 2 to 6.9 min, increase to 80% B to 7.2 min, remaining at 80% B to 9 min and then re-equilibration. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode using the precursor/product ion pairs of *m/z* 284.1/168.1 and *m/z* 284.1/150.9 as quantifier and qualifier for dTGUA and *m/z* 304.1/168 for the internal standard [$^2H_4$]6-thioguanosine. Calibration samples were prepared using dTGUA (Sigma-Aldrich, Milan, Italy) in hydrolysis buffer in the concentration range from 0.1 to 25 pmol per sample. Calibration curves based on internal standard calibration were obtained by weighted (1/*x*) linear regression for the peak–area ratio of the analyte to the internal standard against the amount of the analyte. The concentration of the analyte in unknown samples was obtained from the regression line. Assay accuracy and precision were determined by analysing quality controls that were prepared like the calibration samples. Values were expressed as pmol on μg of DNA (pmol μg^{-1} DNA).

2.13 | Sample preparation and measurements of TPMT activity

To investigate the influence on TPMT activity of bacterial compounds released by *K. pneumoniae* during its growth (conditioned media), 5×10^6 NALM6 and 2.5×10^6 JURKAT cells were seeded in T175 flasks and exposed to RPMI medium (for the investigation of basal conditions), or M9 medium, or the *K. pneumoniae* conditioned media used in the previous tests. At the end of 72 h of incubation, cells were counted by Trypan blue dye exclusion assay. After centrifugation at 600 × *g* for 5 min at room temperature and removal of the supernatant, dried pellets were collected and stored at −80°C for high performance liquid chromatography coupled with a UV detector (HPLC-UV) analyses. Subsequently, dried pellets were thawed, resuspended in phosphate buffer and frozen again at −80°C to enhance the lysis of cells. On the day of the analysis, samples were thawed and sonicated in a water bath for 20 min to achieve the complete lysis; then they were processed as previously described (Schaeffeler et al., 2004), and 25 μl was used for measurements of TPMT activity with the already established method using an Agilent 1200 HPLC coupled with a UV detector (Schaeffeler et al., 2004). In brief, the method is based on the conversion of TG in methylthioguanine (MeTG). Measurements were further normalised to the protein concentration of the samples. Therefore, values were expressed as pmol of MeTG produced per hour, normalised on microgram of proteins.

2.14 | Detection of thiopurines in *K. pneumoniae* pellets by HPLC-UV

Samples containing *K. pneumoniae* exposed or not to thiopurines were diluted 1:10 in fresh M9 medium and centrifuged at 12000 × *g* for 20 min at 4°C to obtain bacterial pellets. Afterwards, bacteria were washed with phosphate buffered saline, centrifuged again at 12000 × *g* for 5 min at 4°C and collected as dried bacterial pellets until HPLC-UV analysis. On the day of the analysis, bacterial pellets were lysed adding 100 μl of solution of acetonitrile and methanol (1:1) (Sigma-Aldrich, Milan, Italy) and incubated at −20°C for 1.5 h. Subsequently, bacterial debris were removed by centrifugation at 12000 × *g* for 20 min at 4°C, and the supernatant was placed under a nitrogen stream. Finally, the dried analyte was resuspended in Milli-Q water (Merck Millipore, Milan, Italy), and 10 μl was injected in the instrument Agilent 1200 HPLC coupled with a UV detector for the detection of AZA, MP and TG in *K. pneumoniae* pellets. Separation of the compounds of interest was achieved using a reverse-phase column Poroshell 120 SB-C8 (2.1 × 150 mm, 2.7 μm, Agilent Technologies, Milan, Italy). The mobile phases consisted of 0.0075% formic acid (Sigma-Aldrich, Milan, Italy) in water (eluent A) and 0.0075% formic acid in acetonitrile (eluent B), and the separation was performed using a gradient at a flow rate of 0.4 ml min^{-1} for 15 min. In particular, at time 0, the flow consisted in 100% eluent A that first linearly decreased at 96.5% (after 2 min) and then at 20% (after 7 min) until 10 min. The last 5 min, the column was reconditioned before the next injection. The column temperature was set at 30°C. Regarding the spectrophotometric conditions, the detection wavelengths were set up at 280, 320 and 340 nm (the peaks of absorbance of AZA, MP and TG, respectively). Calibration curves were constructed in water by preparing of a series of dilutions of the drugs (1, 2.5, 5, 10, 15 and 20 μM) after assessing that there is no signal interference of the matrix in the retention times of the analytes.

Quantification of thiopurine metabolites TGN in red blood cells (RBCs) collected from CD paediatric patients was performed by HPLC-diode array detection, using a previously reported method (Franzin et al., 2022).

2.15 | Metabolomic analyses of *K. pneumoniae* conditioned media, data preprocessing and feature annotation

Metabolomic analyses were performed on *K. pneumoniae*-conditioned media derived from the in vitro exposures, using a 1290 Infinity UHPLC System coupled to a 6550 iFunnel quadrupole time-of-flight mass spectrometer (LC-QTOF-MS) from Agilent Technologies equipped with a Dual Agilent Jet Stream electrospray source. Samples were prepared as previously described (Zimmermann et al., 2019a), and 1 µl was injected into the instrument. Separation was achieved using the same chromatographic conditions used for the detection of thiopurines in *K. pneumoniae* pellets by HPLC-UV using the reverse-phase column Poroshell 120 SB-C8 (2.1 × 150 mm, 2.7 µm, Agilent Technologies, Milan, Italy). Analytical batches were analysed by mass spectrometry in positive and negative mode. The instrument mode was set to extended dynamic range (2 Ghz) and the mass range to low (1700 *m/z*). The mass analyser was calibrated on a daily basis immediately before starting an analytical run. TOF-MS spectra were acquired in centroid mode (intensity threshold 10 counts per 0.001%) at an acquisition rate of 4 spectra s^{-1} from *m/z* 50 to *1650*. Fragment spectra were acquired in samples by auto MS/MS analysis at a rate of 4 spectra s^{-1} for MS1 and MS/MS acquisitions, respectively. MS/MS spectra were triggered from precursors that exceeded an absolute threshold of 200 counts and by selecting maximal three precursors per cycle. Collision energy (V) was adjusted as a function of *m/z* (3.5 × *m/z* × 100–1 + 7), and the quadrupole band-pass for precursor isolation was set to medium (∼4 *m/z*).

To find potential bacterial drug biotransformation products, metabolic features were extracted by Mass Hunter Profinder Batch Recursive Feature Extraction (software version B.08.00, Agilent Technologies). Features that were absent in *K. pneumoniae*-conditioned control media (without drug) were selected as candidate drug metabolites by visual inspection of the data. Assignment of metabolic features to chemical structures was performed by Sirius (Lehrstuhl Bioinformatik Jena, https://bio.informatik.uni-jena.de/software/sirius/) (Dührkop et al., 2019). To increase confidence in the annotation, chemical formulas proposed by Sirius were subjected to the MassHunter Qualitative Analysis Find by Formula (FBF) function, and only compounds achieving a FBF score ≥ 80 were considered as putatively annotated.

Regarding endogenous metabolites, features were extracted by MassHunter Qualitative Analysis Find by Auto MS/MS function and annotated via MassHunter METLIN Metabolite PCDL search (software and PCDL version B.07.00, Agilent Technologies). Based on the putative assigned metabolites, Mass Hunter Profinder Batch Targeted Feature Extraction was performed (software version B.08.00, Agilent Technologies). Results were exported as comma separated value files to perform further data preprocessing and statistical analysis with R 4.2.3 and R studio (http:// www.r-project.org).

2.16 | Collection and sequencing of faecal microbiota samples

Faecal samples were collected from eight CD paediatric patients who had undergone therapy with thiopurine for at least 3 months but no concurrent medication at the Gastroenterology units of IRCCS Burlo Garofolo Hospital (Trieste) and Ca′ Foncello (Treviso). For metagenomic analysis, the stools were processed for total DNA extraction and sequencing with Illumina Nextera WGS shotgun protocol. Metagenomic analysis of fastq files was performed following the previously published guidelines for taxonomic (MetaPhlAn 4.0) profiling of metagenomes (Blanco-Miguez et al., 2023). This pipeline leverages a set of 99,200 high-quality and fully annotated reference microbial genomes spanning 16,800 species. The taxonomic profiling and quantification of organisms' relative abundances of all metagenomic samples were quantified using MetaPhlAn 4.0 with default parameters. In total, we identified 503 microbial species, among which we retrieved 91 genus-genome-binned (GGB) unnamed species. Relative abundances of microbial species underwent transformation (multiplicative replacement followed by centre-log-ratio clr functions; Sci-Kit learn package v1.0.1), then normalisation and standardisation using QuantileTransformer and StandardScaler methods from Sci-Kit learn package v1.0.1. Normalisation using the output_distribution = 'normal' option transforms each variable to a Gaussian-like distribution, while the standardisation results in each normalised variable distribution having a mean of zero and unit variance. These two steps of normalisation and standardisation ensure the proper comparison of variables with different dynamic ranges, such as microbial relative abundances.

2.17 | Quantification and statistical analysis

Statistical tests were conducted only when the group size (number of independent biological samples) was at least $n = 5$. Experiments with group sizes $n < 5$, which were exploratory in nature (i.e. Figures 5, S1–S6 and S11, and the assessment of bacterial modification products in Supporting Information), were not subjected to statistical analysis. Post hoc tests were performed only if F achieved $P < .05$, and there was no significant variance inhomogeneity. Information about immortalised cell lines was not considered to select the experimental condition (e.g. randomisation), and the experimenter was not blinded to treatment conditions. Statistical analyses were carried out using GraphPad Prism software (version 8). In brief, statistical significance was assessed by two-way ANOVA and Bonferroni's post-test for cytotoxicity tests, because data are normally distributed. Welch's *t*-test was carried out for UV analyses and for measurements of dTGUA in DNA. All the results are presented as mean ± standard error (SE). The significance threshold was set at 0.05.

Statistical analyses regarding the measurements of thiopurine metabolites were performed with R software (version 3.2.4) (R Core Team, 2013). After scaling data variables to have a mean value of 0 and a variance of 1, clustering analysis was performed with the complete method and a chi-squared test was performed to assess a difference in the incidence of exposed versus not exposed samples between the groups identified by the clustering. Furthermore, data were analysed by fitting analysis of variance (ANOVA) models (aov function of the stats package), considering each metabolite concentration as the dependent variable and treatment time, drug used and the experimental condition (exposure of drugs to M9 or to *K. pneumoniae*) as independent variables. The significance threshold was set at 0.05.

For metabolomics analyses to screen differentially abundant endogenous metabolites, log2 fold changes were calculated between the median of the abundances of the metabolites derived from the conditioned media by *K. pneumoniae* not exposed to drugs and those exposed to thiopurines. Welch's *t*-test was performed on log2 transformed data using R software (version 4.2.3), setting the significance threshold at 0.05. Graphics were generated using ggplot2 package (Wickham, 2009).

Spearman's test was used to assess the correlation between TGN and the relative abundance of *K. pneumoniae* and *E. coli* in the faecal samples of CD patients ($n = 8$). The significance threshold was set at 0.05. As we evaluated a potential correlation between two variables and did not compare two groups (e.g. treatment vs placebo), no randomisation and blinding were applied.

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.18 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/2022 (Alexander et al., 2021).

3 | RESULTS

3.1 | Bacterial susceptibility to drugs

The Broth Microdilution Susceptibility Test showed that even the highest concentration tested (400 μM) of AZA, MP, TG and methylprednisolone (used for validation), as selected by considering the mean concentration of several drugs in the intestine (Zimmermann et al., 2019a), did not affect the growth of *E. coli*, *S. enterica* and *K. pneumoniae* after 24 h (Table S1). Thus, a concentration of 400 μM of each drug was chosen for the subsequent in vitro exposures.

3.2 | Effects of candidate bacterial species on cytotoxicity of thiopurines

The MTT assay was performed to compare the cytotoxicity of thiopurines exposed or not to bacteria and thus evaluate a potential microbial biotransformation.

The cytotoxic effects on NALM6 cells (Figure 1a–c) after a treatment of 72 h with AZA, MP and TG were significantly lower after incubation of drugs with *K. pneumoniae* (AZA: $IC_{50} > 15$ μM; MP: $IC_{50} = 1.28$ μM; TG: $IC_{50} = 0.95$ μM) compared to the drugs not exposed to bacteria (AZA: $IC_{50} > 15$ μM; MP: $IC_{50} = 1.25$ μM; TG: $IC_{50} = 1.85$ μM). In particular, at the highest concentration of AZA (15 μM) and at concentrations higher than 0.625 μM of MP and of TG, a significant difference between the toxicity of drugs exposed or not to the bacterial strain was observed.

As shown in Figure 1e–f, the cytotoxic effects on JURKAT cells after a 72-h treatment with MP and TG were significantly reduced when the thiopurines were preincubated with *K. pneumoniae* (MP: $IC_{50} = 1.99$ μM; TG: $IC_{50} = 1.67$ μM), compared to the drugs in Minimal Salts Medium M9 (MP: $IC_{50} = 1.48$ μM; TG: $IC_{50} = 0.82$ μM). In particular, a significant difference between drugs (from 1.25 μM for MP and from 0.6 to 1.25 μM for TG) in M9 and the ones exposed to the bacterial strain was observed. Likewise, even if not significant in post-test, a trend in reduced cytotoxicity after the exposure of AZA to the bacterial strain was evident (Figure 1d).

Exposure to *E. coli* and *S. enterica* did not seem to affect the cytotoxicity of the thiopurines tested (Figures S1 and S2).

3.3 | Effects of candidate bacterial species on concentrations of thiopurines

UV spectrophotometry analysis was performed to identify variations in the concentration of thiopurines after the exposure to the bacterial strains.

After incubation of *K. pneumoniae* with 400 μM of thiopurines, the concentration of AZA, MP and TG significantly decreased compared to the drug in M9 (Figure 2a–c). In particular, the concentration was reduced by 23% (315.78 ± 9.05 μM) for AZA, by 46% (218.73 ± 14.36 μM) for MP and by 30% (289.38 ± 13.33 μM) for TG. Furthermore, *Klebsiella pneumoniae* incubated or not with thiopurines grew in logarithmic phase from the concentration of 10^7 to 8.5×10^8 CFU ml^{-1} at the end of the in vitro exposure.

In accordance with the results of the cytotoxicity tests, UV analysis did not show apparent changes of the concentrations of thiopurines exposed to *E. coli* and *S. enterica* (Figure S3) in comparison with drugs in M9. Therefore, further investigations focussed on *K. pneumoniae* were performed.

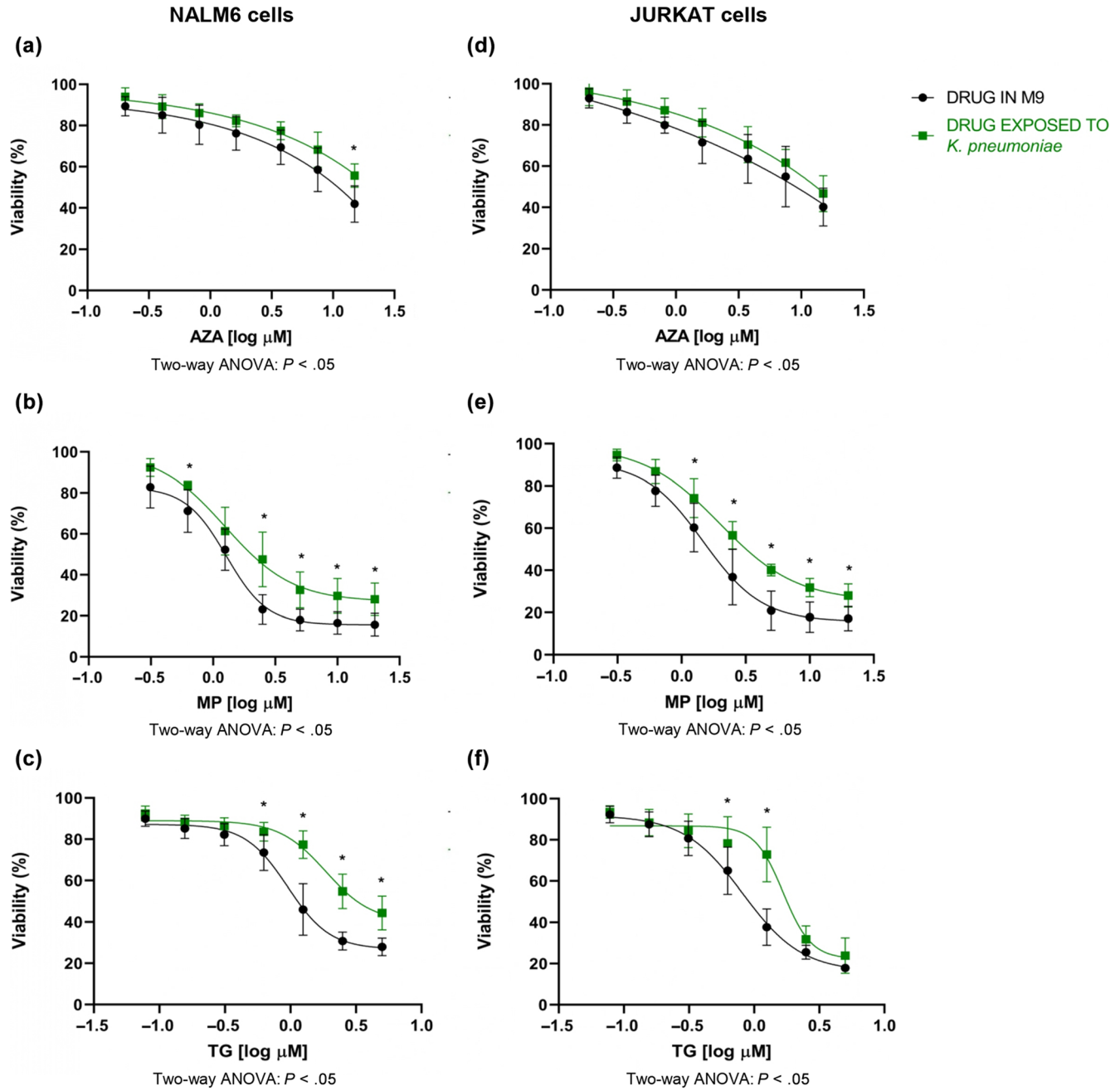

FIGURE 1 Effects on cytotoxicity of thiopurines exposed to *Klebsiella pneumoniae*. Evaluation of cytotoxic effects through MTT assay ($n = 8$) on (a–c) NALM6 and (d–f) JURKAT cells after treatment with different concentrations of AZA (from 0.2 to 15 μM), MP (from 0.3 to 20 μM) and TG (from 0.08 to 5 μM) exposed to *K. pneumoniae* or not. * $P < .05$, two-way ANOVA, Bonferroni post-test.

3.4 | Cytotoxicity of methylprednisolone exposed to *K. pneumoniae*, *E. coli* and *S. enterica*

To validate our results and assess the specificity of the effect of *K. pneumoniae* on thiopurine drugs, in vitro exposure of bacteria to methylprednisolone, a glucocorticoid agent used in IBD treatment, and subsequent cytotoxicity tests were performed. The results, represented in Figure S4, did not evidence variations in the cytotoxic effects of this drug on NALM6 cells when exposed to bacteria, thus suggesting that methylprednisolone is not metabolised by these bacterial species.

3.5 | Cytotoxicity of thiopurines exposed to *K. pneumoniae* CPS

K. pneumoniae strains can produce capsular polysaccharides (CPS). These structures, found on bacterial cell surfaces and secreted from bacteria, mediate the interactions between microorganisms and environment and, to achieve this purpose, can form structures with compounds, such as antimicrobial peptides (Benincasa et al., 2016). To exclude the role of CPS, on cell surfaces or secreted, in forming complexes with thiopurine drugs and therefore in reducing the

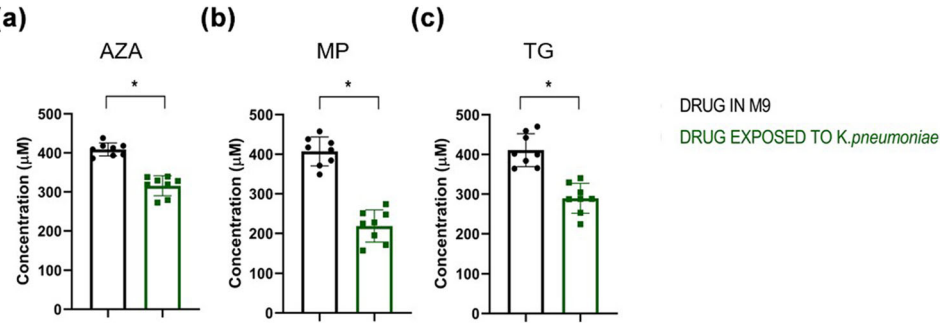

FIGURE 2 Effects on concentration of thiopurines exposed to *Klebsiella pneumoniae*. Concentration of AZA (a), MP (b) and TG (c) (means ± SEM; n = 8) exposed to *K. pneumoniae* or not. Welch's *t*-test (* $P < .05$).

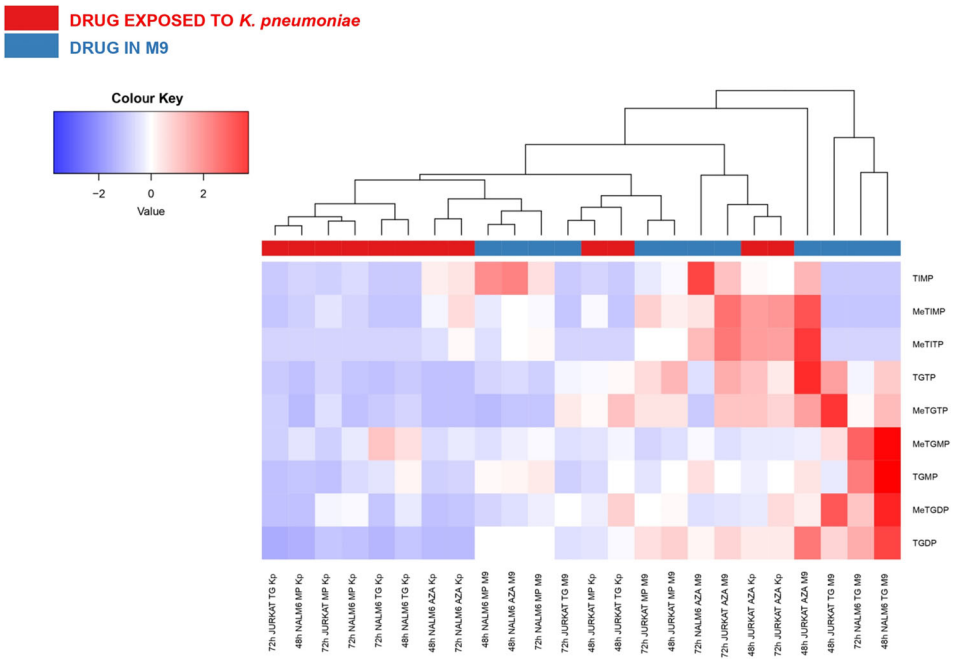

FIGURE 3 Thiopurine metabolites were lower in NALM6 and JURKAT cells treated with thiopurines previously exposed to *Klebsiella pneumoniae*. Heatmap of the normalised thiopurine metabolites measurements ($n = 5$) in NALM6 and JURKAT cells treated for 48 and 72 h with drugs in M9 or exposed to *K. pneumoniae*.

cytotoxicity, in vitro exposure and subsequent cytotoxicity tests were performed with thiopurines exposed to *K. pneumoniae* CPS. This procedure showed that incubating with increased concentrations of CPS did not affect cytotoxicity (Figure S5) and concentrations (Figure S6) of thiopurines.

3.6 | Quantification of thiopurine metabolites in NALM6 and JURKAT cells treated with drugs previously exposed to *K. pneumoniae*

Thiopurine metabolites were measured in NALM6 and JURKAT cells treated with AZA (15 μM), MP (2.5 μM) and TG (1.25 μM) exposed or not to *K. pneumoniae*. The concentrations were chosen on the basis of statistical significance of Bonferroni's analyses on the previously performed cytotoxicity tests. The metabolites quantified were TGMP, TGDP, TGTP, MeTGMP, MeTGDP, MeTGTP, TIMP, TIDP, TITP, MeTIMP, MeTIDP and MeTITP. A summary of thiopurine metabolite measurements and the box plots representing each metabolite concentration depending on treatment time, cell lines and condition (exposed to bacteria or not) are provided in Tables S2–S7 and Figures S7–S9.

Interestingly, thiopurine metabolites were lower in NALM6 and JURKAT cells treated with drugs previously exposed to *K. pneumoniae* (Figure 3). Clustering of metabolite concentrations correctly groups accordingly to exposure status to *K. pneumoniae* ($P = .041$, Chi-squared test).

3.7 | Incorporation of dTGUA in DNA of NALM6 and JURKAT cells treated with thiopurines previously exposed or not to *K. pneumoniae*

Measurements of the dTGUA metabolite in DNA were performed after a 72-h treatment of NALM6 and JURKAT cells with thiopurines exposed or not to *K. pneumoniae*. dTGUA concentrations reflect the

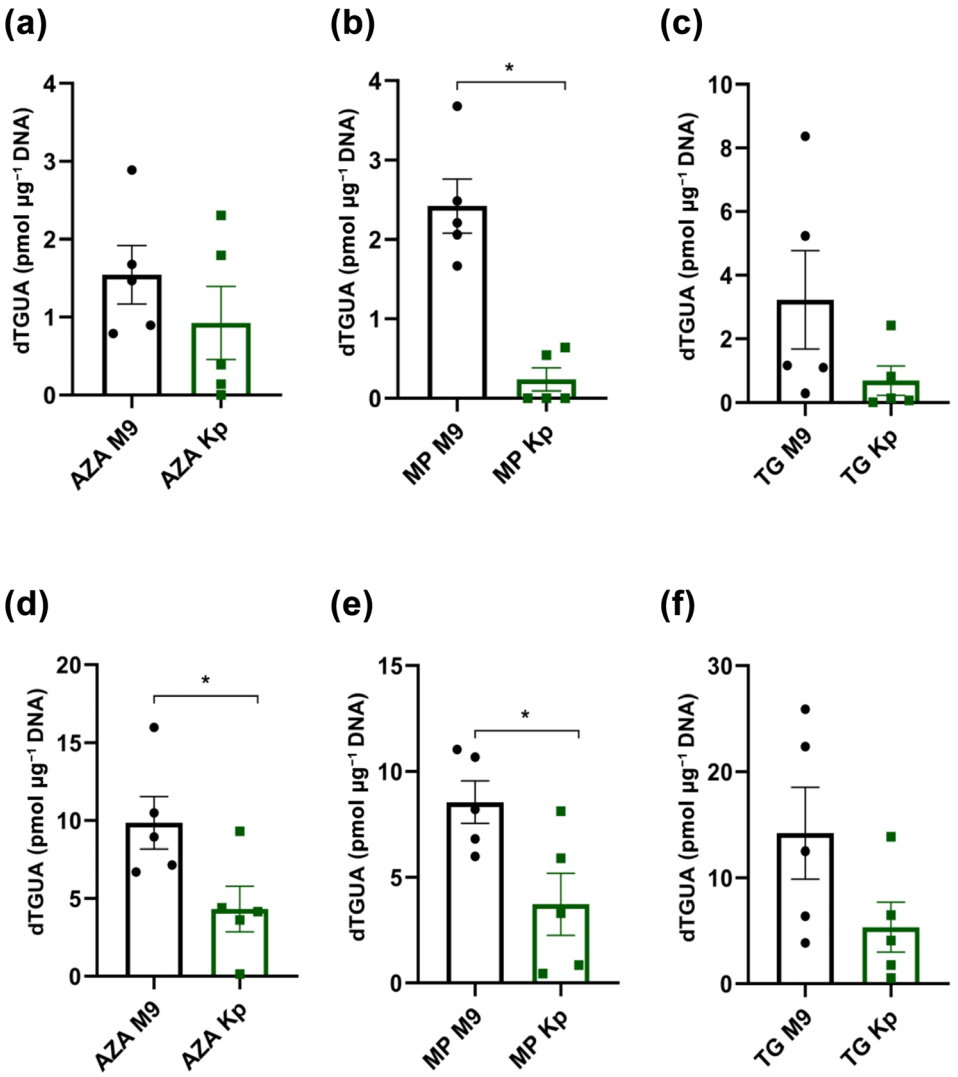

FIGURE 4 Incorporation of dTGUA in DNA of NALM6 and JURKAT cells treated with thiopurines previously exposed to *Klebsiella pneumoniae*. Concentrations of dTGUA (means ± SEM; n = 5) in the DNA, detected after a 72-h treatment of (a–c) NALM6 and (d–f) JURKAT cells with 15 µM AZA, 2.5 µM MP and 1.25 µM TG in M9 (AZA M9/MP M9/TG M9) or M9 previously exposed to *K. pneumoniae* (AZA Kp/MP Kp/TG Kp). Welch's *t*-test (* $P < .05$).

incorporation of dTGTP in DNA and therefore reflect the cytotoxic effects of thiopurines.

Comparing the concentration of dTGUA in DNA of NALM6 and JURKAT cells, the metabolite was more abundant in JURKAT cells' DNA (Figure S10).

As shown in Figure 4b, the detected concentration of dTGUA incorporated in DNA of NALM6 cells treated for 72 h with MP previously exposed to *K. pneumoniae* was lower compared to the concentration quantified in cells treated with the drugs in M9. Furthermore, even if the difference between the experimental conditions was not statistically significant, the same trend was observed for NALM6 treated with AZA and TG previously exposed to the bacterial strain.

Similar to what was observed for NALM6, a 72-h treatment of JURKAT cells with AZA and MP previously exposed to *K. pneumoniae* revealed significantly lower levels of dTGUA in DNA (Figure 4d and e). Moreover, although the decrease was not considered significant by Welch's *t*-test, the same trend was observed for JURKAT treated with TG previously exposed to the bacterial strain.

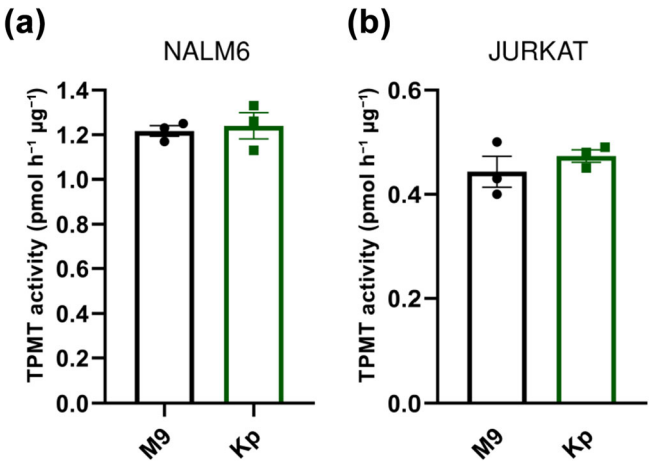

FIGURE 5 **TPMT activity was not influenced by bacterial conditioned media.** TPMT activity (means ± SEM; n=3) of (a) NALM6 and (b) JURKAT cells exposed to M9 and in *Klebsiella pneumoniae* conditioned medium.

TABLE 1 **Thiopurines detection in *Klebsiella pneumoniae*.** Average concentration of AZA, MP and TG (means ± SEM; n=5) detected in *K. pneumoniae* pellets after in vitro exposure of the bacterial strain to thiopurines. n.d. not detected.

Bacterial samples	Concentration of AZA (μM)	Concentration of MP (μM)	Concentration of TG (μM)
K. pneumoniae exposed to AZA	n.d.	6.58 ± 0.11	n.d.
K. pneumoniae exposed to MP	n.d.	194.90 ± 1.80	n.d.
K. pneumoniae exposed to TG	n.d.	n.d.	203.64 ± 1.75

3.8 | TPMT activity in NALM6 and JURKAT cells exposed to bacterial conditioned media

Because NALM6 cells treated with AZA previously exposed to *K. pneumoniae* presented higher levels of MeTIMP (Table S2), and in order to investigate if TPMT activity was influenced by bacterial compounds released by *K. pneumoniae*, the activity of the enzyme in NALM6 and JURKAT cells exposed to M9 and bacterial conditioned medium was measured. TPMT activity was higher in NALM6 compared to JURKAT cells (Figure S11).

As shown in Figure 5, given the fact that data achieved are explorative, there is no apparent change of TPMT activity in both cell lines exposed to M9 or to *K. pneumoniae* conditioned medium.

3.9 | Quantification of thiopurines in *K. pneumoniae*

In order to evaluate if thiopurines are internalised by *K. pneumoniae*, the drug concentration inside bacterial cells was quantified after the in vitro exposure of *K. pneumoniae* to AZA, MP and TG (400 μM) and lysis of the bacterial outer membrane. As shown in the results of Table 1, AZA was not detected in any samples of bacterial lysate; on the contrary, MP was detected in bacteria both after incubation with AZA and with MP, while TG was detected only after incubation with TG.

3.10 | Untargeted metabolomics to assess thiopurine drugs biotransformation and effects on bacterial metabolite secretion in *K. pneumoniae* conditioned media

Targeted analyses of the parent drugs and known human thiopurine metabolites did not entirely explain drug fate by metabolism or internalisation. Therefore, non-targeted metabolomics was used to assess whether the bacteria tested can produce additional thiopurine metabolites that were not covered by our targeted analysis. Furthermore, it was evaluated whether thiopurine treatment differentially affects the secretion of endogenous metabolites. Several metabolites were found to be absent or differently abundant depending on the bacterial conditioned media sample (see succeeding texts).

Data acquired by LC–MS/MS both in positive and negative ionisation mode were processed by screening metabolic features present in *K. pneumoniae* conditioned media after exposure to AZA, MP and TG, but not in untreated controls. A list of putatively annotated compounds with their *m/z* ratio depending on the ionisation mode, retention time, type of bacterial conditioned media in which they are present, proposed chemical formula, scores of identification, mass shift to the original thiopurine compound (Δ*m/z*) and consequent bacterial modification proposed are presented in Supporting Information 2. Noteworthy, after exposure of the bacterial strain to AZA, besides known drug derived metabolites, such as MP or thioxanthine-ribose, several structures corresponding to reduction of AZA, and with subsequent conjugation with a hexose or acetyl group, were putatively annotated. Similar explorative results were achieved after exposure of the bacterial strain to MP and TG. Indeed, a feature that might be thioxanthine-ribose was present in *K. pneumoniae* exposed to MP; instead, features putatively annotated to **xanthine**, as well as TG conjugated with propionic acid, were found in *K. pneumoniae* exposed to TG.

Furthermore, the results obtained from the analysis of endogenous metabolites suggest differences in the abundance of extracellular metabolites secreted by *K. pneumoniae* exposed to AZA, MP and TG (Figure 6).

After exposure of the bacterial strain to AZA, compounds putatively identified as **nicotinic acid**, N-carbamoyl-aspartic acid, **guanine** and **hypoxanthine** were significantly released by *K. pneumoniae*, along with an increasing trend in **tryptophan** levels. In contrast, **ketoglutaric acid** was significantly reduced.

Moreover, the dipeptide composed of **L-proline** and **L-glutamic acid** (Pro-Glu), glutamate, **leucine**, **tyrosine**, **phenylalanine**, hypoxanthine, nicotinic acid, N-carbamoyl-aspartic acid and **orotic acid** were significantly released in *K. pneumoniae* exposed to MP; on the other hand, features putatively identified as **malic acid**, **phenyllactic acid**, itaconic acid, hydroxyisovaleric acid and ketoglutaric acid were less present.

Lastly, *K. pneumoniae* exposed to TG showed significantly higher levels of features putatively identified as N-carbamoyl-aspartic acid, nicotinic acid, tyrosine, leucine, guanine and orotic acid, along with reduced levels of malic acid, phenyllactic acid and ketoglutaric acid.

3.11 | Association between *K. pneumoniae* abundance and concentrations of TGN in CD paediatric patients

The faecal microbiota of paediatric IBD patients was sequenced to investigate if the Enterobacteriaceae, whose effect on drugs was examined in vitro, could affect the bioavailability of thiopurine drugs.

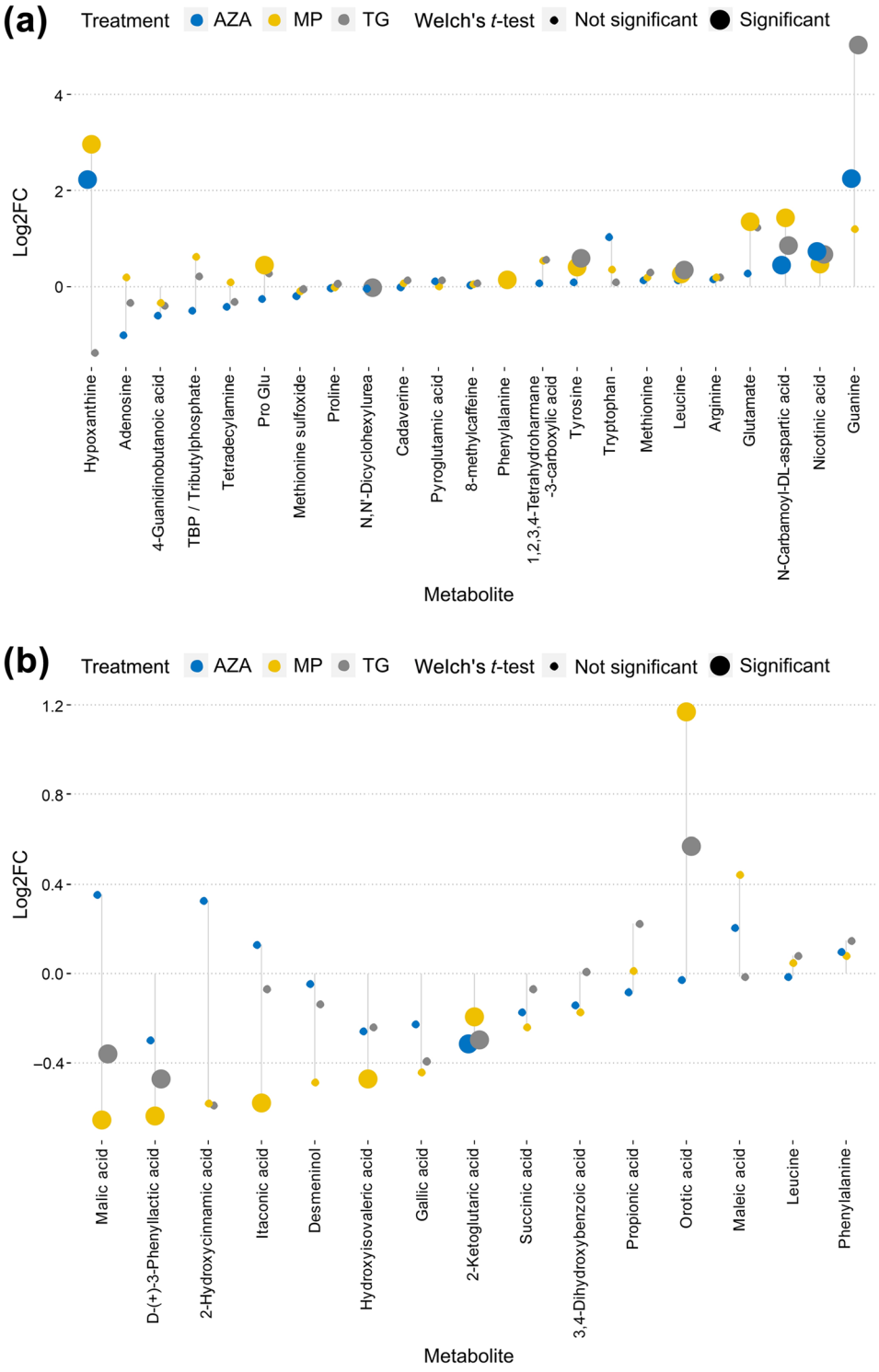

FIGURE 6 Differences in the abundance of extracellular metabolites secreted by *Klebsiella pneumoniae* exposed to thiopurines. Results of metabolomic analysis ($n = 5$) of differences in extracellular metabolites secreted by *K. pneumoniae* exposed to AZA (blue), MP (yellow) and TG (grey) obtained in (a) positive ionisation mode and in (b) negative ionisation mode. The points in the graph show the log2 fold change (Log2FC) calculated between the median of the untreated control group (*K. pneumoniae* not exposed to drugs) and the respective experimental condition (*K. pneumoniae* exposed to AZA, MP or TG). Differences considered significant by Welch's *t*-test ($P < .05$) are shown with large dots, whereas the ones considered non-significant are shown with small dots.

In particular, the relative abundance of the bacterial species *K. pneumoniae*, *E. coli* and *Salmonella enterica* was correlated with the concentrations of the TGN thiopurine metabolites, measured in red blood cells (RBCs) and commonly associated with clinical response to these drugs.

This pilot study included eight paediatric CD patients, undergoing AZA therapy for at least 3 months. Three (37.5%) were males; the median age was 13.5 (interquartile range [IQR]: 3.7); the median dose normalised on the weight (mg kg^{-1}) was 2.3 (IQR:0.76); the median duration of the therapy was 15.8 months (IQR:11.0). Disease was located in ileocolon (L3) in one patient, in the terminal ileum and upper gastrointestinal tract (L1, L4) in one patient, in the colon (L2) in three patients and in the ileocolon and in the gastrointestinal tract (L3, L4) in three patients. Extraintestinal manifestations, such as

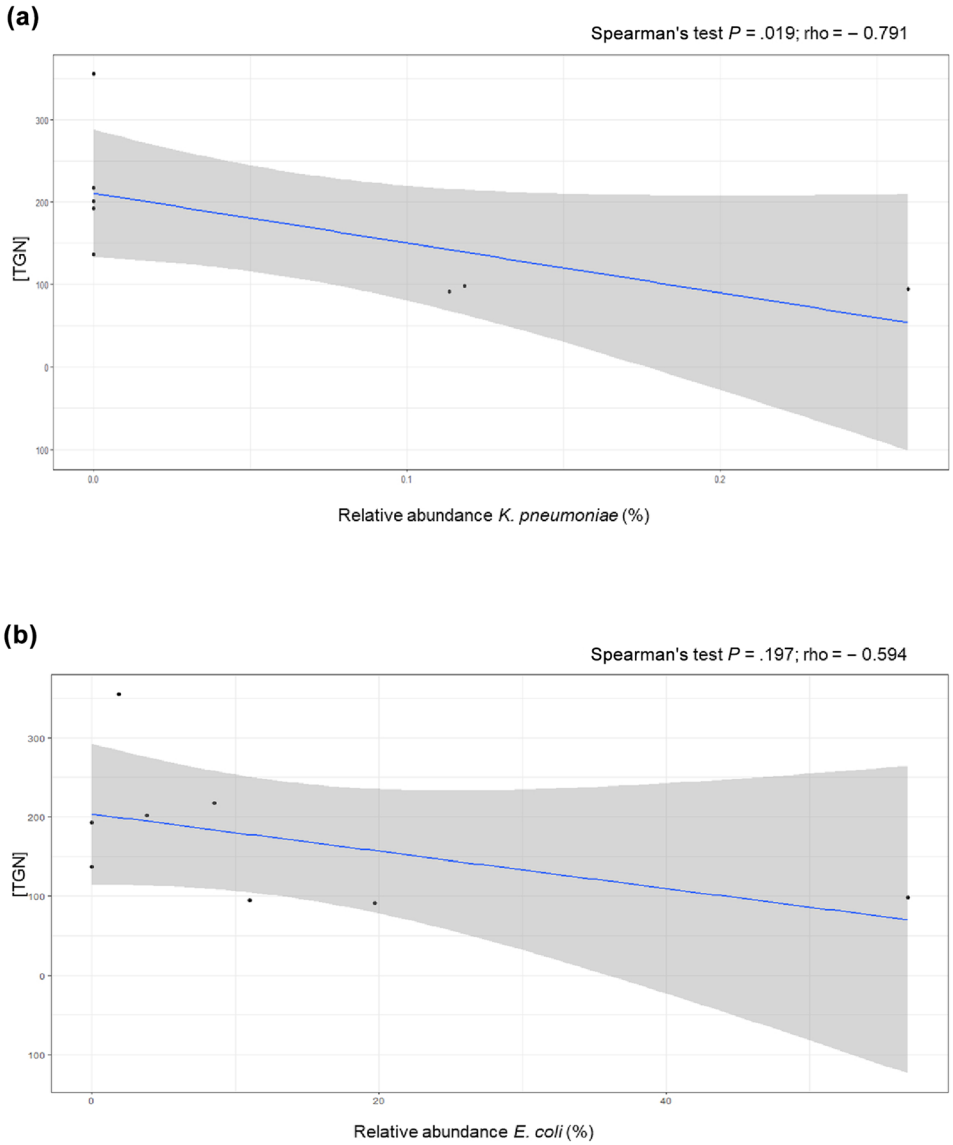

FIGURE 7 ***Klebsiella pneumoniae* was associated with lower concentrations of TGN in CD paediatric patients.** Correlation between the relative abundance (%) of (a) *K. pneumoniae* and (b) *E. coli* in faecal microbiota of eight CD patients with TGN concentration in RBCs (pmol/8 $\times$ 10^8 RBCs). Spearman's correlation test ($P = .019$ rho $= -0.791$ *K. pneumoniae*; $P = .197$ rho $= -0.524$ *E. coli*). Data correspond to the black points in the graph. The blue line represents the linear regression between the two variables with the 95% confidence interval (grey area).

hepatocholangitis and erythema nodosum, were present at the disease onset in two patients. All patients were in remission with the median disease activity score, in particular the Paediatric Crohn's Disease Activity Index (PCDAI), and calprotectin levels corresponding to 1.25 (IQR:3.75) and 53 mg kg^{-1} (IQR: 347), respectively. No other treatment, except for the thiopurine, was administered to the patients. Prior treatment consists in steroids and ursodeoxycholic acid.

Noteworthy, a significant negative correlation between the relative abundance of *K. pneumoniae* and the concentrations of TGN measured in RBCs, expressed as pmol/8 $\times$ 18^8 RBCs (Figure 7a), was shown, contrary to what was observed for *E. coli* (Figure 7b). *S. enterica* resulted not to be present in the faecal samples analysed.

4 | DISCUSSION

Bacteria belonging to the Enterobacteriaceae family are abundant in the intestinal microbiota of patients suffering from paediatric IBD, giving rise to a reduced diversity of gut bacterial species, a condition known as dysbiosis (Khan et al., 2019; Lucafo et al., 2020; Sundin et al., 2017). Thiopurines are drugs commonly used in the maintenance of remission of this disease but, despite their widespread use, therapeutic failure and adverse effects are relatively common (Franca et al., 2019; Konidari & Matary, 2014). Given the growing evidence of the influence of intestinal bacteria in drug therapies, the present study investigated in vitro the role of three bacterial species, belonging to the Enterobacteriaceae family, known to be enriched and potentially harmful in the intestinal microbiota of IBD patients in reducing the

response to thiopurines (Geller et al., 2017; Zimmermann et al., 2019a).

Our studies revealed that thiopurines (50–400 μM) do not exert an antimicrobial activity towards *E. coli*, *S. enterica* and *K. pneumoniae*, contrary to what was previously observed with other bacteria such as *Mycobacterium paratuberculosis* (Shin & Collins, 2008). This finding suggests that patients undergoing thiopurine treatment probably do not experience a reduction of these bacterial species in the intestinal microbiota. Thus, our experiments, performed exposing bacteria to a concentration of thiopurines which did not inhibit the bacterial growth, have no bias because of bacterial susceptibility to drugs.

After exposure of thiopurines to bacterial species, only *K. pneumoniae* caused a reduction in the cytotoxicity of thiopurines on the NALM6 and JURKAT lymphocytic cell lines, whereas exposure to *E. coli* and *S. enterica* did not affect them. In a previous paper, Oancea and colleagues described the ability of *E. coli* and other bacterial species to metabolise TG, after detecting thiopurine metabolites in bacterial cultures incubated with the drug (Oancea et al., 2017). However, no data showing the consequences on mammalian cells are available. Moreover, the concentration of TG (1 mM) used for the incubation was higher than the one used in the present study (Oancea et al., 2017).

In order to validate our experiments, in vitro exposure and subsequent cytotoxicity tests were performed with the glucocorticoid methylprednisolone, which is used in IBD treatment and has a mechanism of action and chemical structure different from thiopurines. Comparing the cytotoxicity of this drug exposed or not to bacteria, we concluded that methylprednisolone was not subjected to biotransformation by the bacterial strains tested, as other corticosteroids were (Yadav et al., 2013). Indeed **prednisolone**, **beclomethasone dipropionate** and **budesonide** were biotransformed by bacteria obtained from faecal slurries of three healthy volunteers (Yadav et al., 2013); one of the proposed mechanisms of biotransformation and consequent loss of biological activity was the hydroxylation at position 6, a reaction for which methylprednisolone could not be a substrate because of its chemical structure (Yadav et al., 2013).

According to UV analyses, the exposure to *K. pneumoniae*, but not to *E. coli* and *S. enterica*, reduced the concentrations of all thiopurine drugs, suggesting that the reduction of cytotoxicity observed for MP and TG was because of a lower amount of drugs in bacterial-conditioned media. All the subsequent experiments were therefore focussed on *K. pneumoniae*. Contrary to what was observed in our study, the work of Oancea suggested a reduction of TG concentration after incubation with *E. coli*, because of TGN production (Oancea et al., 2017). This may be because of the conversion of small amounts of the drug in metabolites (20 μM of TGN generating from 1 mM of TG), which was not evidenced by the single use of the UV spectrophotometer because of its low sensitivity (Oancea et al., 2017). *K. pneumoniae* strains can produce 77 different capsular polysaccharides (CPS) and, thanks to their specific composition, CPS can form adducts with other compounds, such as antimicrobial peptides (Benincasa et al., 2016). To exclude the role of the CPS of *K. pneumoniae* in the reduction of thiopurines cytotoxicity, the effect of drugs exposed to increasing concentrations of the CPS produced by the bacterial strain used was evaluated. Results from cytotoxicity tests and UV analyses did not evidence any variation compared to the drug incubated in M9; therefore, we excluded a role of CPS in mediating the in vitro effects of thiopurines.

Another important factor came out from measurements of thiopurine metabolites in NALM6 and JURKAT cells. Noteworthy, thiopurine nucleotides were lower when cells were treated for 48 and 72 h with AZA, MP and TG previously exposed to *K. pneumoniae*. This result confirmed that the lower cytotoxicity noticed after bacterial incubation is associated with a reduced metabolite level.

Furthermore, when NALM6 cells were treated with AZA exposed to bacteria, differences not only in the abundance but also in the percentage of each metabolite on the total content of thiopurine metabolites were evidenced in comparison with the quantifications in cells treated with AZA in M9. In particular, the methylated metabolite MeTIMP was higher after treatment with the drug exposed to *K. pneumoniae* compared to the control. Therefore, we tested the activity of the TPMT enzyme, responsible for the methylation of thiopurines (Franca et al., 2019), after exposure of cells to *K. pneumoniae*-conditioned media. Interestingly, our results showed that TPMT activity did not change after the exposure of cells to *K. pneumoniae*-conditioned media, suggesting that there is no stimulation of the activity of this enzyme in NALM6 cells by compounds secreted by this bacterial strain. Higher levels of the methylated compound MeTIMP in NALM6 cells treated with AZA exposed to bacteria could be because of stimulation by a bacterial metabolite produced after exposure to AZA or to S-methyl-4-nitro-5-thioimidazole, derived by the conversion of AZA to MP (Lazarević et al., 2022). Further experiments are required to better understand this finding.

We also performed measurements of dTGUA in NALM6 and JURKAT cells DNA. dTGUA could be a biomarker of thiopurine efficacy alternative to the thiopurine metabolites TGN and MMPN: indeed, dTGUA concentrations in DNA reflects the levels of dTGTP incorporated instead of deoxyguanosine triphosphate in the DNA, provoking cell arrest and apoptosis (Toyonaga et al., 2021). Comparing the concentrations of dTGUA in DNA, the concentrations of the metabolite were lower in both cell lines treated with thiopurines previously exposed to *K. pneumoniae*. These results were in line with measurements of thiopurine metabolites and highlighted that, after bacterial exposure to drugs, there was not only a lower metabolism but also a lower incorporation in DNA of NALM6 and JURKAT cells.

In this context, to understand if bacteria can internalise the thiopurines, as previously reported for other drugs (Klünemann et al., 2021), the concentrations of the drugs in *K. pneumoniae* were evaluated after exposure to thiopurines. MP was quantifiable in bacteria after incubation with AZA and MP, and TG after incubation with TG. *K. pneumoniae* can internalise MP and TG, removing them from the external environment and determining a reduction of cytotoxicity when cells are treated with bacterial conditioned media. Indeed, when ammonia, the preferred nitrogen source, is limited, certain *K. pneumoniae* strains are capable of assimilating the purines **adenine**

and guanine, to convert them to hypoxanthine and xanthine through deamination, finally transforming the compounds first in **uric acid**, then in allantoin and finally in CO_2 and ammonia (de la Riva et al., 2008). Because MP and TG are purine analogues, bacteria could use the same catabolic pathway to obtain nitrogen sources.

MP was found in low concentration also in *K. pneumoniae* exposed to AZA, indicating that this bacterial strain can convert AZA to MP and the compound S-methyl-4-nitro-5-thioimidazole, enzymatically through glutathione S-transferase (GST) activity or non-enzymatically through nucleophiles. Indeed, the scientific literature reports that some strains belonging to the Proteobacteria phylum have GST activity (Vuilleumier & Pagni, 2002). Unfortunately, the concentration of MP found inside bacteria did not justify the reduction of the corresponding concentration of AZA in the bacterial conditioned media. Therefore, we cannot exclude the contribution of metabolites secreted by *K. pneumoniae* after exposure to AZA in mediating the cytotoxicity of the drug. Even if further experiments will be needed, we hypothesised that AZA could be converted in less toxic metabolites, putatively identified by metabolomics, through glycosylation, acetylation and reduction reactions.

Metabolomic analyses suggested putatively differences in the presence of metabolites produced and secreted by *K. pneumoniae* in bacteria when exposed to AZA, MP and TG. Noteworthy, several metabolites derived from thiopurines were putatively identified in each bacterial conditioned media, when *K. pneumoniae* was exposed to drugs suggesting potential bacterial biotransformation. According to these results, *K. pneumoniae* seems to be able to metabolise through deconjugation, reduction, glycosylation, acetylation and conjugation with other functional groups, such as propionic acid, as previously reported in scientific literature (Zimmermann et al., 2019a). The production of new drug-derived compounds probably indicates that the bacterial strain can biotransform the original molecules, thus reducing their concentration and making them less cytotoxic.

Furthermore, when comparing the abundance of metabolites, differences in substances attributable to tricarboxylic acid (TCA) cycle and in pyrimidine biosynthesis seem to emerge. For example, metabolites annotated as malic acid, itaconic acid and ketoglutaric acid belonging to TCA cycle were less present in media conditioned by *K. pneumoniae* previously exposed to thiopurines, whereas amino acids such as glutamate, tyrosine, phenylalanine, leucine and tryptophan, substances involved in anaplerotic reactions of TCA cycle, seem to be present in higher levels. Therefore, we speculated that if substances released are proportional to intracellular metabolites, incubation with thiopurines slowed down TCA cycle in bacteria, determining lower concentration of compounds being part of it and higher concentration of compounds used to replenishing it. Indeed, because *K. pneumoniae* grew in M9 medium, there was a unique carbon source derived from glucose and thus glycolysis and TCA cycle pathway were used to generate energy and reducing power (Cabelli, 1955). Providing thiopurines to *K. pneumoniae* determined novel sources of nitrogen for bacteria that could be metabolised and substitute as alternative source to glucose contained in M9. Therefore, the results obtained could suggest the metabolism of thiopurines by *K. pneumoniae*.

Metabolites belonging to de novo pyrimidine biosynthesis pathway such as N-carbamoyl aspartic acid and orotic acid, already known to be produced by several bacterial strains including *K. pneumoniae* (Lipowska et al., 2019), were present in higher extent in bacteria exposed to thiopurines. Interestingly, the synthesis of N-carbamoyl aspartic acid and orotic acid is reported to be stimulated by nitrogen sources: in particular, the formation of these compounds leading to de novo pyrimidine biosynthesis is initiated by the presence of ammonia (Visek, 1992). As previously mentioned, *K. pneumoniae* strains can metabolise purines in several steps to CO_2 and ammonia (de la Riva et al., 2008), and we speculated they are able to convert thiopurines as well, producing ammonia and stimulating de novo pyrimidine biosynthesis. Moreover, this could suggest the metabolism of thiopurines by *K. pneumoniae*.

In the present pilot study, *K. pneumoniae* relative abundance was related to lower concentrations of TGN in paediatric CD patients, indicating an impact of this bacterial strain on reducing drug metabolites, thus potentially influencing drug efficacy. Indeed, lower TGN concentrations are known to be associated with a reduced therapeutic efficacy in IBD paediatric patients (Lucafò et al., 2019). However, this feature could not be investigated in the pilot study because all patients were in remission. To the authors' knowledge, this is the first time that a bacterial strain was linked to lower bioavailability of thiopurines in humans affected by CD. Indeed, measurements of drug metabolites to assess bacterial biotransformation of the original drugs was performed in vitro and in faecal slurries of mice with colitis (Oancea et al., 2017). Furthermore, the role of *K. pneumoniae* abundance has never been studied in IBD before; the increased abundance of this bacterial species was mainly reconducted to episodes of bacteraemia (Shimasaki et al., 2019). Contrary to what was observed for *K. pneumoniae*, besides not altering the cytotoxicity of thiopurines in vitro, *E. coli* did not influence TGN concentrations in the paediatric CD patients analysed.

5 | CONCLUSIONS

In the present study, we showed the role of *K. pneumoniae* in reducing the effects of thiopurines in vitro and elucidated the mechanisms of interference of *K. pneumoniae* towards MP and TG. In particular, the lower cytotoxicity of MP and TG was caused by the internalisation of these drugs by the bacteria, determining a lower availability for cells treated with these bacterial conditioned media.

The reduction of cytotoxicity of AZA could be partially explained by the conversion of AZA to MP and in the internalisation of the latter in bacteria, but we cannot exclude the contribution of bacterial metabolites released following the exposure to the drug.

This pilot study also showed a correlation between *K. pneumoniae* abundance and thiopurine metabolite TGN concentration in CD paediatric patients, suggesting its possible role in reducing clinical efficacy of these drugs in vivo. Because our cohort of patients is in remission, our study has not assessed the effect of *K. pneumoniae* abundance on treatment efficacy. We do not even know if other bacterial species,

within the IBD-altered microbiota composition, could be able to internalise or metabolise thiopurines or if the resolution of inflammation in IBD responding to thiopurine therapy is associated with microbial changes. Our preliminary data need to be confirmed on a larger cohort of IBD patients. However, it is important to underline that *K. pneumoniae* is only one of the species that proliferate abundantly in the gut of paediatric IBD patients and that our research does not represent the complex situation of intestinal microbiota of paediatric IBD patients.

Another limitation of our study consists in testing only NALM6 and JURKAT immortalised cell lines, which are not able to reflect completely the in vivo situation, to underline differences in cytotoxicity. Furthermore, even if our speculations of these results are in line with the previous ones, metabolomics data are obtained by putative identification.

AUTHOR CONTRIBUTIONS

M. Franzin: Conceptualization (lead); data curation (equal); formal analysis (equal); investigation (lead); methodology (equal); project administration (equal); visualization (equal); writing—original draft (lead). **C. Lagatolla:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (lead); writing—original draft (equal). **S. S. Forgiarini:** Data curation (equal); formal analysis (equal); writing—original draft (equal). **M. Haag:** Formal analysis (equal); resources (equal); writing—review and editing (equal). **S. K. Neef:** Data curation (equal); formal analysis (equal); writing—original draft (equal). **M. Comar:** Formal analysis (equal); methodology (equal); software (equal). **E. Schaeffeler:** Investigation (equal); methodology (equal); validation (equal). **B. Bellich:** Data curation (equal); investigation (equal); validation (equal). **M. Bramuzzo:** Resources (equal); validation (equal). **G. Decorti:** Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); visualization (equal); writing - review and editing (equal). **M. Lucafò:** Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (lead); visualization (equal); writing—review and editing (equal). **U. Hofmann:** Funding acquisition (equal); resources (equal); writing—review and editing (equal). **M. Schwab:** Funding acquisition (equal); project administration (equal); resources (equal); supervision (lead); writing—review and editing (equal). **G. Stocco:** Conceptualization (lead); funding acquisition (equal); project administration (lead); resources (equal); supervision (equal); visualization (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors have nothing to report.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions. Furthermore, sequencing data are openly available in NCBI SRA repository at BioProject ID PRJNA977201. Local ethical committee approval for the study (protocol CEUR-2022-OS-61-BURLO) was provided, and appropriate informed consent was obtained from patients and/or their parents or guardians.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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