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Unraveling the cellular mechanisms of thiopurine-induced pancreatitis in pediatric inflammatory bowel disease: Insights from induced pluripotent stem cell models

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ABSTRACT

Thiopurines are effective drugs for inflammatory bowel disease, but their use is limited by side effects such as pancreatitis, whose mechanism remains unknown and may be more severe in children. This study investigated in a personalized way thiopurine-induced pancreatitis mechanism using induced pluripotent stem cells from pediatric inflammatory bowel disease patients.

Ten pediatric patients, five developing pancreatitis (cases) and five without it (controls), were enrolled. Patient-specific stem cells and their pancreatic differentiated counterparts were used to evaluate thiopurine cytotoxicity, to quantify metabolites levels by liquid chromatography-tandem mass spectrometry, and to assess thiopurine pharmacodynamics by western-blot assay. Statistical analyses were performed applying Student's t-test or two-way ANOVA followed by Bonferroni's post-hoc test for multiple comparisons. Cytotoxicity assays revealed higher thioguanine cytotoxicity in stem and pancreatic cells from cases; pancreatic cells from cases were also more sensitive to mercaptopurine. Moreover, thioguanine treatment on stem cells produced thioguanosine monophosphate and its methylated form, but their concentration did not differ significantly between the groups. In addition, higher *TPMT* gene expression was observed in stem cells from cases, but no differences were observed in pancreatic cells. No significant differences were detected in *HPRT*, *NUDT15*, *ITPA*, or *PACSIN2*

Abbreviations: 6-MMPR, 6-methylmercaptopurine riboside; AZA, Azathioprine; CD, Crohn's disease; CT, Contrast-enhanced computed tomography; DMSO, Dimethyl sulfoxide; EDTA, Ethylenediaminetetraacetic acid; FGF7, Fibroblast growth factor 7; GST, Glutathione S-transferase; IBD, Inflammatory bowel disease; IPSCs, induced pluripotent stem cells; LC-MS/MS, Liquid chromatography-mass spectrometry; Me-TGMP, methyl-thioguanosine monophosphate; MP, Mercaptopurine; MTT, 3–4,5-Dimethylthiazol-2-yl-2,5-Diphenyltetrazolium Bromide; PBS, Phosphate-buffered saline; ROS, Reactive oxygen species; TG, Thioguanine; TGMP, Thioguanosine monophosphate; TIP, Thiopurine-induced pancreatitis; TNF-α, Tumor necrosis factor-α; TPMT, Thiopurine methyltransferase; UC, Ulcerative colitis.

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expression. Lastly, Rac1 protein concentration was similar in stem cells from cases and controls, but pancreatic cells from cases exhibited significantly higher Rac1 expression. These findings suggest that thiopurine cytotoxicity differences might be linked to pharmacokinetics in stem cells, while altered Rac1 expression in pancreatic cells might contribute to pancreatitis, implicating distinct mechanisms between stem and differentiated cells.

1. Introduction

Inflammatory bowel disease (IBD), characterized by relapsing and remitting gastrointestinal inflammation [1], comprises Crohn's disease (CD) and ulcerative colitis (UC), which differ in severity and response to treatment [2]. IBD causes are unknown, but it is believed to be an immune-mediated condition due to genetic, immunologic, environmental, and infective factors [3]. UC and CD affect men and women equally and may occur in adults, children, and adolescents [4]. Patients with pediatric-onset IBD have more extensive anatomic involvement and often require more aggressive treatment [5]. Moreover, adverse effects of treatments may have long-term consequences severely affecting quality of life in pediatric IBD patients [6].

IBD treatment involves immunomodulatory drugs such as corticosteroids, aminosalicylates, and thiopurines. Patients with more severe disease require biological drugs, among which the tumor necrosis factor-α (TNF-α) inhibitors infliximab and adalimumab [7,8]. TNF-α inhibitors are mainly used to induce and maintain a remission state, while aminosalicylates and thiopurines are used especially for remission [9]. The therapy aims to induce and maintain remission of symptoms, to prevent and treat complications, and achieve mucosal healing [10]. Thiopurines, including azathioprine (AZA) and mercaptopurine (MP), play an important role in IBD therapy. Azathioprine is non-enzymatically converted into mercaptopurine by reduced glutathione [11], and enzymatically by glutathione S-transferase (GST) in the liver [12]; in turn, mercaptopurine is converted into thioguanine nucleotides after the generation of intermediate metabolites [13]. Thioguanine is instead directly converted into thioguanine nucleotides, without intermediate metabolites [14]. Azathioprine and mercaptopurine are used for IBD treatment, while thioguanine prolonged use is limited by the risk of severe adverse events [15]. Thiopurines present different side effects, among which are nausea, vomiting, and some more severe reactions, such as myelosuppression, hepatotoxicity, and pancreatitis [16]. Thiopurine-induced pancreatitis (TIP), affecting about 3 % of thiopurine-treated IBD patients [17], often leads to therapy interruption and generally manifests more severely in pediatric patients [18–20]. The causes of TIP are unknown, possibly linked to genetic predisposition [21], abnormalities of the immune response, or alteration in thiopurine biotransformation [22]. In addition, CD patients have a higher risk of developing TIP than those with UC [23]. To date, no fully validated biomarker of TIP is available.

Due to the clinical relevance of TIP, it is important to elucidate its mechanism and to identify predictive markers. Current models for studying TIP often fail to capture patient-specific variations, underscoring the need for innovative approaches. Induced pluripotent stem cells (iPSCs) offer a patient-specific platform to explore TIP mechanisms, particularly in pediatric IBD patients. iPSCs are stem cells generated by reprogramming differentiated cells, usually adult somatic cells [24,25]. iPSCs can be differentiated into any tissue of the human body, including pancreatic tissue [26], and iPSC-differentiated pancreatic cells allow to study TIP in a personalized way. In this context, an efficient protocol for differentiating of iPSCs into pancreatic exocrine cells was developed by Takizawa-Shirasawa and colleagues [27].

The objective of this study was to investigate TIP mechanism using iPSCs from pediatric IBD patients. We demonstrated that iPSCs and iPSC-derived pancreatic exocrine cells from IBD patients with TIP are more sensitive to thiopurine cytotoxic effects compared to cells from patients without pancreatitis. The differences in cytotoxicity might be related to pharmacokinetics in iPSCs, while the pharmacodynamic thiopurine target Rac1 seems to be involved in the higher cytotoxicity in TIP pancreatic cells. These findings suggest that the cytotoxicity mechanisms of thiopurines might differ between iPSCs and pancreatic exocrine cells.

2. Methods

2.1. Study design and sample collection for iPSC generation

This case-control study enrolled inflammatory bowel disease patients that developed TIP during azathioprine therapy as cases. For each case, a control patient who tolerated azathioprine therapy without developing TIP was enrolled. Cases and controls were matched on age at the time of azathioprine exposure and gender to ensure comparability. Pancreatitis was defined as a severe abdominal pain with serum amylase and lipase levels greater than three times the upper limit of normal, or an asymptomatic increase of amylase greater than three times the upper normal limit, along with echography alterations.

Patients were enrolled between June 2017 and September 2020 in the pediatric departments of Napoli, Ravenna, Treviso, and Trieste (Italy) hospitals. Blood was collected in tubes with lithium heparin as anticoagulant, and samples were processed within 24 h for iPSC generation (see supplemental material [28]). The study was approved by the Ethical Committee of the Institute of Maternal and Child Health IRCCS Burlo Garofolo (Trieste), with approval number 1556 (internal ID of the study RC 44/22).

2.2. Cell culture

iPSCs were cultured using StemMACS iPS-Brew XF medium (Miltenyi Biotec). Cell culture plates were pre-coated with diluted Matrigel® (Corning) (1:60 Matrigel®-DMEM/F-12) to ensure cell adhesion. Cells were passaged twice a week after reaching about 80 % of confluence, using standard procedures allowing the maintenance of the clusters. Briefly, cells were exposed to Versene (ThermoFisher Scientific) for 2 min to detach them and re-plated in fresh medium at the desired confluence. Cell cultures were maintained in a humidified incubator at 37 °C and with 5 % CO_2.

2.3. Differentiation of iPSCs into pancreatic exocrine cells

iPSCs were differentiated into pancreatic exocrine cells following a stepwise protocol developed by Takizawa-Shirasawa and colleagues [27] with slight modifications [29]. Briefly, iPSCs were treated with Activin A (100 ng/mL, Merck) and CHIR99021 (3 μM, Merck) for 4 days to generate definitive endoderm cells (stage I). FGF-7 (50 ng/mL, Abnova) addition for 3 days stimulated the formation of primitive gut tube cells (stage II), and the subsequent addition of cyclopamine (0.25 μM, Merck), noggin (50 ng/mL, Invitrogen), and all-trans retinoic acid (2 μM, Merck) for 3 days led to the formation of pancreatic progenitors (stage III). Lastly, FGF-7 (50 ng/mL, Abnova), glucagon-like peptide 1 (100 ng/mL, RayBiotech), and nicotinamide (10 mM, Merck) for 3 days led to obtain pancreatic exocrine cells (stage IV). In each stage of the differentiation, B27 1X (ThermoFisher Scientific) was added. During the differentiation process, cells were grown in RPMI 1640 medium (EuroClone) added with 1 % of penicillin-streptomycin (EuroClone) and 2 mM L-glutamine (EuroClone).

2.4. Cytotoxicity assay (MTT)

Patient-derived iPSCs were seeded (10 $\times 10^3$ cells/well) in flat-bottom 96-well plates, pre-coated with diluted Matrigel® (Corning) (1:60 Matrigel®-DMEM/F-12), in a final volume of 100 μL/well in the presence of rock inhibitor Y-27632 10 μM (Miltenyi Biotec). The following day, cells were treated with thiopurines for 72 h (supplemental material).

For MTT analysis of pancreatic cells, patient-derived iPSCs were differentiated in flat-bottom 96-well plates and treated with thiopurines for 72 h (supplemental material).

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Merck) 0.5 mg/mL was added to the culture medium and the plates were incubated at 37 °C with 5 % CO_2 for 4 h. After incubation, the medium was removed and the salts dissolved with 100 μL/well of DMSO. Absorbance values at 540 and 630 nm were obtained using the spectrophotometer FLUOstar® Omega microplate reader (BMG LABTECH, Germany).

2.5. [^{3}H]-thymidine incorporation assay

iPSCs were seeded (10 $\times 10^3$ cells/well) in flat-bottom 96-well plates, pre-coated with diluted Matrigel® (Corning) (1:60 Matrigel®-DMEM/F-12), in a final volume of 100 μL/well in the presence of rock inhibitor Y-27632 10 μM (Miltenyi Biotec). The next day, cells were treated with thiopurines for 72 h (see supplemental material for thiopurine treatments). After 72 h, [^{3}H]-thymidine 4.5 μCi/mL (PerkinElmer) was added to the culture medium. The plates were incubated for 5 h at 37 °C with 5 % CO_2. The medium was then removed, the cells detached using 50 μL/well of Versene (ThermoFisher Scientific) and transferred on a filter plate (Corning) previously washed with PBS. After two washes with PBS, 25 μL/well of scintillation liquid (Optiphase "Super Mix" Perkin Elmer Life Science) were added and the plate was read with the Wallac 1450 Microbeta liquid scintillation counter (Perkin Elmer).

2.6. Quantification of thiopurine metabolites by LC-MS/MS

Quantification of thiopurine metabolites was performed by liquid chromatography-mass spectrometry (LC-MS/MS), as reported in more detail in supplemental material. Patient-derived iPSCs were seeded (6 $\times 10^6$ cells) and, the day after, treated with thioguanine 2.5 $\times$ 10^{-7} M for 48 h. After 48 h, cells were detached and living cells were counted using the Trypan Blue (Merck) assay. Cells were then centrifuged at 400 xg at 4 °C for 5 min and cell pellets were snap-frozen in liquid nitrogen and stored at −80°C until the LC-MS/MS analysis. A mixture of 250 μL of EDTA 50 mM, 15 μL of dithiothreitol solution 30 mg/mL, and 10 μL of internal standard working solution 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 xg for 20 min, 10 μL of the supernatant were used for LC-MS/MS analysis, as previously described [30], on an Agilent 6460 A triple quadrupole mass spectrometer (Agilent, Waldbronn) coupled to an Agilent 1290 Infinity HPLC system.

2.7. Assessment of gene expression of thiopurine metabolizing enzymes

RNA extraction and reverse transcription methods are reported in the supplemental material. The expression of thiopurine metabolizing enzymes was assessed by real-time PCR with the following TaqMan primer and probe sets: Hs02800695_m1 (HPRT), Hs00738803_m1 (ITPA), Hs04399328_g1 (TPMT), Hs01087148_m1 (NUDT15), and Hs00200589_m1 (PACSIN2). β-actin (Hs01060665_g1) was used as a housekeeping gene. All the probes were purchased from ThermoFisher Scientific.

The reaction mix was prepared using 5 μL of 2X TaqMan® Universal Master Mix II no UNG (ThermoFisher Scientific), 0.5 μL of TaqMan® Gene Expression Assay (containing the primers and the specific probe), 2.5 μL of cDNA, and nuclease-free water to a final volume of 10 μL.

The thermal protocol used was the following: 95° C 10 min (95°C 15 sec, 60°C 1 min) x 40 cycles.

2.8. Immunoblotting

For immunoblot analysis, equal protein amounts (20 μg for each sample; lysate preparation and quantification reported in supplemental material) were mixed with 1X NuPAGE LDS Sample Buffer (ThermoFisher Scientific), 1X NuPAGE Sample Reducing Agent (Invitrogen, ThermoFisher Scientific), and RIPA buffer to a final volume of 40 μL. Proteins were separated using a NuPAGE 4–12 %, 1.0 Mini Protein Gel (ThermoFisher Scientific), using 1X Bolt MES SDS Running Buffer (ThermoFisher Scientific), and transferred to nitrocellulose membranes (ThermoFisher Scientific) using a wet transfer system (Bio-Rad). After incubation with 5 % non-fat milk in Tris-buffered saline (10 mM Tris-HCl, pH= 7.5, 150 mM NaCl) added with 0.1 % Tween-20 (TBS-T), membranes were incubated overnight at 4 °C with rabbit anti-β-actin (1:3000 dilution, Abcam) or mouse anti-Rac-1 (1:500 dilution, Cytoskeleton) primary antibodies. Membranes were then incubated with HRP-conjugated secondary anti-rabbit IgG (1:10000 dilution, Merck) or HRP-conjugated secondary anti-mouse IgG (1:8000 dilution, Cell Signaling Technology) for 1 h at 4 °C. Immunocomplexes were visualized using the LiteAblot TURBO Extra Sensitive Chemiluminescent Substrate (EuroClone) by the ChemiDoc Imaging System (Bio-Rad).

2.9. Statistical analysis

The differences in patient-specific cells sensitivity to thiopurine cytotoxicity have never been evaluated in a case-control study involving this patient population. Based on a preliminary analysis of 4 cases and controls, differences in the sensitivity to thiopurines for iPSCs and pancreatic progenitor cells have been preliminary observed. Statistical power simulations indicate that analyzing 5 patients for each group, if the differences and standard deviations remain consistent with those previously observed, the result would be fully statistically significant (power 80 %; $p < 0.05$, *t*-test).

Statistical analyses were performed using the GraphPad Prism software (San Diego, CA, USA) applying Student's *t*-test or two-way ANOVA followed by Bonferroni's post-hoc test for multiple comparisons. ImageJ software has been used for the quantification of the immunoblot bands.

3. Results

3.1. Patients' characteristics

This case-control study enrolled 10 pediatric IBD patients: 5 developed thiopurine-induced pancreatitis (TIP) and 5 controls, without TIP. All TIP cases matched at least two of the three diagnostic criteria for pancreatitis. All cases presented with mild pancreatitis according to the clinical classification. Since none of the cases showed severe pancreatitis, contrast-enhanced computed tomography (CT) was not required for diagnosis, and ultrasonography alone was employed [31,32]. Among the TIP cases, 3 had UC and 2 had CD; the control group had the same distribution. The mean age at blood sampling was 15.3 ± 3.4 years for the TIP group and 14.5 ± 3.1 years for the controls ($p > 0.05$, *t*-test). All TIP cases were male; the control group included 4 males and 1 female. All participants had been treated with azathioprine, though the TIP group tolerated a lower dose (2.0 ± 1.0 mg/kg/day) compared to the controls (2.8 ± 0.2 mg/kg/day) ($p > 0.05$, *t*-test). Due to the adverse event, the average duration of azathioprine therapy was 78.8 ± 94.6 days in the TIP group and 1105.4 ± 819.8 days in the control group ($p < 0.05$, *t*-test). Four TIP cases presented with symptomatic

pancreatitis, and 1 had asymptomatic hyperamylasemia. TIP cases started azathioprine therapy at 8.3 ± 5.1 years, while therapy for control patients started at 11.4 ± 2.6 years. TIP cases developed pancreatitis at an average age of 12.0 ± 2.6 years.

3.2. Cytotoxicity of thiopurines on patient-derived iPSCs

MTT assays on patient-derived iPSCs after 72 h of treatment with thiopurines showed a higher cytotoxicity in TIP cells in comparison to no-TIP cell lines after the treatment with thioguanine ($p < 0.001$, two-way ANOVA; Fig. 1). Significant difference in the cytotoxicity was noticed at the concentration of 2.5×10^{-7} M ($p < 0.001$, Bonferroni post-hoc test; Fig. 1). Instead, no statistically significant differences in the cytotoxicity between TIP and no-TIP iPSCs were noticed after the treatments with azathioprine and mercaptopurine ($p > 0.05$, two-way ANOVA; Fig. 1).

The EC_{50} values for each drug for TIP and no-TIP iPSCs are reported in Table 1.

Table 1
Cytotoxicity EC_{50} values for no-TIP and TIP iPSCs for azathioprine, mercaptopurine, and thioguanine.

	EC_{50} no-TIP iPSCs	95 % C.I. no-TIP	EC_{50} TIP iPSCs	95 % C.I. TIP
AZA	$1.54x10^{-6}$ M	$1.33x10^{-6}$ to $1.78x10^{-6}$ M	$1.19x10^{-6}$ M	$9.68x10^{-7}$ to $1.48x10^{-6}$ M
MP	$4.33x10^{-7}$ M	$3.12x10^{-7}$ to $5.98x10^{-7}$ M	$2.77x10^{-7}$ M	$1.98x10^{-7}$ to $3.87x10^{-7}$ M
TG	2.25×10^{-7} M	$1.94x10^{-7}$ to $2.61x10^{-7}$ M	$1.30x10^{-7}$ M	$1.10x10^{-7}$ to $1.53x10^{-7}$ M

Average EC_{50} values and 95 % C.I. calculated for no-TIP and TIP iPSCs treated with azathioprine, mercaptopurine, and thioguanine after 72 h of exposure. Data are reported as means of at least 3 independent experiments performed in triplicate and 95 % C.I. AZA: azathioprine; MP: mercaptopurine; TG: thioguanine; TIP: thiopurine-induced pancreatitis.

3.3. Cytotoxicity of thiopurines on iPSC-derived pancreatic exocrine cells

MTT assays on iPSC-derived pancreatic exocrine cells after 72 h of treatment with thiopurines showed higher cytotoxicity of TIP patients in comparison to no-TIP after mercaptopurine ($p < 0.01$, two-way ANOVA; Fig. 2) and thioguanine ($p < 0.01$, two-way ANOVA; Fig. 2) exposure. For thioguanine, the significant difference in the cytotoxicity between TIP and no-TIP patients was noticed at the concentration $1.6x10^{-5}$ M ($p < 0.05$, Bonferroni post-hoc test; Fig. 2). No statistically significant differences in the cytotoxicity between TIP and no-TIP iPSCs were noticed after azathioprine treatment ($p > 0.05$, two-way ANOVA; Fig. 2).

The EC_{50} values for each drug for TIP and no-TIP pancreatic exocrine cells are reported in Table 2.

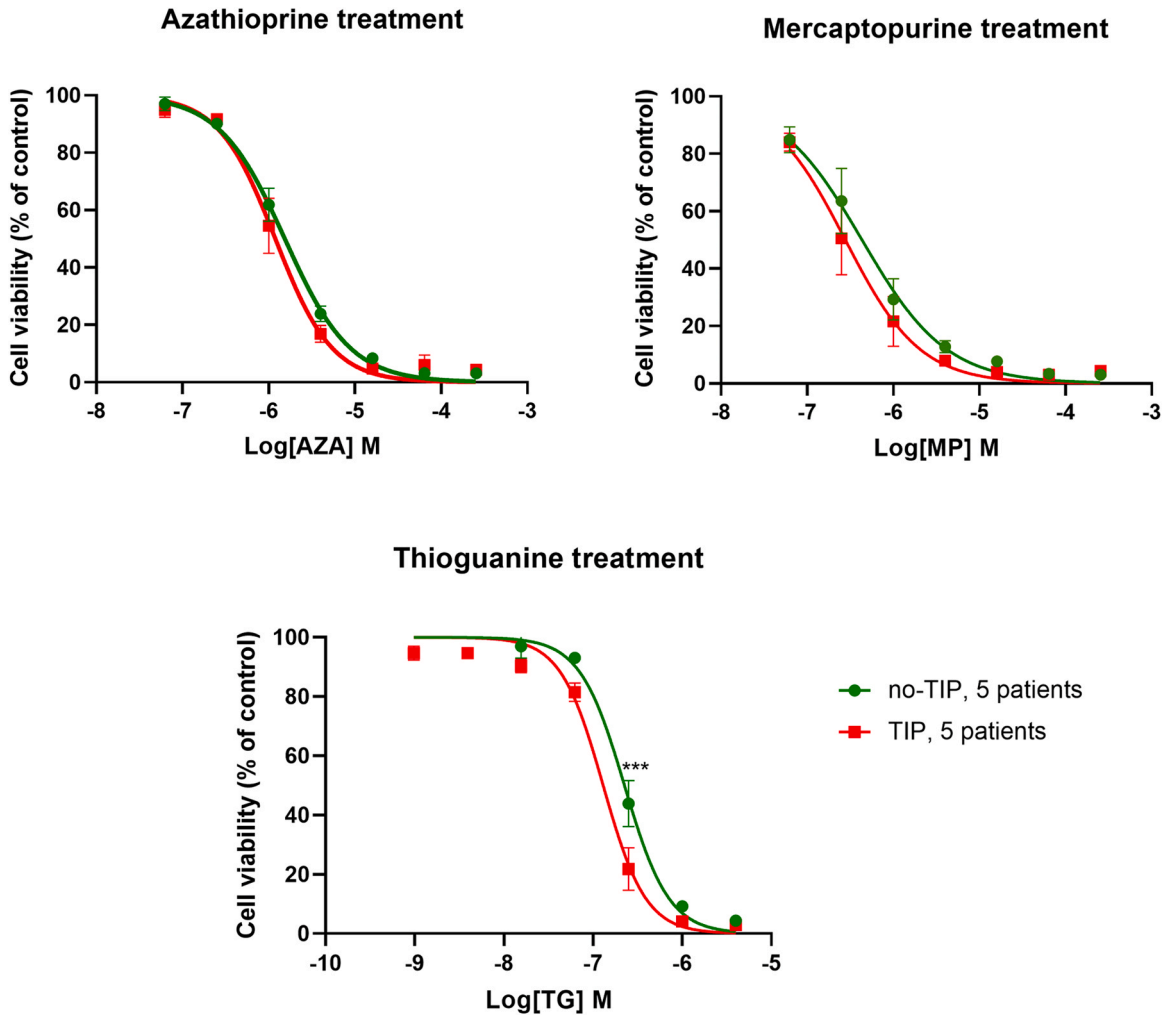

Fig. 1. Effects of thiopurine treatment for 72 h on patient-derived iPSCs. Statistical analysis TIP vs no-TIP iPSCs: $p < 0.001$, two-way ANOVA. ***$p < 0.001$, Bonferroni post-hoc test (significant concentration: $2.5x10^{-7}$ M). For this analysis, at least three biological replicates per cell line have been performed.

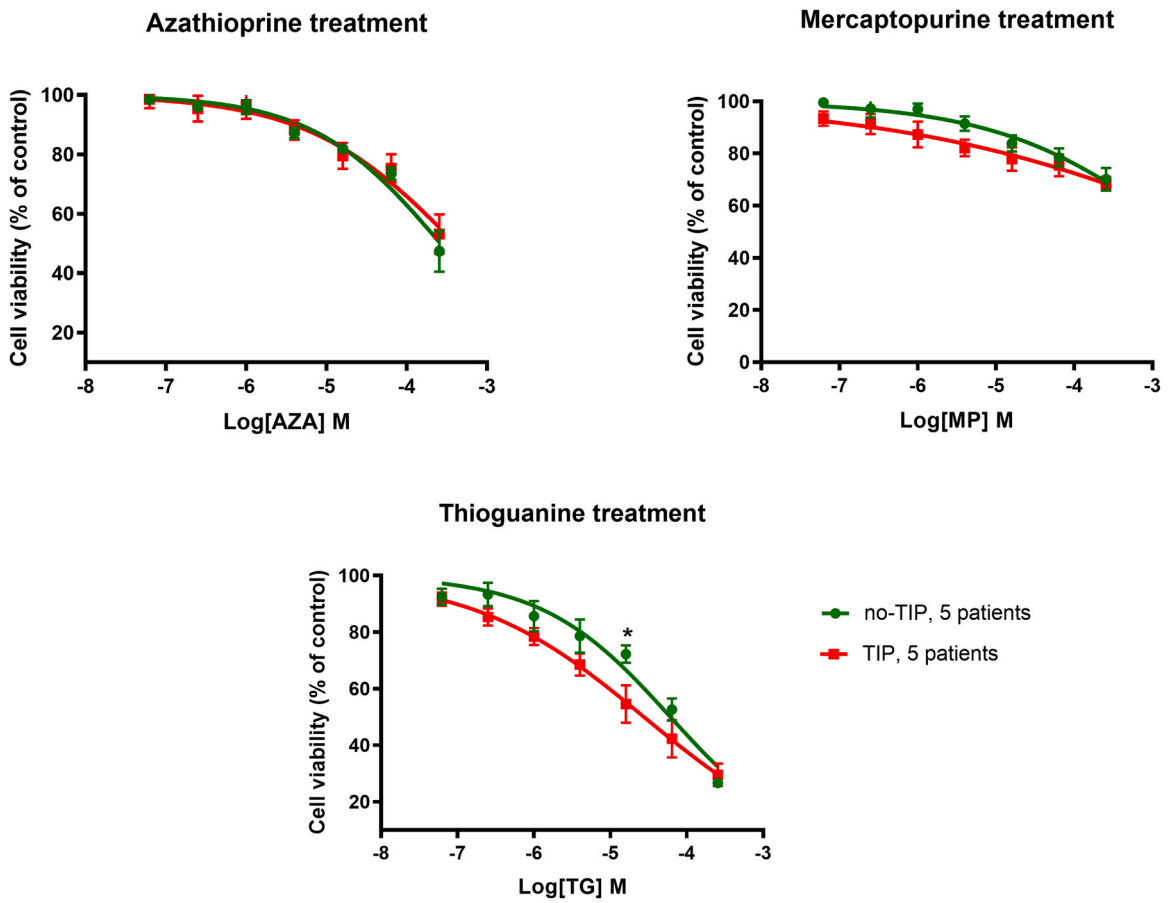

Fig. 2. Effects of thiopurine treatment for 72 h on iPSC-derived pancreatic exocrine cells. Statistical analysis TIP vs no-TIP cells after mercaptopurine: $p < 0.01$, two-way ANOVA, and thioguanine: $p < 0.01$, two-way ANOVA. *$p < 0.05$, Bonferroni post-hoc test (significant concentration: $1.6x10^{-5}$ M). For this analysis, at least three biological replicates per cell line have been performed.

Table 2
Cytotoxicity EC_{50} values for no-TIP and TIP pancreatic exocrine cells for azathioprine, mercaptopurine, and thioguanine.

	EC_{50} no-TIP pancreatic exocrine cells	95 % C.I. no-TIP	EC_{50} TIP pancreatic exocrine cells	95 % C.I. TIP
AZA	$2.64x10^{-4}$ M	$1.84x10^{-4}$ to $4.32x10^{-4}$ M	$3.27x10^{-4}$ M	$1.72x10^{-4}$ to $9.52x10^{-4}$ M
MP	$2.16x10^{-3}$ M	$8.54x10^{-4}$ to $9.97x10^{-3}$ M	$9.44x10^{-3}$ M	$1.63x10^{-3}$ to $3.80x10^{-1}$ M
TG	$6.12x10^{-5}$ M	$4.22x10^{-5}$ to $9.29x10^{-5}$ M	2.75×10^{-5} M	$1.78x10^{-5}$ to 4.45×10^{-5} M

Average EC_{50} values and 95 % C.I. calculated for no-TIP and TIP pancreatic exocrine cells treated with azathioprine, mercaptopurine, and thioguanine after 72 h of exposure. Data are reported as means of at least 3 independent experiments performed in triplicate and 95 % C.I. AZA: azathioprine; MP: mercaptopurine; TG: thioguanine; TIP: thiopurine-induced pancreatitis.

3.4. [^{3}H]-thymidine incorporation assay on patient-derived iPSCs

[^{3}H]-thymidine incorporation assay on patient-derived iPSCs after 72 h of treatment with thiopurines showed no significant differences in cellular proliferation between TIP and no-TIP iPSCs ($p > 0.05$, two-way ANOVA; Fig. 3). Moreover, no statistically significant differences were noticed between the proliferation rate of TIP and no-TIP untreated controls ($p > 0.05$, *t*-test; Fig. 3).

The EC_{50} values for each drug for TIP and no-TIP iPSCs are reported in Table 3.

3.5. LC-MS/MS thiopurine metabolite analysis on patient-derived iPSCs

The analysis of thiopurine metabolites by LC-MS/MS after exposure for 48 h to $2.5x10^{-7}$ M thioguanine showed only the presence of thioguanosine monophosphate (TGMP) and its methylated form (me-TGMP). No significant differences in the concentrations of these metabolites were noticed between TIP and no-TIP cells ($p > 0.05$, *t*-test; Fig. 4).

3.6. Thiopurine metabolizing enzymes expression analysis

The assessment of thiopurine metabolizing enzymes by real-time PCR showed no significant differences in the mRNA expression levels of *HPRT, ITPA, NUDT15*, and *PACSIN2* between TIP and no-TIP iPSCs ($p > 0.05$, *t*-test; Fig. 5). On the other hand, no-TIP iPSCs showed a significant higher expression of TPMT compared to TIP iPSCs ($p < 0.05$, *t*-test; Fig. 5).

On the contrary, no statistically significant difference in *TPMT* expression was noticed between TIP and no-TIP pancreatic exocrine cells ($p > 0.05$, *t*-test; Fig. 5).

3.7. Immunoblot analysis of patient-derived iPSCs

Immunoblot analysis on TIP and no-TIP iPSCs showed no significant difference in the protein expression of Rac1 ($p > 0.05$, *t*-test), as shown in Fig. 6.

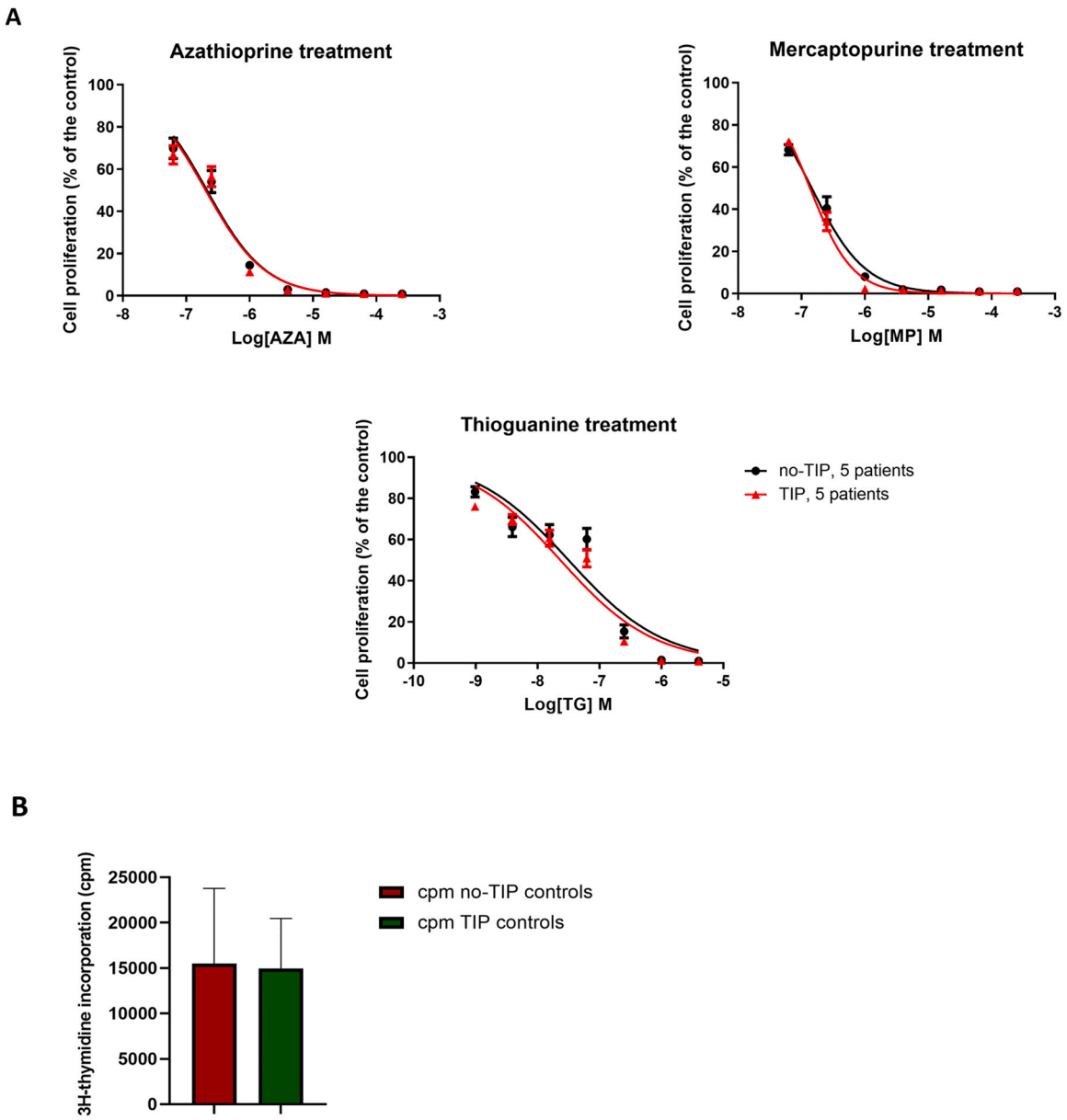

Fig. 3. Proliferation rate analyzed by [^{3}H]-thymidine incorporation assay on patient-derived iPSCs after 72 h of thiopurine exposure. A) Effects of azathioprine, mercaptopurine, and thioguanine on the proliferation rate of patient-derived iPSCs. B) Bar chart of [^{3}H]-thymidine incorporation rate in TIP and no-TIP untreated controls. For this analysis, at least three biological replicates per cell line have been performed. [^{3}H]-thymidine incorporation into DNA is expressed as counts per minute (cpm).

Table 3
Proliferation rate EC_{50} values for no-TIP and TIP iPSCs for azathioprine, mercaptopurine, and thioguanine.

	EC_{50} no-TIP iPSCs	95 % C.I. no-TIP	EC_{50} TIP iPSCs	95 % C.I. TIP
AZA	$2.12x10^{-7}$ M	$1.73x10^{-7}$ M to $2.59x10^{-7}$ M	$2.02x10^{-7}$ M	$1.61x10^{-7}$ M to 2.54×10^{-7} M
MP	$1.45x10^{-7}$ M	$1.25x10^{-7}$ M to $1.69x10^{-7}$ M	$1.37x10^{-7}$ M	$1.23x10^{-7}$ M to $1.51x10^{-7}$ M
TG	$3.17x10^{-8}$ M	$2.01x10^{-8}$ M to $5.00x10^{-8}$ M	$2.34x10^{-8}$ M	$1.61x10^{-8}$ M to $3.39x10^{-8}$ M

Proliferation rate EC_{50} values and 95 % C.I. calculated for no-TIP and TIP iPSCs after azathioprine, mercaptopurine, and thioguanine after 72 h of exposure. Data are reported as means of at least 3 independent experiments performed in triplicate and 95 % C.I. AZA: azathioprine; MP: mercaptopurine; TG: thioguanine; TIP: thiopurine-induced pancreatitis.

3.8. Immunoblot analysis of iPSC-derived pancreatic exocrine cells

Immunoblot analysis on TIP and no-TIP iPSC-derived pancreatic exocrine cells showed a significant difference in the protein expression of Rac1 ($p < 0.05$, *t*-test), as shown in Fig. 7.

4. Discussion

IBD, comprising UC and CD, is a chronic condition for which no cure is available [33]. Thiopurines are used to manage symptoms but may cause TIP, a severe adverse effect [34]. Since the molecular mechanism of TIP is unknown and it often leads to therapy interruption [18,19], patient-derived iPSCs represent a valuable tool for elucidating TIP mechanism and for the discovery of clinical biomarkers. Indeed, iPSCs and iPSC-derived pancreatic exocrine cells could help researchers in elucidating TIP mechanisms, which are unattainable to be explored with

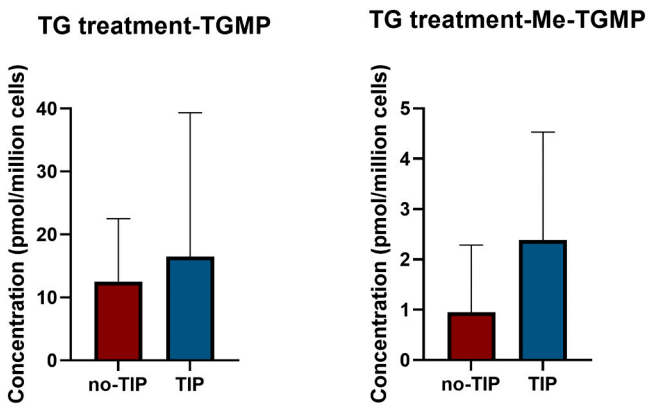

Fig. 4. LC-MS/MS thiopurine metabolite analysis after thioguanine treatment for 48 h. The concentration of the metabolites detected is expressed as pmol/million cells. For this analysis, one biological replicate per cell line has been performed and the results are presented as the average value of the five TIP and the five no-TIP cell lines, respectively.

traditional cell lines or animal models.

In this case-control study, bidimensional pancreatic exocrine models were generated from iPSCs of ten pediatric IBD patients, five presenting TIP and five without it, to study TIP in a personalized way. We first investigated any possible differences in thiopurine cytotoxicity between TIP and no-TIP iPSCs and iPSC-derived pancreatic exocrine cells by MTT assay. Both TIP iPSCs and pancreatic cells were significantly more sensitive to thioguanine treatment compared to no-TIP iPSCs; in addition, iPSC-derived pancreatic cells from TIP patients were also significantly more sensitive to mercaptopurine than no-TIP cells. Conversely, no differences emerged after azathioprine treatment, possibly due to its additional cytotoxicity mechanisms, being a glutathione-depleting drug leading to an increase of reactive oxygen species (ROS) [35,36], and consequent cellular stress. These effects might be responsible, at least in part, for azathioprine cytotoxicity in TIP and no-TIP cells, and could manifest similarly in the two groups, explaining the absence of significant differences. To our knowledge, no other examples of the study of TIP using patient-derived iPSCs and iPSC-derived pancreatic exocrine cells are reported in the literature.

Thiopurines' cytotoxic effects are strictly related to their cell cycle-specific action, interfering with DNA synthesis during the S phase [37]. We assessed how thiopurines can affect the proliferation rate of TIP and no-TIP iPSCs. No statistically significant difference was found between TIP and no-TIP cells, probably due to their similar proliferation rate (untreated controls). However, compared to other stable cell lines, iPSCs were highly sensitive to thiopurines with low EC_{50} values. Since the S phase of the cell cycle of iPSCs is particularly long compared to non–stem cell lines [29,38], the high cytotoxic effects of thiopurines can be explained by their action in the S phase, incorporating thioguanine nucleotides into the newly formed DNA.

Thiopurines exert cytotoxicity through various mechanisms, including apoptosis induction. Indeed, thiopurines disrupt mitochondrial function, causing loss of mitochondrial transmembrane potential

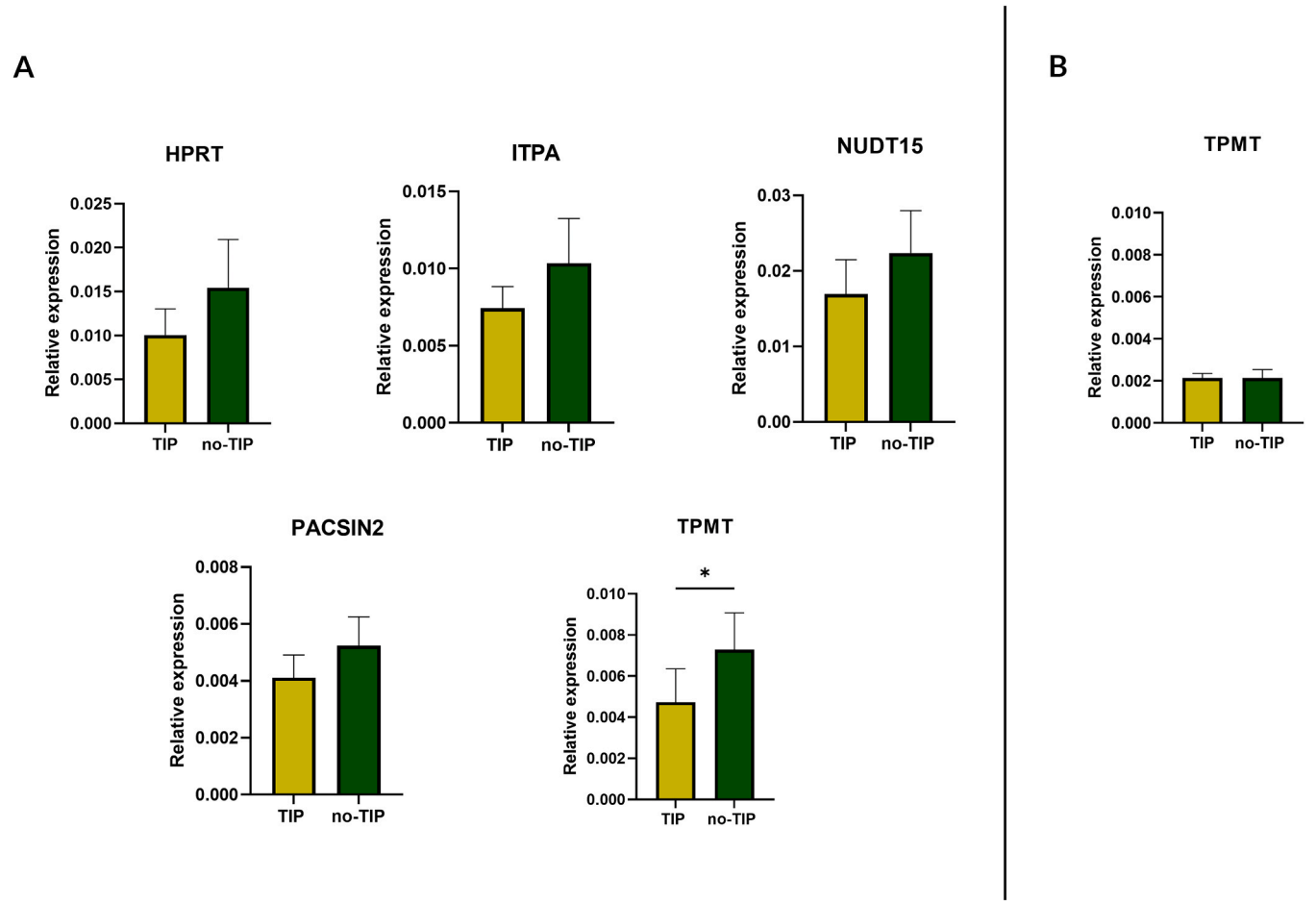

Fig. 5. A) Thiopurine metabolizing enzymes expression analysis in TIP and no-TIP iPSCs. B) TPMT expression analysis in TIP and no-TIP pancreatic exocrine cells. Relative expression values are expressed as $2^{-\Delta Ct}$; β-actin was used as a housekeeping gene. Statistical analysis TIP vs no-TIP TPMT: *$p < 0.05$, *t*-test. For this analysis, one biological replicate per cell line has been performed and the results are presented as the average value of the five TIP and the five no-TIP cell lines, respectively.

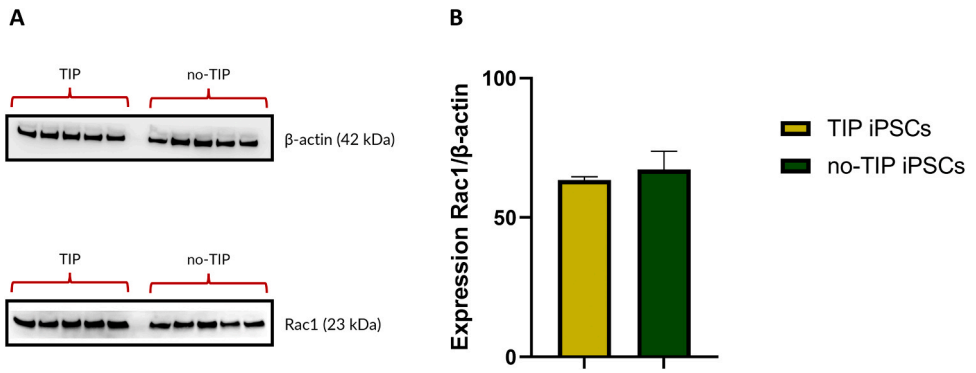

Fig. 6. Immunoblot analysis of Rac1 protein expression in TIP and no-TIP patient-derived iPSCs. A) Immunoblot bands of β-actin and Rac1 in TIP and no-TIP patient-derived iPSCs; B) Bar chart of the protein expression levels of Rac1 in TIP and no-TIP patient-derived iPSCs. β-actin has been used as a housekeeping, on which the expression levels have been normalized. Data have been obtained considering at least two replicates per cell line. Full-length blots are presented in Supplementary Figure 1.

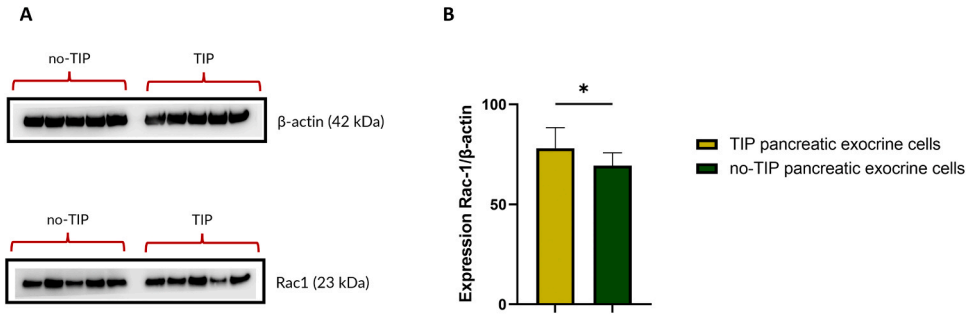

Fig. 7. Immunoblot analysis of Rac1 protein expression in TIP and no-TIP iPSC-derived pancreatic exocrine cells. A) Immunoblot bands of β-actin and Rac1 in no-TIP and TIP pancreatic exocrine cells; B) Bar chart of the protein expression levels of Rac1 in no-TIP and TIP pancreatic exocrine cells. Statistical analysis TIP vs no-TIP: *$p < 0.05$, *t*-test. β-actin has been used as a housekeeping, on which the expression levels have been normalized. Data have been obtained considering at least two replicates per cell line. Full-length blots are presented in Supplementary Figure 1.

and increased production of ROS, which play a significant role in mediating thiopurine-induced apoptosis [39]. Since no differences in proliferation were observed between TIP and no-TIP iPSCs after thiopurine treatment, the differences in cytotoxicity between the two groups might be related to apoptotic mechanisms.

To better investigate the mechanisms responsible for the differences in thiopurine cytotoxicity between TIP and no-TIP cells, we analyzed thiopurine metabolites in patient-derived iPSCs by LC-MS/MS. No statistically significant difference in thiopurine metabolites concentration was noticed between TIP and no-TIP cells, suggesting that pharmacokinetics is not responsible for the cytotoxicity differences. Therefore, thiopurine metabolite levels are not predictive of TIP manifestation, and our findings are in line with other studies focusing on other thiopurine-related adverse effects. For instance, Dubinsky and colleagues [40] found no correlation between mercaptopurine metabolite levels and pancreatitis. Similarly, another study [41] detected no correlation between 6-methylmercaptopurine riboside (6-MMPR) and pancreatitis. In addition, while dose-dependent thiopurine adverse effects (e.g. myelotoxicity) are associated with thiopurine metabolism [42], TIP is an idiosyncratic dose-independent reaction [43]. Therefore, it is plausible to speculate that thiopurine metabolites are not responsible for its onset. To further confirm this hypothesis, we analyzed the expression of the enzymes related to thiopurine metabolism in TIP and no-TIP iPSCs by real-time PCR. We also investigated the expression of *PACSIN2*, since polymorphisms in this gene are related to impaired TPMT activity and increase in gastrointestinal toxicity [44]. No statistically significant differences in the expression of *HPRT*, *ITPA*, *NUDT15*, and *PACSIN2* between TIP and no-TIP cells emerged, however no-TIP iPSCs expressed significantly higher levels of the *TPMT* gene compared to TIP cells. To date, most of the studies in the literature focus on the relationship between single nucleotide polymorphisms of this gene and thiopurine side effects, such as myelotoxicity, and hepatic toxicity, but no correlation with TIP has been found [45]. Previous studies [46,47] showed that cells with higher *TPMT* levels are less sensitive to thioguanine. This finding is in line with our results, and the lower levels of *TPMT* in TIP cells could explain the significant higher sensitivity of these cells compared to no-TIP lines after thioguanine treatment. Regarding mercaptopurine cytotoxicity, research [46–48] demonstrated that cells expressing higher *TPMT* levels are more sensitive to mercaptopurine effects. However, this finding does not explain the effects noticed in our study after mercaptopurine treatment, suggesting that mercaptopurine cytotoxicity might involve additional mechanisms beyond *TPMT* expression, which warrants further investigation. On the other hand, no statistically significant differences in *TPMT* gene expression were noticed between TIP and no-TIP pancreatic exocrine cells, suggesting that the mechanisms of thiopurine cytotoxicity might vary between iPSCs and differentiated cells. Generally, further studies are needed to better comprehend the role of TPMT, such as the evaluation of its protein concentration, and enzymatic activity.

We also investigated the pharmacodynamic component, focusing on Rac1, a small GTPase member of the Rac subfamily of the Rho GTPases [49], important for the regulation of many cellular functions, such as cell-cell adhesion, and cellular migration [50,51]. Rac1 is one of the molecular targets of thiopurines, which inhibit its activity in T lymphocytes, leading to apoptosis [52]. Increased Rac1 expression, due to single nucleotide polymorphisms, seems to be related to an increased inflammatory response in the colon [53], suggesting a potential role of Rac1 in IBD pathogenesis. To investigate the involvement of Rac1 in

thiopurine cytotoxicity, we performed immunoblot analyses. No differences in Rac1 protein expression between TIP and no-TIP iPSCs emerged, but TIP pancreatic cells expressed higher levels of this protein compared to no-TIP cells. This might suggest different thiopurine cytotoxicity mechanisms between iPSCs and differentiated cells, with Rac1 potentially involved in cytotoxicity in pancreatic exocrine cells. In this context, the only example reported in the literature is the one provided by Tél and colleagues [54], who demonstrated that azathioprine inhibits Rac1 leading to an impaired localization of the cystic fibrosis transmembrane conductance regulator at the plasma membrane in murine pancreatic ducts, possibly contributing to TIP. Despite the need for further investigation, our results seem to be in line with these findings. Thus, the higher Rac1 levels in pancreatic exocrine cells from TIP patients could, at least partially, explain TIP mechanism. Further studies should include proteomic analysis of Rac1 activity in TIP and no-TIP pancreatic exocrine cells, as well as functional assays to determine its role in apoptosis.

Our study, based on iPSCs and iPSC-derived pancreatic exocrine models, underlines the importance of using personalized assays to better understand TIP mechanisms. One of the major limitations of this exploratory study is the high variability that was observed, especially for the thiopurine metabolites and the Rac1 protein expression. Therefore, by considering a larger number of patients, and further investigating the role of Rac1 (e.g. assessing its activity), it will be possible to verify our hypothesis and evaluate this protein as a clinical biomarker for the prediction of TIP. Nonetheless, more investigations are needed, such as genomic, transcriptomic, and proteomic analyses.

5. Conclusions

Our findings highlight that both iPSCs and pancreatic exocrine cells from TIP pediatric patients exhibit higher cytotoxicity to thiopurines compared to cells from pediatric patients without TIP. These differences might be related to pharmacokinetics in iPSCs, and especially to the higher expression of *TPMT* in no-TIP cells which could lead to a greater detoxification of the cytotoxic metabolites. Conversely, the higher Rac1 expression in TIP pancreatic exocrine cells could promote thiopurine cytotoxicity in differentiated cells of TIP patients. Thus, we hypothesize different thiopurine cytotoxicity mechanisms between iPSCs and pancreatic cells.

Informed consent and ethics

The study was approved by the Ethical Committee of the Institute of Maternal and Child Health IRCCS Burlo Garofolo, with approval number 1556 (internal ID of the study RC 07/14 and RC 44/22).

Title of the approved project: " Patient-derived induced pluripotent stem cells for personalizing therapy: the paradigm of thiopurine pancreatitis in Crohn's disease" and "Comparison of 2D and 3D patient-derived pancreatic exocrine models for the study of thiopurine induced pancreatitis in pediatric IBD patients: an innovative approach therapy personalization"; Name of the institutional approval committee: Ethical Committee of the Institute of Maternal and Child Health IRCCS Burlo Garofolo (Trieste, Italy); Approval number: 1556; Date of approval: 14.07.2014.

Parental informed consent was obtained from all subjects involved in the study.

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CRediT authorship contribution statement

Conceptualization: GD, ML, and GS. Data curation: PR, EG, MF, RMF, ELM, MB, SM, EM, MM, FM, and MP. Formal analysis: PR and EG. Funding acquisition: GD and GS. Investigation: PR, EG, ML, and GS. Methodology: PR, EG, FY, MF, UH, RMF, ELM, GP, and MP. Project administration: GD, ML, and GS. Resources: SCG, MS, GD, ML, and GS. Supervision: UH, MS, FY, SCG, MP, GD, ML, and GS. Patients' enrollment: MB, SM, EM, MM, and FM; Writing—original draft: PR and EG. Writing—review and editing: PR, EG, FY, KJ, MF, UH, MS, RMF, ELM, SCG, GP, MB, SM, EM, MM, FM, MP, GD, ML, and GS.All the authors read and approved the final 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.

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The authors declare that they have not used AI-generated work in this manuscript.

Consent for publication

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.2025.118539.

Data availability

Data will be made available on request.

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