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Review Article

Review Article

A Systematic Review and Meta-Analysis of 6-Thioguanine Nucleotide Levels and Clinical Remission in Inflammatory Bowel Disease

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Abstract

Background and Aims: Thiopurines are widely used in the management of inflammatory bowel diseases. However, their minimum effective dose and dose-response relationship remain undefined, and evidence about their use in clinical practice is mostly heterogeneous. This systematic review and meta-analysis aimed: i] to assess the clinical value of 6-thioguanine nucleotide thresholds; and ii] to compare mean 6-thioguanine nucleotide concentrations between patients in clinical remission *vs.* those with active disease.

Methods: A systematic literature search was carried out using four databases. Statistical heterogeneity was assessed with the I^2 statistic followed by subgroup and sensitivity analyses. Odds ratios were computed using the random-effects model.

Results: A total of 1384 records were identified in the systematic search, of which 25 were retained for further analysis: 22 were used in the cut-off comparisons and 12 were used in the 6-thioguanine nucleotide mean differences analysis. The global odds ratio for remission in patients with 6-thioguanine nucleotide levels above the predefined thresholds was 3.95 (95% confidence interval [CI], 2.63–5.94; $p < 0.001$]. When considering the different thresholds individually, the odd ratios were significant for values above 235 pmol/8 × 10^8 and 250 pmol/8 × 10^8 red blood cells [2.25 and 4.71, respectively]. Mean 6-thioguanine nucleotide levels were higher among patients in clinical remission, with a pooled difference of 63.37 pmol/8 × 10^8 red blood cells [95% CI, 31.81–94.93; $p < 0.001$].

Conclusions: This study reinforces the link between 6-thioguanine nucleotide levels and clinical remission in inflammatory bowel diseases, also exploring the validity of specific 6-thioguanine nucleotide thresholds to predict clinical outcomes.

Key Words: Thiopurines; inflammatory bowel disease; clinical outcome

1. Introduction

Inflammatory bowel diseases [IBD] are complex, multifactorial and known to arise in response to a complex interplay of individual genetics, environmental triggers, and changes in the intestinal microbiome. The combination of those factors may stimulate an unbalanced immune response, which in turn leads to a chronic intestinal inflammation.[1] IBDs have a substantial impact on patients' health-related quality of life [HRQoL], given their early onset, main symptoms, fluctuating course, and lack of curative options. Moreover, IBD monitoring and treatments carry considerable expense to health care systems.[2]

Thiopurines, comprising azathioprine [AZA], mercaptopurine [MP], and thioguanine [TG], are part of the therapeutic armamentarium used in IBD treatment. Their pharmacologically active metabolites are 6-thioguanine nucleotides [6-TGN], and their immunosuppressive effect is attributed to their incorporation into nucleic acids and consequent inhibition of lymphocyte proliferation, as well as to their important role in inducing apoptosis.[3] Indeed, these drugs specifically target the Vav1/Rac1 signalling pathway of T lymphocytes: 6-thioguanine triphosphate [one of the downstream metabolites] binds to Rac1 as a competitive antagonist of guanosine triphosphate [GTP] and converts a co-stimulatory signal into an apoptotic one.[4,5]

These drugs are steroid-sparing agents and are indicated after surgery in Crohn's disease [CD], in case of failure of maintenance therapy with 5-ASA [5-aminosalicylic acid] in ulcerative colitis [UC], and as concomitant immunosuppressive drugs during therapy with biologic agents.[6] Notwithstanding, the moment of introduction of these immunomodulators in IBD therapy is being progressively anticipated to earlier stages of the disease evolution—in fact, the latest therapeutic approaches favour a top-down strategy as an attempt to alter the natural history of these conditions.[7]

Traditionally, thiopurines dosing is weight-based, starting at a low dosage and being then gradually increased until reaching full therapeutic levels [2.0 to 2.5 mg/kg/day of AZA or 1.0 to 1.5 mg/kg/day of MP], following haematological monitoring.[8] However, this conventional dosing strategy has been associated with intolerance, inefficacy, and adverse effects, leading to the cessation of therapy in 9% to 25% of patients.[8,9] An alternative dosing strategy based on thiopurine S-methyltransferase [TPMT] activity may prevent early leukopenia, but the other causes of intolerance and adverse effects remain apparently constant.[10]

According to the literature, therapeutic response to thiopurines is influenced by many factors, including genetic differences, age, and disease duration and severity, as well as comorbidities. An inadequate response to these drugs is, in most cases, related to underdosing and/or poor compliance.[11] On the other hand, drug adverse reactions may be related to drug metabolism or be of an idiosyncratic origin, the latter accounting for 1% to 7% of all cases.[10] As such, surveillance plays a key role for prompt identification of loss of response and toxicity.[11]

The latest developments on thiopurines' pharmacokinetics and pharmacodynamics, allied to a growing clinical experience, has allowed the optimisation of dosing regimens with a positive impact on efficacy and safety.[10] Nonetheless, the minimum effective dose is not yet consensual, and the dose-response relationship is still controversial.[9]

The usefulness of TPMT phenotyping and genotyping and also of therapeutic drug monitoring [through analysis of 6-TGN levels, blood count measurements, or evaluation of erythrocyte mean corpuscular volume as a surrogate marker of 6-TGN concentration] have recently been addressed.[6,12,13] However, due to studies' heterogeneity and conflicting results, its application to clinical practice is still debatable.[14] In fact, according to the current European and American guidelines [ECCO and AGA], the available scientific evidence is insufficient to recommend the routine measurement of 6-TGN metabolites.[15,16]

This paper's aim was to systematically review all published evidence regarding the use of thiopurines in the treatment of IBD. The specific objectives were: i] to determine whether there was a relationship between the 6-TGN thresholds and clinical outcomes; and ii] to analyse the differences of mean 6-TGN concentrations between patients with active disease and those achieving remission. To do so, all results from selected articles were quantitatively integrated in a detailed meta-analysis.

2. Materials and Methods

2.1. Search strategy

This study was conducted following the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses [PRISMA] Guidelines,[17] as well as the Cochrane Collaboration Guidelines for reporting meta-analyses.[18] The published studies were retrieved after a literature search including four electronic databases: PubMed [https://www.ncbi.nlm.nih.gov/pubmed/], Web of Science [http://www.isiwebofknowledge.com], ScienceDirect [www.sciencedirect.com], and CENTRAL—Cochrane Central Register of Controlled Trials [http://www.mrw.interscience.wiley.com/cochrane/cochrane_clcentral_articles_fs.htm]. The literature search was carried out in December 2016 using the following words or medical subject heading terms: (['thiopurine*'] OR ['azathioprine'] OR ['6-mercaptopurine'] OR ['6-thioguanine nucleotide*']) AND (['inflammatory bowel disease*'] OR ['Crohn's disease'] OR ['colitis, ulcerative']) AND (['clinical response'] OR ['remission'] OR ['disease activity'] OR ['outcome*']). In order to ensure that all pertinent articles were included, the reference lists of the studies selected from the databases were manually reviewed.

2.2. Eligibility and inclusion/exclusion criteria

Any study enrolling adult or infant patients previously diagnosed with IBD using clinical, endoscopic, radiological, and/or pathological features, and using a commonly accepted method to assess disease progression, was considered eligible for inclusion in this systematic review. Moreover, the following studies' designs were considered: randomised controlled trials [RCT], cohort studies, and case series. The inclusion criteria were: i] articles studying the association between 6-TGN concentrations and clinical outcomes [active disease or remission]; and ii] English language. No restriction in terms of publication dates was applied. Whenever full-text articles were unavailable, abstracts were included if considered critically relevant.

Exclusion criteria included: i] systematic reviews or guidelines; ii] studies involving patients with diseases other than IBD; or iii] studies involving patients receiving thiopurines in association with biological agents or with a low dose of allopurinol. Indeed, none of the studies where allopurinol had been added to thiopurine treatment was eligible as they all failed the criterion of relating 6-TGN levels to clinical outcomes. Animal studies were also excluded, as recommended in the Cochrane Handbook of Systematic Reviews of Interventions.

2.3. Study selection and data collection process

The studies identified using the databases or the reference lists were independently screened by two reviewers. Any study in which title and abstract clearly indicated that it failed to meet the previously

described selection criteria, was immediately excluded from further analysis. In all the other studies, the full text was considered in order to determine its inclusion or exclusion.

The following information was collected from the selected studies: journal and authors' names, publication year, article type, study design, cohort's geographical origin, number of patients enrolled, cohort's age group [paediatric *vs*. adult], IBD type, type of thiopurine used and treatment duration, 6-TGN serum levels, 6-TGN cut-offs, methodology used in 6-TGN quantification, definition of clinical outcomes, and proportion of patients in remission or with active disease above and below the 6-TGN cut-off defined. Patients labelled as 'partial responders' were considered to have active disease.

2.4. Quality assessment

A funnel plot was used as a visual aid to detect potential publication bias and/or systematic heterogeneity. The quality of the included studies was further independently assessed by two investigators, following the quality assessment tool [QATSDD] described by Sirriyeh *et al.* [2012].[19] This assessment tool includes 16 items scored from 0 to 3, which reflect the intelligibility of description of aims and setting, data quality, methodology, and self-assessment. Two of the items concern only qualitative studies, and therefore were not evaluated in this study. For each paper, the scores were added and divided by the maximum possible score [42] to obtain the paper's overall quality score.

2.5. Statistical analysis

The main variable analysed in this meta-analysis was the clinical response, defined as remission *vs*. active disease. The proportion of patients in remission with concentrations of 6-TGN above and below the defined threshold values was extracted or calculated from each article included. These two groups were then compared using random-effects meta-analysis and following Cochran–Mantel–Haenszel statistics to estimate the odds ratio [OR] for remission and its 95% confidence interval [95% CI]. This methodology assumes that the effects estimated in the different studies are not identical but are similar, and follow some distribution.[20] All estimates were re-computed from the descriptions provided in the original articles, which might result in values that are slightly different from the original ones. Moreover, random-effects models were used to test whether mean 6-TGN concentrations differ among patients in remission or with active disease.

All *p*-values are two-sided and have a 5% significance level. Review Manager version 5.3 was used to calculate the ORs and the corresponding 95% CIs, to generate the forest and funnel plots, and to evaluate the mean 6-TGN differences. Statistical heterogeneity was assessed using the I^2 statistic [values above 50% indicate a substantial level of heterogeneity] and by performing subgroup analyses on the following variables: i] paediatric/adult population; ii] single/multiple 6-TGN measurements; iii] 6-TGN determination methodology; iv] duration of treatment; v] patient's origin; and vi] tools used to evaluate clinical remission. The stability of the combined ORs and the weight of each study in the heterogeneity analysis was assessed by performing a sensitivity analysis omitting one study at a time in a stepwise fashion.

3. Results

3.1. Bibliographic search and study selection

The selection strategy followed is summarised in Figure 1. The initial electronic database search yielded 1384 results, of which 289 were excluded: 141 for being neither observational studies nor controlled trials; 105 for being duplicates; and 43 for not involving humans. Of the remaining studies [*n* = 1095], 1034 were excluded after screening their titles and abstracts: 915 did not relate predefined 6-TGN thresholds with clinical outcomes; five were guidelines; six enrolled patients with comorbidities; six where abstract and full text were not available; and 102 were written in a language other than English. A total of 61 papers were then considered for full-text analysis, from which 36 were excluded: 19 did not present 6-TGN mean values nor defined cut-offs; eight assessed an outcome not considered in our meta-analysis [ie different from clinical remission/disease activity]; five did not depict 6-TGN levels; two had 6-TGN levels expressed in units other than those used in our analyses; one where the definition of remission was endoscopic; and one that was unavailable.

Overall, 25 studies matched the inclusion criteria of this systematic review, which included a total of 3515 patients with IBD [2093 in remission and 1422 with active disease] [Table 1]. From the 25 studies included, 22 were used in the comparison of 6-TGN cut-off values [as three[8,21,22] did not have all the information needed for this], and 11 were used to compute the pooled 6-TGN levels among patients in remission *vs*. those with active disease.

3.2. Study descriptions

The 25 selected studies varied widely regarding their country of origin and their publication year [from 1996 to 2016], as well as the number of patients enrolled [between 25 and 240] and their age [seven[8,23–28] studied paediatric populations; 16 included only adults; two[29,30] included both children and adults]. Regarding the IBD type, four studies[23,31–33] included patients with CD, one[8] studied patients with UC, 18 evaluated CD and UC patients (among which five[25,28,30,34,35] also considered indeterminate colitis [IC]), and two[36,37] did not specify the IBD type. However, the impact of IBD type in the results could not be assessed, as only three studies[33,38,39] had the results stratified by this criterion. Moreover, selected studies also varied widely regarding disease severity definition. Indeed, four different classification systems were used for patients with CD (Harvey-Bradshaw Activity Index [HBI], Crohn's Disease Activity Index [CDAI], Paediatric Crohn's Disease Activity Index [PCDAI] ,and Inflammatory Bowel Disease Questionnaire [IBDQ]), and for UC this number was even higher. Also, three[29,30,35] studies used a clinical remission definition based on the clinicians' global assessment.

According to the literature,[40] 6-TGN concentration in erythrocytes is related to the extent of incorporation of this nucleotide in peripheral blood leukocyte DNA, being a surrogate marker for the assessment of thiopurine therapy. Multiple per-patient measurements of 6-TGN levels were performed in nine studies,[21,25–28,30,32,36,37] whereas in 16 studies only one determination per patient was carried out. These determinations were made by high-performance liquid chromatographic [HPLC] assays except in the studies carried out by González-Lama *et al.* [2011][41] and Kim *et al.* [2014],[5] where liquid chromatography–mass spectrometry [LC-MS] was applied. Concerning HPLC assays, 13[21–26,31,34–36,38,42,43] of the 25 studies followed the procedures described by Lennard and Singleton [1992],[44] five[27,28,30,32,33] followed the methodology published by Dervieux and Boulieu [1998],[45] and two followed the procedures described by Erdmann *et al.* [1990].[46] Two[29,37] studies failed to mention the determination methodology. In order to compare the data obtained from different methodologies and following a previously recommended strategy,[47,48] the Lennard assay was used as 'standard' and the conversion factor of 1.6 was applied to the results obtained[49,50] using the Erdmann method, whereas a conversion factor of 2.6 was applied to

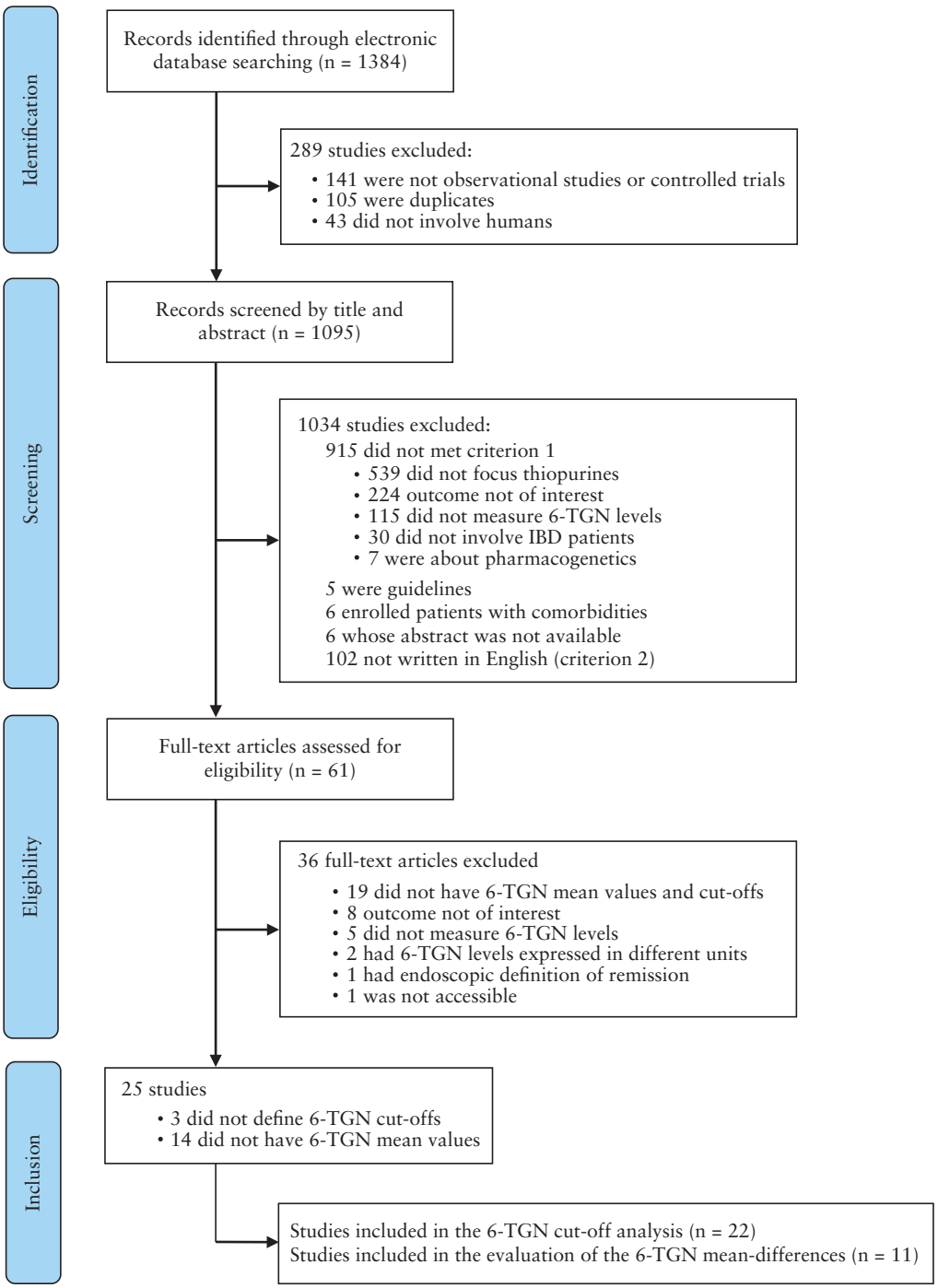

Figure 1. Flow diagram concerning article selection and data collection process.

the results obtained using the Dervieux and Boulieu method. These three methodologies determine 6-TGN levels following monophosphate, diphosphate, and triphosphate hydrolysis and are based on HPLC coupled with MS or UV detection. Even though the results vary according to the assay, due to the extent of hydrolysis, it was been demonstrated that there is a high degree of correlation among methodologies.[14,49]

There was considerable variability concerning the definition of 6-TGN threshold levels: 200 pmol/8 × 10^8 red blood cells [RBC], 225 pmol/8 × 10^8 RBC, 230 pmol/8 × 10^8 RBC, 235 pmol/8 × 10^8 RBC, 250 pmol/8 × 10^8 RBC, and 260 pmol/8 × 10^8 RBC were the thresholds considered in one,[30] one,[33] three,[31,41,43] 10,[25,26,29,32,34–37,39,50] nine,[23,24,27,28,31,34,38,42,49] and two[35,41] studies, respectively. Four[31,34,35,41] of the 22 studies used for 6-TGN cut-offs comparison considered two threshold values. In these cases, the lowest threshold value was used in the pooled results, whereas both cut-offs were considered in the per-'6-TGN level' analysis.

The computation and analysis of a forest plot revealed a considerable amount of variability between studies, even though the funnel plot does not suggest the existence of substantial publication bias.

The results of the methodological assessment are presented in the last column of Table 1 and are expressed as the percentages of

Table 1. Characteristics of the studies included in the meta-analysis.

Study	Country	Study design	Population	Disease	Treatment regimen and duration	6-TGN			6-TGN > cutoff		Definition of remission	QAT score [%]
						Measurement per patient	Method	Cut-off pmol/8 × 10^8RBC	Remission	Active disease		
Cuffari *et al.*, 1996[23]	USA	Prospective	25 patients, paediatric	CD	6-MP > 4 months	Single	HLPC [Lennard and Singleton]	250	10/20 = 50%	1/5 = 20%	HBI < 5	69.05
Cuffari *et al.*, 2000[24]	USA	Prospective	82 patients, adults	59 CD, 23 UC	AZA, 6-MP > 2 months	Single	HLPC [Lennard and Singleton]	250	32/42 = 76.19%	9/40 = 22.50%	HBI < 5 [CD]; Lichtiger's score < 10 [UC]	64.29
Dubinsky *et al.*, 2000[25]	Canada	Prospective	92 patients, paediatric	79 CD, 8 UC, 3 IC	AZA, 6-MP > 4 months	Multiple	HLPC [Lennard and Singleton]	235	69/103 = 67%	20/70 = 28.57%	HBI < 5 [CD]; TWAI < 5 [UC]	80.95
Belaiche *et al.*, 2001[31]	Belgium	Prospective	28 patients, adults	CD	AZA, 6-MP > 3 months	Single	HLPC [Lennard and Singleton]	250 230	5/6 = 83.33% 6/8 = 75%	1/6 = 16.67% 2/8 = 25%	CDAI < 150	71.43
Cuffari *et al.*, 2001[42]	USA	Prospective	82 patients, adults	63 CD, 19 UC	AZA > 12 weeks	Single	HLPC [Lennard and Singleton]	250	31/45 = 68.89%	5/37 = 13.51%	HBI < 5 [CD]; Lichtiger's score < 10 [UC]	73.81
Gupta *et al.*, 2001[26]	USA	Retrospective	101 patients, paediatric	72 CD, 29 UC	AZA > 4 months	Multiple	HLPC [Lennard and Singleton]	235	37/73 = 50.68%	32/87 = 36.78%	PCDAI < 10 [CD]; TWAI < 5 [UC]	78.57
Lowry *et al.*, 2001[49]	USA	Prospective	170 patients, adults	130 CD, 40 UC	AZA, 6-MP > 3.5 months	Single	HPLC [Erdmann *et al.*]	250	20/114 = 17.54%	10/56 = 10.86%	IBDQ ≥ 170	64.29
Achkar *et al.*, 2004[35]	USA	Retrospective	60 patients, adults	49 CD, 8 UC, 3 IC	AZA, 6-MP > 3 months	Single	HLPC [Lennard and Singleton]	260 235	16/24 = 66.67% 19/24 = 79.17%	13/36 = 36.11% 8/36 = 22.22%	Global assessment	59.52
Cuffari *et al.*, 2004[21]	USA	Prospective	101 patients, adults	65 CD, 36 UC	AZA > 4 months	Multiple	HLPC [Lennard and Singleton]	Not defined	—	—	HBI < 5 [CD]; TWAI < 5 [UC]	73.81
Goldenberg *et al.*, 2004[50]	Canada	Retrospective	74 patients, adults	56 CD, 18 UC	AZA, 6-MP > 10 weeks	Single	HPLC [Erdmann *et al.*]	235	7/15 = 46.67%	22/59 = 37.29%	HBI < 5 [CD], PTAI ≤ 3 [UC]	71.43
Wusk *et al.*, 2004[29]	Switzerland	Prospective	158 patients, paediatric and adults	119 CD, 63 UC	AZA, 6-MP, 6-TG > 1 month	Single	Not stated	235	67/83 = 80.72%	44/75 = 58.67%	Global assessment	52.38

Table 1. *Continued*

Study	Country	Study design	Population	Disease	Treatment regimen and duration	6-TGN			6-TGN > cutoff		Definition of remission	QAT score [%]
						Measurement per patient	Method	Cut-off pmol/8 × 10^8RBC	Remission	Active disease		
Roblin *et al.*, 2005[38]	France	Prospective	106 patients, adults	70 CD, 36 UC	AZA > 1 month	Single	HLPC [Lennard and Singleton]	250	77/90 = 85.56%	Not provided	CDAI < 150 [CD]; UCDAI < 2 or Lichtiger's score < 10 [UC]	80.95
Ooi *et al.*, 2007[36]	Australia	Retrospective	56 patients, paediatric	42 CD, 4 UC, 10 IC	AZA, 6-MP > 1 month	Multiple	HPLC [Lennard *et al.* modifed]	235	58/158 = 36.71%	32/168 = 19.05%	PCDAI < 15	66.67
Andoh *et al.*, 2008[39]	Japan	Prospective	83 patients, adults	42 CD, 41 UC	AZA, 6-MP > 4 months	Single	HPLC [Pike *et al.*]	235	28/42 = 66.67%	18/41 = 43.90%	CDAI < 150 [CD]; Rachmilewitz's clinical activity index < 4 [UC]	57.14
Kwan *et al.*, 2008[43]	USA	Retrospective	39 patients, adults	23 CD, 16 UC	AZA, 6-MP > 6 months	Single	HPLC [Lennard *et al.* modifed]	230	10/16 = 62.50%	6/18 = 33.33%	HBI < 5 [CD]; SCCAI [UC]; global assessment	69.05
Hanai *et al.*, 2010[22]	Japan	Prospective	170 patients, 14–68 years old	UC	6-MP > 12 weeks	Single	HPLC [Lennard *et al.* modifed]	Not defined	—	—	Rachmilewitz's clinical activity index < 4	64.29
Waljee *et al.*, 2010[37]	USA	Retrospective	240 patients, adults	IBD	AZA, 6-MP > 3 months	Multiple	Not stated	235	94/216 = 43.52%	55/179 = 30.72%	mHBI < 4; [CD] mUCDAI < 4 [UC]	85.71
González-Lama *et al.*, 2011[41]	Spain	Prospective	95 patients, adults	CD, UC	AZA, 6-MP > 2 months	Single	LC-MS [Dervieux *et al.*]	230 260	49/86 = 56.98% 49/86 = 56.98%	8/11 = 72.73% 8/11 = 72.73%	CDAI < 150 [CD]; mTWAI < 11 [UC]	90.48
Gilissen *et al.*, 2012[34]	The Netherlands	Prospective	100 patients, adults	57 CD, 40 UC, 3 IC	AZA, 6-MP > 3 months	Single	HPLC [Lennard *et al.* modifed]	235 250	35/59 = 59.32% 32/59 = 54.24%	15/41 = 36.59% 12/41 = 29.27%	CDAI < 150 [CD]; CAI < 8 [UC]	78.57
Nguyen *et al.*, 2013a[27]	France	Retrospective	86 patients, paediatric	59 CD, 26 UC, 1 IC	AZA > 2 months	Multiple	HPLC [Dervieux and Boulieu]	250	145/173 = 83.82 %	182/267 = 68.16 %	PCDAI ≤ 10 [CD]; PUCAI ≤ 10 [UC]	80.95
Nguyen *et al.*, 2013b[28]	France	Retrospective	78 patients, paediatric	52 CD, 26 UC	AZA > 3 months	Multiple	HPLC [Dervieux and Boulieu]	250	24/57 = 42.11%	5/21 = 23.81%	PCDAI ≤ 10 [CD]; PUCAI ≤ 10 [UC]	76.19
Smith *et al.*, 2013[30]	UK	Retrospective	189 patients, paediatric and adults	134 CD, 50 UC, 5 IC	AZA, 6-MP > 1 month	Multiple	HPLC [Dervieux and Boulieu]	200	218/260 = 83.85%	11/67 = 16.42%	Global assessment	64.29

Table 1. *Continued*

Study	Country	Study design	Population	Disease	Treatment regimen and duration	6-TGN			6-TGN > cutoff		Definition of remission	QAT score [%]
						Measurement per patient	Method	Cut-off pmol/8 × 10^8RBC	Remission	Active disease		
Kim *et al.*, 2014[8]	Korea	Retrospective	109 patients, paediatric	87 CD, 22 UC	AZA > 3 months	Single	LC-MS [Dervieux *et al.*]	Not defined	—	—	PCDAI ≤ 10 [CD]; PUCAI ≤ 10 [UC]	71.43
Liu *et al.*, 2016[32]	China	Prospective	69 patients, adults	CD	AZA > 3 months	Multiple	HPLC [Dervieux and Boulieu]	235	44/60 = 73.33%	4/9 = 44.44%	CDAI < 150; CRP levels	71.43
Fangbin *et al.*, 2016[33]	China	Prospective	70 patients, adults	CD	AZA, 6-MP > 12 weeks	Single	HPLC [Dervieux and Boulieu]	225	39/47 = 82.98%	11/23 = 47.83%	CDAI < 150 [CD]; Southland Index ≤ 2 [UC]	76.19

6-TGN, 6-thioguanine; RBC, red blood cells; CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; IC, indeterminate colitis; 6-MP, 6-mercaptopurine; AZA, azathioprine; HLPC, high-performance liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; CRP, C-reactive protein; HBI, Harvey-Bradshaw Activity Index; TWAI, Truelove and Witts' Activity Index; CDAI, Crohn Disease Activity Index; PDCAI, Paediatric Crohn's Disease Activity Index; PTAI, Powell-Tuck Activity Index; IBDQ, Inflammatory Bowel Disease Questionnaire; UCDAI, Ulcerative Colitis Disease Activity Index; SSCAI, Simple Clinical Colitis Activity Index; CAI, Clinical Activity Index; PUCAI, Paediatric Ulcerative Colitis Activity Index; QAT Score, Quality Assessment Tool Score.

the maximum possible score obtained for each included study, considering the 14 criteria of the quality assessment tool [QATSDD]. Studies' scores ranged from 52.38% (Wusk *et al.* [2004])[29] to 90.48% (González-Lama *et al.*, [2011[51]]), yielding an average quality score for all papers of 71.43 ± 9.39%. The studies with lower methodological quality scores were those where IBD severity was defined through global assessment and where the assay used for 6-TGN measurements was not stated. Almost all evaluated studies had the maximum possible score for the parameters: 'explicit theoretical framework', 'statement of aims/objectives', 'clear description of research setting', and 'description of procedure for data collection', whereas the lowest scores were found for criteria: 'evidence of sample size considered in terms of analysis' and 'evidence of user involvement in design'.

3.3. Cut-offs of 6-TGN and clinical remission

A pooled analysis of the 22 studies [including the different thresholds ranging 200-260 pmol/8 × 10^8 RBC] revealed that 63.04% [95% CI, 56.32–69.88] of the patients in clinical remission [n = 1791] had 6-TGN levels above the considered cut-offs, a situation shared with 37.65% [95% CI, 30.67–44.63] of the patients with active disease [n = 1344]. Overall, patients with 6-TGN levels above the predefined thresholds were nearly four times more likely to be in clinical remission [OR = 3.95, 95% CI, 2.63–5.94; p < 0.001] [Figure 2]. When considered individually, statistical significance was reached in 15[24,25,28–31,33–39,42,50] of the 22 studies.

The studies considered in this analysis had a significant degree of heterogeneity [p < 0.001; I^2 = 79%]. As such, sensitivity analyses were performed. However, the exclusion of each study individually has neither eliminated the heterogeneity nor changed considerably the pooled OR. Subgroup analyses were then performed to further explore this issue. Considering that the number of subgroups must be reduced to avoid spurious statistical findings, the following comparisons were made: i] studies enrolling adults *vs.* those with paediatric cohorts [p = 0.130; I^2 = 55%]; ii] single *vs.* multiple 6-TGN measurements [p = 0.198; I^2 = 34%]; iii] the three most used methodologies for 6-TGN determination [p = 0.170; I^2 = 43%]; iv] duration of treatment [p = 0.760; I^2 = 0%]; v] continent of origin [p = 0.440; I^2 = 0%]; and vi] tools used for the definition of IBD severity. A significant degree of heterogeneity was found to be associated with the tools used for disease severity classification for UC [p < 0.001; I^2 = 83.5%], but not for CD [p = 0.211; I^2 = 34%] [Supplementary Figures 2 and 3, available as Supplementary data at *ECCO-JCC* online]. None of the other comparisons revealed a considerable heterogeneity degree [Supplementary Figures 4 and 5, available as Supplementary data at *ECCO-JCC* online].

Studies were stratified into six groups according to their cut-offs, and the OR for remission was separately computed for each of these groups [Figure 3]. Two threshold values were used in a single study: 200 pmol/8 × 10^8 RBC[30] and 225 pmol/8 × 10^8 RBC,[33] and their ORs for remission were 26.42 [95% CI, 12.79–54.51] and 5.30 [95% CI, 1.7-16.3], respectively. The pooled ORs for remission were: 2.12 [95% CI, 0.41–10.88; p = 0.370] for a cut-off of 230 pmol/8 × 10^8 RBC [Figure 3A]; 2.66 [95% CI, 1.94–3.66; p < 0.001] for a cut-off of 235 pmol/8 × 10^8 RBC [Figure 3B]; 4.71 [95% CI, 2.31–9.62; p < 0.001] for a cut-off of 250 pmol/8 × 10^8 RBC [Figure 3C]; and 1.39 [95% CI, 0.20–9.55; p = 0.740] for a cut-off of 260 pmol/8 × 10^8 RBC [Figure 3D].

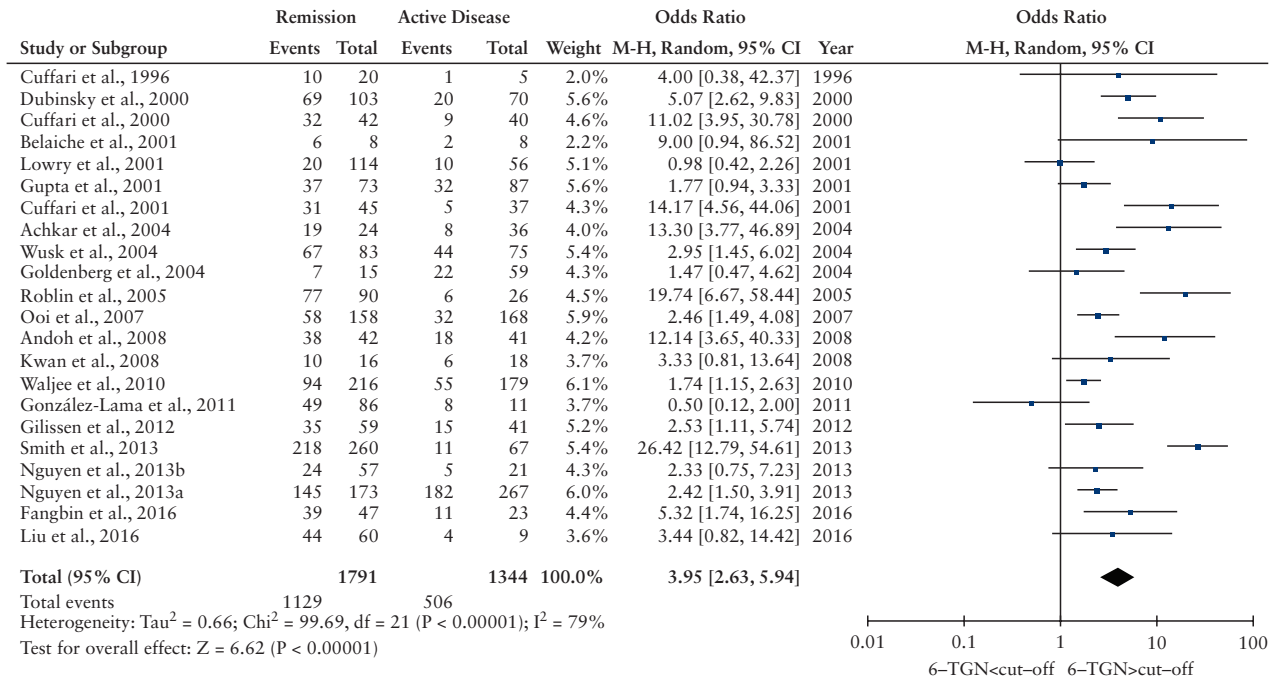

Figure 2. Forest plot representing the odds ratio [OR] for clinical remission associated with 6-thioguanine [6-TGN] levels over a predefined cut-off [global analysis].

3.4. Differences in 6-TGN levels between patients in remission and active disease

Figure 4 depicts the analysis of the mean differences reported for the 6-TGN levels between patients in clinical remission and patients with active disease. This information was extracted from eight[29,32,35,36,39,43,49,50] articles used for cut-off analysis and from three studies[8,21,22] that were solely used in this section. A statistical assessment revealed significant heterogeneity among the 11 considered studies [$p < 0.001$; $I^2 = 92\%$], which could not be eliminated through an individual exclusion approach [sensitivity analysis]. Overall, the mean 6-TGN levels were higher among patients in remission than in those with active disease, with a pooled difference of 63.37 pmol/8 × 10^8 RBC [95% CI, 31.81–94.93; $p < 0.001$] [Figure 4].

4. Discussion

Thiopurines are widely used as immunosuppressive drugs in the treatment of IBD, and their efficacy has been demonstrated in a broad range of clinical presentations.[52] However, a few questions remain unanswered concerning intolerance, loss of therapeutic action, and adverse effects, and these missing links may preclude further drug administration or may even be life-threatening.[53] An association between 6-TGN and clinical remission was described for the first time by Cuffari *et al.* [1996],[23] who reported a significant inverse relationship between disease activity and nucleotide levels. And in fact, further studies have consistently related thiopurines' clinical efficacy with serum 6-TGN levels of 235-450 pmol/8 × 10^8 RBC.[25] Notwithstanding, and despite the theoretical pertinence of monitoring thiopurine metabolites for therapy optimisation, thiopurine measurement is not recommended by the main guidelines for IBD management, and therefore only a small number of IBD gastroenterologists do it as part of their daily practice.[51] Moreover, the existence of a 6-TGN cut-off level clearly and consistently linked with a clinical outcome remains controversial, given the marked inter- and intra-individual variability of 6-TGN levels. As far as the authors know, this is the first meta-analysis that considers different thresholds of 6-TGN that have been reported in the literature [200, 225, 230, 235, 250 and 260 pmol/8 × 10^8 RBC]. This analysis aimed to overcome the lack of evidence for recommending a specific 6-TGN cut-off as a marker of clinical remission.[54]

The global analysis concerning the association between 6-TGN levels and patient clinical status unveiled that those patients with 6-TGN levels above the different cut-off levels were 3.95 times more likely [95% CI, 2.63–5.94] to achieve clinical remission. This value is similar to others obtained previously: a meta-analysis published in 2006,[48] including six studies and 437 patients, described an OR for remission of 3.27 [95% CI, 1.71–6.27], and a more recent study from 2014,[14] including 17 studies and 2049 patients, reported an OR of 3.15 [95% CI, 2.41–4.11].

When considering the different thresholds separately, the highest pooled OR was found for the threshold of 250 pmol/8 × 10^8 RBC, with a value of 4.71 [95% CI, 2.31–9.62; $p < 0.001$]; followed by that obtained for the cut-off of 235 pmol/8 × 10^8 RBC [OR = 2.66, 95% CI, 1.94–3.66; $P < 0.001$]. The ORs polled for 6-TGN levels above 230 and 260 pmol/8 × 10^8 RBC were lower and not statistically significant [$p > 0.05$].

Even so, it must be noted that 36.96 ± 17.95% of the patients who achieved remission in our study cohort did not have 6-TGN values above the considered thresholds, suggesting that other factors besides these metabolite levels play a role in the response to MP and AZA. Indeed, and even though 6-TGN monitoring provides an insight on thiopurine metabolism, factors like age, gender, disease duration and severity, comorbidities, and concomitant medication have been reported to play a role in these drugs' efficacy.[55] In addition, preliminary reports suggest that there are other parameters that may be more reliable in the prediction of therapeutic efficacy, like the 6-MMPR:6-TGN ratio.[56] More recently, emphasis has been laid on the relevance of monitoring the pharmacodynamics of thiopurines

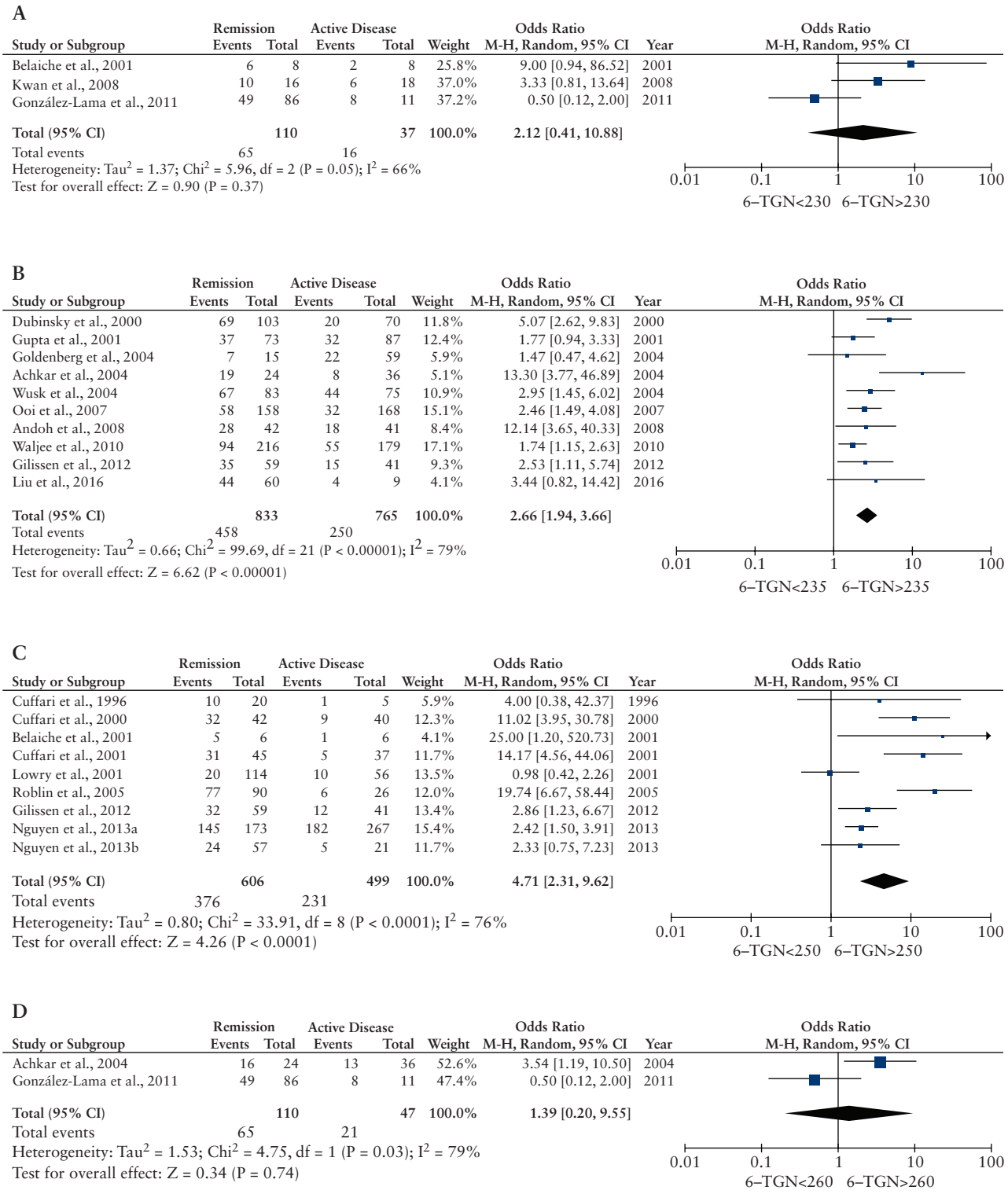

Figure 3. Forest plots representing the odds ratio[OR] for clinical remission associated with6-thioguanine [6-TGN] levels over different threshold values: a] 230; b] 235; c] 250 and d] 260 pmol/8 × 10^8 RBC.

directly in leukocytes through the determination of Rac1-GTP and other downstream molecular mediators.[57]

The analysis of the difference between means has shown that 6-TGN levels of patients in clinical remission are significantly higher than those observed in patients with active disease, with a mean pooled difference of 63.37 pmol/8 × 10^8 RBC [95% CI, 31.81–94.93; $p < 0.001$]. This value is consistent with the one obtained by Osterman *et al.* [2006],[48] who reported a pooled difference of 66 pmol/8 × 10^8 RBC when considering mean and median 6-TGN concentrations from eight studies published between 2000 and 2004. At this point it is important to highlight that we have chosen to include only mean values that were explicitly provided by

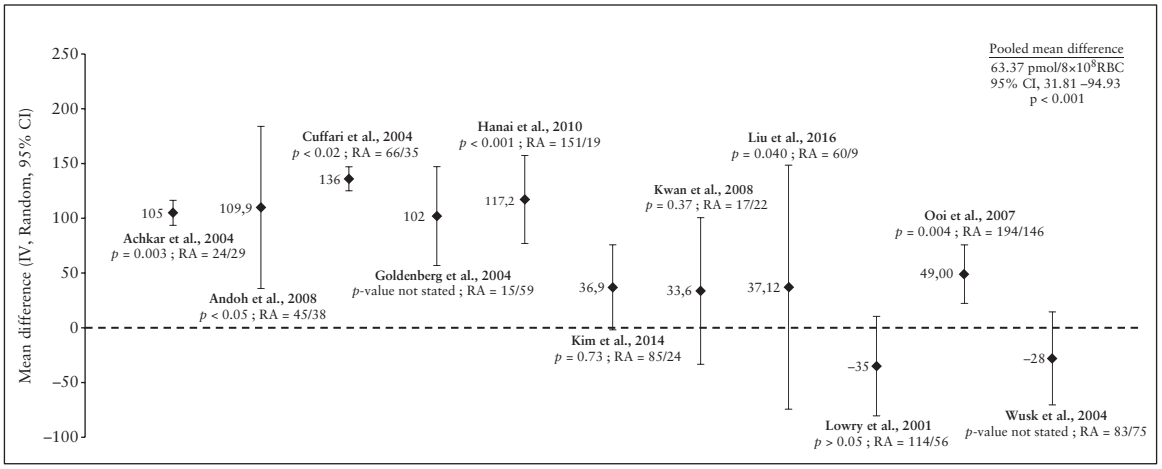

Figure 4. Mean differences between 6-thioguanine [6-TGN] levels in patients in clinical remission and patients with active disease across different published studies. RA, number of patients in remission/patients with active disease].

the authors in their studies, and made no estimation for those that reported only the median or range, in order to avoid possible interferences. Interestingly, two of the studies[29,49] found that the 6-TGN levels were actually higher in patients with active disease when compared with those in clinical remission. Even though those results did not differ statistically [95% CI includes zero], this inverted pattern may be due to the use of cohorts with particularly severe disease conditions—in which a higher serum concentration would be the result of a higher dosage motivated by the severity of the symptoms, or in which the time spent from the beginning of the medication to the study assessment was not long enough to allow the positive effects of medication to take place.

The studies used to compute ORs for clinical remission and 6-TGN mean values were significantly heterogeneous, and this heterogeneity could not be eliminated through sensitivity analysis. A subgroup analysis was carried out to further explore this issue, and no observable subgroup effect was found regarding the variables: paediatric/adult population; methodology used for dosing the 6-TGN; or the use of single/multiple 6-TGN measurements. This last point is particularly important, as according to a prospective cohort study,[58] patients on a stable AZA dose may present variable levels of 6-TGN over time, bringing into question the value of a single measurement. Interestingly, and opposite to our results, a previous meta-analysis on the same topic reported that heterogeneity between studies could be assigned to differences in the analytical methodologies used for 6-TGN measurements. This discrepancy with our results may be due to differences in the studies included. Indeed, and when compared twiththe report published in 2014,[14] there are 10 additional studies included in ours, using five different procedures for the quantification of nucleotide levels [four using HPLC and one with LC-MS], whereas in the former only three methodologies were compared.

The therapeutic effects of thiopurines are reported to take place between 12 to 17 weeks after their initiation.[10] The delayed onset of action may be due to the existence of a certain latency between treatment initiation and thiopurine-induced apoptosis, as well as to the fact that T cells whose cycle is already arrested are still able to have effector cell function, further enhancing a pro-inflammatory environment.[5,59] Even so, no significant subgroup effect regarding the minimal time [below or above 3 months] between treatment initiation and 6-TGN determination was unveiled in the present study. Moreover, and even though the activity of the enzymes involved in thiopurine metabolism is known to vary among ethnic groups,[60] no significant heterogeneity was found between studies enrolling patients from different continents [America, Asia, or Europe]. This is in agreement with what has been previously reported by other authors.[14,60]

On the other hand, heterogeneity between studies may be associated with differences in the tools used for disease severity definition in the case of UC, for which the diversity of classification systems used was higher than for CD. Indeed, upon splitting data into subgroups according to the classification criteria, a quantitative interaction—with constant direction but a variable size of the effect—was observed. This finding corroborates the pertinence of developing a validated and pragmatic severity classification to guide current therapeutic strategies for IBD, as recently highlighted by Peyrin-Biroulet *et al.* [2016].[61]

This study has a few methodological limitations that should be taken into consideration. First, it is important to consider that clinicians may be more prone to order 6-TGN testing in patients whom disease remains active despite the pharmacological treatment. Accordingly, the proportion of non-responders in this meta-analysis [74% of the 3292 patients] is clearly higher than that observed in clinical practice and in clinical trials.[62] Second, and due to the limited availability of data from the included studies, the results of patients with CD and UC were pooled together. The analysis of the impact of the type of disease could have been important—in fact, Fangbin *et al.* [2016][33] reported that 6-TGN levels correlated well with clinical responsiveness in CD patients but not in UC patients. Third, some parameters whose assessment was out of the scope of this review may have had an unpredictable effect on clinical outcomes. Among these, one should highlight different treatment regimens and durations, disease-related factors, and genetic polymorphisms. Fourth, the variability in design and methodology of the included studies and the lack of randomisation and blinding may have influenced study outcomes and conclusions. Also, most of the included studies were prospective, and the existence of bias related to losses to follow-up cannot be disregarded. Finally, the clinical outcome definition was heterogeneous among studies. As to avoiding the variability inherent in clinical assessment scales, the use of endoscopic and/or objective biomarkers would have been preferable and should be considered in future studies. Also, the utility of measuring 6-TGN concentrations in the setting of combination with biological agent therapy remains poorly understood.[13]

Currently, therapeutic drug monitoring is almost reserved for particular cases, namely in patients who fail to respond to standard thiopurine doses or when patient non-compliance or toxicity is suspected.[32,56] The results obtained in this meta-analysis reinforce that 6-TGN levels are related to clinical remission, and provide an insight regarding the cut-offs that may be used to guide clinical decision making.

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Conflict of Interest

FM served as speaker and received honoraria from Merck Sharp & Dohme, Abbvie, Vifor, Falk, Laboratorios Vitoria, Ferring, Hospira, and Biogen. The other authors have no conflict of interest to disclose.

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Author Contributions

MME and JA were involved in acquisition, analysis, and interpretation of data and drafting of the manuscript; IR, PL, ET and LC helped with data interpretation and manuscript drafting; CC coordinated the statistical analysis; and FM was involved in study concept and design, data interpretation, and manuscript drafting. All authors read and approved the final manuscript.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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