

Format for ANSWERING REVIEWERS

November 5th, 2014



Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 14405-edited.doc).

Title: Thiopurine metabolites variations during co-treatment with aminosaliclates: effect of N-acetyl transferase polymorphisms

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The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

(1) The manuscript "Variations of thiopurine metabolites during co-treatment with aminosaliclates in young patients with inflammatory bowel disease: effect of N-acetyl transferase polymorphisms" report two results: the first is the concentration of 6-TGN before and after the interruption of the aminosaliclate and the second is the correlation between NAT1 genotypes and 6TGN concentration. Comments: The first results is not original because various studies have reported elevated 6-TGN levels caused by co-administration of 5-ASA during thiopurine therapy (Szumlanski and Weinshilboum, 1995; Lewis et al., 1997; Lowry et al., 2001; Dewit et al., 2002; Xin et al., 2005; Hande et al., 2006; De Boer et al., 2007b).

Reply to reviewer: we agree with the reviewer that the results about a decrease of TGN concentration after aminosalicylate interruption is not novel; however the number of study in the pediatric population is still limited and, moreover, the main aim of this study was to evaluate the integration of pharmacokinetic and pharmacogenetic information.

The underlying pharmacological mechanism remains to be verified. Furthermore, a dose dependent effect was observed for two different 5-ASA doses (2 g followed by 4 g, both daily during 4 weeks) on total levels of 6-TGN and 6-MMPR metabolites. This effect is not mentioned in this manuscript.

As requested by the reviewer we have added the above mentioned study to the discussion, with the sentence "Furthermore, a dose dependent effect was observed for two different 5-ASA doses on thiopurine metabolite levels {de Graaf, 2010}. In our study all patients were treated with a dose of 5-ASA of 50 mg/kg, equivalent to the higher dose reported by de Graaf et al. In our study, after interruption of 5-ASA we observed an effect on TGN and not on MMPN concentration, even with a high dose of 5-ASA. This may be due to the different populations considered: TPMT activity indeed is significantly higher in wild-type children (0.08-17 years) than in wild-type adults (aged 18-68 years) {Serpe, 2009}".

The second result concerned a few number of patients (12) and can't be considered to evaluate the role of genetic polymorphisms on the evolution of 6-TGN concentration.

We agree with the reviewer that the number of patients is limited; therefore we have added to the conclusion of the abstract and of the paper that this has to be considered a pilot study.

The results of previous studies concerning the frequency of NAT 1 and NAT2 polymorphisms in IBD is not mentioned in this manuscript (proportion of slow and rapid acetylators in the group of patients with IBD than in the group of healthy subjects.)

As requested by the reviewer, we have mentioned, in the introduction, the previous study about the incidence of NAT1 and NAT2 polymorphisms in IBD.

(2) This is an interesting and clinically relevant paper. Obviously the study has a small sample size and thus it is best described as a pilot study.

We agree with the reviewer that the number of patients is limited; therefore we have added to the conclusion of the abstract and of the paper that this has to be considered a pilot study.

I have the following concerns for the authors to please address: 1. For the patients enrolled in this study, what was the clinical reason for ceasing the aminosalicilate and then restarting it – if no clinical reason, then are there not ethical concerns if the aminosalicilate was part of the medication regimen for these patients and then ceased (albeit relatively briefly) with any reason but for the purposes of this study? That is, were the participants put at clinical risk by participation in this study?

In our study all patients interrupted the aminosalicilate because of clinical reasons and not for the purposes of this study, and no patient restarted aminosalicylates; indeed, our study had a retrospective design. Clinical reason was mostly simplification of therapy to increase compliance, which is particularly useful in pediatric patients. Moreover for Crohn's disease patients the use of aminosalicilate in relapse prevention is not evidence-based, in particular in patients treated with other immunosuppressants. For ulcerative colitis, at the time of the study (2005-2006) the chemopreventive action was not well established. We have clarified these aspects in the first paragraphs of Materials and Methods and Results.

2. The study is premised on the hypothesis that NAT1 is inherent to 5ASA inactivation and this appears to be based on only one paper published 25 years ago! (Allgayer et al, Gastroenterol 1989) Can the authors please further enlighten the reader on why NAT1 is thought to be integral to 5ASA inactivation?

We have added more recent papers demonstrating the role of NAT1 on 5ASA inactivation to the introduction.

3. There are only 12 patients in this study providing an opportunity for more data transparency by inserting another table including each of the participants, their age, their disease, their thiopurine and 5ASA dose and their before/after 6TGN and percentage change in 6TGN. This would inform the reader about what happened in each case, according to NAT1 status.

We have added the requested Table to the manuscript.

4. Was there a dose-dependent effect of either the 5ASA or the thiopurine on the change in 6TGN? This is important given that if so, hypothetically a clinician may be able to dose-reduce the 5ASA (eg from 4g to 2g daily) maintaining theoretical chemopreventive benefits, rather than cease it in someone with adverse NAT1 status and high 6TGN levels...

The dose of aminosalicilates was consistent among patients and not significantly associated with TGN concentrations. Therefore a dose dependent effect could not be ascertained in our study. However, we appreciate the comment of the reviewer and have added this possibility in the discussion among the potential developments of the research.

5. Using the linear mixed effects model for most analyses you have presented p values, but I think you should also present the coefficient for each analysis also (with p value following in brackets). I am a little surprised that you can derive such statistically significant results with such a small sample size?

We added the coefficient and their confidence intervals to the p-values, as suggested by the reviewer. Given the difference in means and the standard deviation distribution of preliminary data, the minimum sample size to identify a statistically significant ($p = 0.05$, power 80%) result is 9 for the paired test comparing azathioprine metabolites during aminosalicilate treatment and after the suspension. For the analysis comparing thiopurine metabolites concentration in NAT1 fast acetylators compared to slow acetylators, the minimum number of patients to detect a statistically significant ($p = 0.05$, power 80%) result is 5 for each NAT1 activity status. We added a paragraph on the power analysis to the statistical analysis section of the manuscript.

6. Minor typographic/ grammatical errors: ? Short title "aminosalicylates" should read "aminosalicylates" – same error also in Results/ Conclusions sections of Abstract and multiple times in rest of manuscript – please correct ? Last sentence of 1st paragraph in Material and Methods "parents or tutors" – do you mean "parents or guardians"? ? Last sentence of 2nd paragraph in Materials and Methods "The ratio between TGN and the dose of azathioprine was calculated considering for each individual measurement the dose the patients was taking the day the blood sample for the metabolites assessment was collected," could instead read "The ratio between TGN and the dose of azathioprine was calculated to account for the respective dose each patient was taking on the day that the metabolite testing was performed." ? Last sentence of Statistical analysis paragraph "in order to adjust the normality of the distribution," could instead read "in order to achieve normality of the distribution." ? 2nd sentence of 2nd paragraph of Results section "MMPN concentration where not affected.." should read "MMPN concentration were not affected..." ? There may be others, please check carefully!

Typographic and grammatical errors have been corrected.

(3) The authors have attempted to explore the interactions of 5ASA drugs and thiopurines including the effect of enzymes thought to be important in metabolizing 5ASA drugs. The results of this small study are of interest in that there seems to be some pharmacokinetic interaction between 5ASA drugs and thiopurines, there also seems to be a potentially important and previously unexplored effect of N-acetyl transfersase polymorphisms. The main issue regarding this study is the small size and although several statistical analysis have been applied, there is lack of clarity over the precise aim and sample size required of this study. Major points. 1. There are 12 patients but 36 samples distributed across the 2 time points. This seems a very unusual design and I very much suspect this was not how the study was designed.

Given the study design, a minimum of two blood samples for thiopurine metabolites measurement had to be taken one month before and one month after 5-ASA interruption as indicated in the Materials and Methods. However, due to clinical reasons, for 8 patients it was not possible to collect two samples before 5-ASA interruption and two samples after 5-ASA interruption: therefore 12 samples (5 before and 7 after) were missing for the analysis; however each patient had

at least one sample before and one after 5-ASA interruption. We clarified these results in the "Patients enrolled and samples collected" paragraph.

There really should only be 2 samples used per subject, otherwise the risk of bias increases. The results do need to be recalculated with only 2 data points per subject.

As requested by the reviewer, we performed analysis considering only 2 sample per patient, the closest to the 5-ASA interruption, and the results are still significant both for the effect of 5-ASA interruption on TGN concentration (p-value = 0.046) and for the effect of NAT1 acetylator status on TGN concentration (p-value = 0.038).

2. The methods should be explicit in terms of what was the primary intended outcome for this study and how the sample size and power we calculated from this.

The primary intended outcome of this study was to evaluate variations of the concentration of thiopurine metabolites after 5-ASA interruption and the role of genetic polymorphisms of NAT 1 and 2. Given the difference in means and the standard deviation distribution of preliminary data, the minimum sample size to identify a statistically significant ($p = 0.05$, power 80%) result is 9 for the paired test comparing azathioprine metabolites during aminosalicylate treatment and after the suspension. For the analysis comparing thiopurine metabolites concentration in NAT1 fast acetylators compared to slow acetylators, the minimum number of patients to detect a statistically significant ($p = 0.05$, power 80%) result is 5 for each NAT1 activity status. We added a relevant paragraph to the statistical analysis section of the manuscript.

3. The potential effect of NAT1 genotype on thiopurine levels is very interesting but and although data figures are provided it would be very interesting to know the absolute mean values of fall of red cell 6TGNs over the study period and absolute 6TGN levels depending on NAT genotype.

A table with clinical data of patients, metabolite levels and NAT1 status has been added. Moreover, mean values of fall of red cells 6TGN (109 pmol/8x10⁸ erythrocytes) has been added to the abstract and results. Mean values were 269.3 and 400.8 respectively in patients with fast and slow NAT1 status before 5-ASA interruption, and 181.9 and 261.6 after 5-ASA interruption; this sentence has been added to the results.

4. Given the possible mechanisms of NAT1 interacting thiopurine metabolism, the authors really seem to have only performed half a study now. The study would be greatly enhanced by the inclusion of a separate group taking thiopurines but no exposure to 5ASA drugs within a much longer period (say 3 months) and this would help determine if NAT1 does directly influence thiopurine metabolism or whether these effects reported in the current study are predominantly influenced by red cell TGN levels at the initial point in the study and the level of decrease is determined more by red cell life span.

At this point unfortunately we do not have NAT1 acetylator status in a cohort of patient not taking 5-ASA. This could be an interesting development of the study in patients with IBD treated with azathioprine. A sentence in the discussion describing this development was added.

3 References and typesetting were corrected
This was done.

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

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