Name of journal: World Journal of Gastroenterology

ESPS Manuscript NO: 5434

Columns: BRIEF ARTICLES

Dear Associate Editor of World Journal of Gastroenterology,

We thank the reviewers for finding the results of our study interesting and for reading in detail our work entitled "**Thiopurine-methyltransferase variants in inflammatory bowel disease: prevalence and toxicity in Brazilian patients"**. We understand that the corrections and modifications suggested continue to support and confirm the previous findings, and we agree that our study is even more consistent after the changes, contributing for the manuscript improvement.

Reply to the reviewers

Reviewers' Comments:

Reviewer #00503176

I have reviewed the manuscript - *Thiopurine-Methyltransferase variants in inflammatory bowel disease: prevalence and toxicity in Brazilian patients*. It is one of the many papers on the topic, but is of interest since it attempts to relate the genotype to phenotype. Also, is informative since it reports on the prevalence of SNPs in a population for which this has not been reported so far. However, the manuscript requires a major revision.

Major comments

1. The table currently labeled as Table 2 – should be labeled Table 1 – it contains data on primers and is the first table to be mentioned in the manuscript (Patients and Methods section).

We agree with this reviewer and changes were performed.

2. Why do the Results start with "Genotype analysis"? And not with "Patient characteristics"? – This should be changed – The first subsection of the Results should be "Patient characteristics" and the table that is currently labeled Table 1 (contains patient data), should be labeled Table 2, and be referred to in the text.

We understand this reviewer's point of view. But, actually, patients characteristics were already described in Methods session of the manuscript (now Table 2).

3. The first sentence in the Subsection of the Results – "Genotype Analysis" – is difficult to understand. It reads: "The distribution of the selected *TPMT* gene polymorphism TPMT*2 (C238G), TPMT*3A (G460A/A719G), and TPMT*3C (A719G) genotypes was 21.6%, 32.4%, and 46%, respectively, which corresponds to 3.6%, 5.4%, and 7.7% of the patients, respectively". "Distribution of genotypes" implies that what is reported is proportion of patients having each of the tested SNPs (ie., are at least heterozygous). So, how many were (at least) heterozygous at locus 238 (C238G) – 21.6% or 3.6%? Table 3 shows that out of 214 patients, only 8 WERE NOT a "wild type", i.e., 8 were heterozygous at this locus (i.e., had the SNP) – 8/214= 3.7%. The same goes for the other 3 loci. What is meant to be said here?

We agree with this reviewer and changes were performed. Further explanation was added to text.

4. According to Table 3, not ALL subjects were genotyped at all loci – this should be clearly stated. As I mentioned earlier, the first subsection of the Results should be Patient characteristics, and the number of actually genotyped should be declared.

We agree with this reviewer, and we apologize for the mistake, and the correction was carried out.

5. Table 5 – the number of subjects in headings of columns with wild-type homozygous and polymorphic heterozygous subjects should be given.

We agree with this observation, and the text was corrected accordingly.

6. Although the Fischer exact test for proportions, e.g., for pancreatitis in UC patients – 0/69 wild-type vs. 1/2 with SNP (locus 238) was significant – I WOULD NOT CONCLUDE "too much" on this finding – there was overall ONLY ONE SUBJECT with pancreatitis! And 0/69 and 1/2 could have been by chance. Let's assume that the prevalence of pancreatitis in "wild-type" is 2/100 (0.02). There is still 25% probability to find 0 cases among 69 subjects. Let's assume that the prevalence of pancreatitis among SNP carriers is just slightly higher – eg., 0.025. The probability of finding exactly 1 case out of 2 patients is still 5%. In other words – this was a small sample! The findings might be indicative, but I would not draw too many conclusions on such a small sample combined with this low event rate.

We agree with this reviewer's comment, and we understand his/her point of view. We tried to temper our conclusions, rephrasing sentences in the manuscript.

7. The part on statistics in the Patients and Methods section – mentions logistic regression. I do not see that this method was employed anywhere. Footnotes to Table 5, 6 and 7 – point-out that Fischer exact was used to compare proportions of wild-type and "polymorphic" subjects in respect to each of the listed phenomena that, presumably, were AZA adverse events. For which analysis was logistic regression used?

This reviewer is correct. We apologize for the inconvenience and the sentence was removed.

8. Generally, statistical processing of the data seems insufficient. For example, all "genotype-adverse event" associations are based on simple, univariate tests (serial comparisons between wild-type and "SNP" patients for each "adverse effect" using a simple univariate test) that cannot account for any confounding. For example – if there is an association between ONE SNP and one adverse effect, how do you know that there was no confounding by the (possibly) co-existing SNP, or, for example, age, or level of disease activity, or stage or….. Another problem is the number of serial univariate tests. For example, "myelosuppression" and "neutropenia" and "flu-like symptoms" and nausea and vomiting" were each compared SEPARATELY – and

actually, all those phenomena could be ONE SAME ADVERSE EVENT – there is myelosuppression and neutropenia goes with it, and flu-like symptoms actually are a part of the "general infectious syndrome" – and could simply be due to neutropenia…just like nausea and vomiting, etc. This brings-up the problem of multiplicity. Strictly speaking, to keep the "experiment-wise" type I error-rate at 0.05, the comparison-wise error rate needs to be adjusted, e.g., by the Bonferroni or any other adjustment method. For a set of 5 univariate comparisons for the outcomes that are closely related, to keep the overall type I error rate at 0.05, the comparison-wise type I error rate should be 0.05/5= 0.01. By this criterion, none of the comparisons reported "significant" – are no longer "statistically significant". Overall, also – "breaking-down" the whole cohort into disease-type and further "breaking-down" by a clinical condition results in "annihilation" of the sample into "little pieces".
I suggest that in place of the comparisons displayed in tables 5-7 the following is done:
- DO NOT separate CD and UC; do NOT separate ADVERSE EVENTS. Count all patients with at least ONE adverse event that could be considered "likely" AZA-related. The count will likely be around 40 or 50. So, you have a sample of 196 patients WITH ALL LOCI GENOTYPED and among them, 40 phenotypical "cases" – and do the LOGISTIC REGRESSION: dependent variable is "adverse effect, yes/no" and predictors are: genotypes at each of the 3 loci (3 separate independents), DISEASE STAGE and AGE (what is the pharmacokinetics of the metabolite that most likely accounts for toxicity? Eliminated by the kidneys? If yes – than eGFR should be a covariate) – AND THEN TRY TO identify at least "indications" of an independent association. Also, the informative thing would be to see how combination of the polymorphic heterozigocity at all three loci is associated with the presumed adverse events.

We greatly appreciate the comments from this reviewer. We completely agree with his/her comment on statistical analysis. But at this moment (and with the present data, with limited numbers), instead of adding Bonferroni adjustment and/or running additional multivariate comparisons, we decided to keep the manuscript in a more descriptive style, preserving the exploratory nature of the data presentation. For this purpose, we merged the Tables as suggested (5, 6, and 7), and removed all p values. In the discussion, we tried to temper the conclusions regarding isolated findings).

9. There are too many tables. Tables 3 and 4 could be ONE table. And H-W equilibrium could be reported for all patients, and by disease. Tables 5, 6 and 7 could also be one table. But, as mentioned, they should only be descriptive and a multivariate analysis should be attempted to at least explore the possibility of independent associations.
I feel free to insert a table showing how simultaneous existence of SNPs at different loci can be displayed:

We understand this reviewer's point of view, and we managed to present H-W equilibrium by disease (Table 3), and merged Tables 5, 6 and 7 together, removing all p values.

Minor comments
Throughout the text - Why thiopurine-methyl-transferase – with capitalization? Why Azathioprine with capitalization?
We apologize for the mistake, and the correction was performed.

Patients and Method – Details on detection of individual SNPs – no need to repeat the first sentence about PCR.
Although it may appear repetitive, actually we are describing different moments of the work. We were concerned about explaining in detail each step of our protocol.

The second paragraph of the Discussion – repeats what was already said in the Introduction.
Remove – focus on the study – strengths and weaknesses (limitations), the main findings and there potential repercutions.
We tried to follow this reviewer's suggestions.

Abstract – adjust according to the revision of the main text.

After all changes made, we corrected the abstract accordingly.

We again thank this reviewer for his/her attentive and detailed analysis of the manuscript. We believe that his/her comments give us the opportunity to greatly improve our presentation and the manuscript as a whole. We tried to follow, as much as possible, directions from this reviewer.

Reviewer #00503539

ESPS 5434 Ms. Title: Thiopurine-methyltransferase variants in inflammatory bowel disease: prevalence and toxicity in Brazilian patients by Ana Teresa P, et al. General comments : The authors firstly investigated the TPMT*2, TPMT*3A and TPMT*3C genotypes in Brazilian patients with IBD and demonstrated that the prevalence of TPMT gene polymorphisms is relatively high among Brazilian patients, including two genetic variants, TPMT*2 and TPMT*3C, that have been associated with pancreatic toxicity in IBD patients taking azathioprine. These results were interesting and important in clinical pretreatment of IBD.

The major problem is, however, that any representative data of PCR assay were not demonstrated as figures.

We understand this reviewer's point of view, and we added a figure representative of the PCR assay.

Specific comments:

1. Abstract: line 9; TPMP → TPMT?

We apologize for the mistake and text was corrected, as suggested.

2. Abstract: line 17 and Results: page 9, line 26; "was 21.6%, 32.4%, and 46%," "46%"; The number of effective figure should be considered.

We apologize for not being clear enough. We meant to summarize and simplify the description of results. We followed this reviewer's suggestion, and we amended the manuscript text accordingly (results session).

3. Material and Methods: page 8, lines 21 & 28; "was performed as previously describe"; Appropriate references should be indicated.

We apologize for the inconvenience, and the reference was highlighted in text, as suggested (ref. 19).

We thank this reviewer for the comments and the attentive reading and comprehension of our work and efforts. We also appreciate the suggestions, which give us the opportunity to include some additional literature to the references and improve the quality of our manuscript.

Reviewer # 00004011

The manuscript is interested and well written. Few grammatical errors should be corrected. Recent references ie J clin Pharm Ther 2010 35(1):93-7 should be included and discussed

We thank this reviewer for the comments and the comprehension of our work. We also appreciate the suggestions with regards to recent literature concerning the subject.

Reference and comments were added to the manuscript as suggested.

Reviewer #00029041

The prevalence of TPMT genotypes among control population should be assessed for comparison.

We thank this reviewer for analyzing our study. We understand his/her point of view regarding the interest of having a control population for comparison. Unfortunately, as our study was designed we cannot provide data on a healthy control population at this moment. However, we believe that such task would be beyond the scope of this manuscript.

The objective of this study was to analyze the prevalence of TPMT genotypes among our patients with IBD, and then analyze possible genotype-phenotype associations. Most of our patients with Crohn's and a relevant number of UC patients use azathioprine as maintenance therapy, and potential toxicity is an actual concern. Previous similar works on the subject also focused on the TPMT among patients on medication only (Naughton et al., Rheumatology 1999;38:640-4; Cao et al., Digestion 2009;79:58-63).

Once again we thank this and all reviewers for giving us the opportunity of responding to questions, trying to clarify critical points in our manuscript, in order to improve the overall quality of our manuscript.

Sincerely,

Heitor SP de Souza October 16th, 2013