Dr. Tiago Villanueva

Editor-Chefe

Acta Med Port

Dear Sir,

Enclosed, please find the revised version of our manuscript #9696 entitled “*Molecular staging of patients with colon cancer. The C-Closer-II study: a multicentre experience in Portugal*”. We have carefully addressed all concerns raised by reviewers. We thank editor and reviewers for their time and efforts in providing to us valuable comments, suggestions, and recommendations for changes, which have greatly improved the contribution of our work. Please, find in the response to the reviewers a point-by-point itemized list of changes to address each of the raised comments. Changes in the manuscript were highlighted as coloured text.

Editor:

1. Conforme estrutura do corpo do manuscrito, também o resumo e o abstract deverão iniciar-se pela secção "Introdução/ Introduction"

***As the Editor specified, the text has been modified in both Abstract and Manuscript.***

1. De igual modo, para que reflictam fielmente a estrutura do artigo, o resumo e o abstract deverão incluir um parágrafo independente relativo ao capítulo "Discussão"

***Likewise, a Discussion section have been included in the Abstract.***

1. O resumo e o abstract não deverão incluir abreviaturas.

***As the Editor indicated, the acronyms have been removed from the Abstract.***

1. Na listagem final de referências deverão ser identificados os seis primeiros autores das obras consultadas, e só depois fazer-se uso da expressão "et al"

***We agree with the Editor; we are afraid that references did not meet the guidelines. The format of the references has been changed.***

Reviewer A:

The title is adequate and the abstract is well structured and very complete without being overly lengthy. The methods were adequate to their objectives and I did not find any methodological failures. The results are clear and important for diagnosis practice. Conclusions were consistent with the results. The references are in order and follow AMP’s guidelines. The vast majority of references are recent although I feel the reference list should be longer. I did not identify any conflicts of interest.

Reviewer C:

Molecular staging of patients with colon cancer. The C-Closer-II study: a multicentre experience in Portugal

The paper focuses on the molecular staging of post-surgery patients through lymph node analysis using two methods – histology and OSNA. Although the methodology is not novel and similar studies have been performed in other countries, it is still a relevant and interesting study as there is no data from Portuguese Hospitals regarding this subject. This work could improve the methodology and potentiate changes in technical approaches for diagnosis of LN metastases. In this sense, the manuscript adds to the current knowledge in healthcare.

Comments on the Manuscript

Title: The title is short, concise and descriptive of the study.

Abstract: The abstract reflects the general contents and findings of the study and summarises it fairly efficiently.

Introduction: The introduction is well structured and is informative.

Objectives and relevance of the study are also summarised.

Methods: The methods are appropriately described.

Results: The authors present all data in tables, which is sometimes confusing and less intuitive than graphs and charts would be. Nevertheless, the data match the objectives set by the authors. The results are clear and although the n numbers are too low, as it is pointed out by the authors, there is an indication that OSNA is in fact a more robust technique for diagnosis, compared to histology.

Discussion: The discussion describes the limitations of the paper, such as low patient numbers and low lymph node numbers. It also summarises the findings adequately.

Conclusions: The conclusions are relevant and in line with the findings and objectives of the paper.

References: The literature used is adequate and number of references also adequate.

Acknowledgments: The authors include details of financial funding for the experiment and do not report conflicts of interest.

The length of the manuscript is adequate and the paper is reasonably presented.

It is my recommendation that this paper is published in AMP after minor corrections.

Questions for the authors and changes:

1. In figure 2, the authors should include, in addition to the number of patients from each hospital, the number of lymph nodes tested in each case.

***As the reviewer suggested, we have modified Figure 2, which now includes the number of lymph nodes (LN) analyzed in each hospital. Due to this revision, a mistake according to the total number of LN analysed, has been checked and corrected in paragraph #2 and #3 of ‘Results’ section, and also in table 1 and 2.***

1. The text should specify the number of OSNA positive LNs per patient and the variability of results presented in table 3.

***We have included it in paragraph #3 of ‘Results’ section. On the other hand, as far as we are concerned the variability of table 3 results is already explained in the above-mentioned paragraph. In accordance with literature, our results are in-line with the ratios of molecularly up-staged patients who had pathological-negative nodes (pN0) (15.3%1; 25.2%2; 11.3%3), as mostly up-staged patients retrieved a single OSNA-positive node (71% 1; 39%4; 73%2) or few (1-3 LN) and mainly with low molecular tumour burden (micrometastases) (74%2).***

1. Additionally, the data suggest that 71% of the patients “had a single LN affected”. If this is the case, could the low number of LN collected underestimate the number of patients requiring upstaging? Could some patients have been misdiagnosed with negative OSNA due to reduced number of LNs tested?  The authors should comment on this.

***Certainly, some patients may not benefit from OSNA assay rather than misdiagnose due to low ratio of freshly-collected LN. As mention in last paragraph of Discussion section, fresh dissection of LN needed for OSNA analysis depends on pathologists’ expertise. Indeed, the lower fresh LN dissected, the poorest chance for patient’s staging improvement by OSNA assay. A training period should be carried-out to warrant meting the guidelines for CRC staging with a minimum of 12 LN examined. Nonetheless, our LN collected rate is similar to other OSNA CRC studies (42% pts <12LN,2). Actually, despite the importance of dissection expertise, all authors agreed to point out the potential benefit of precise staging by OSNA outweighs such drawbacks. Noteworthy, nodes smaller than 50 mg but freshly dissected are not suitable for OSNA assay independently of pathologist’s expertise, which also affect the rate of LN assessed with OSNA. Nonetheless, recently studies showed that the improvement of patient staging by OSNA is beyond the number of positive LN. The total tumour burden of CRC patients given by OSNA assay not only correlates with N stage but also is a prognostic factor of relapse and survival 4–7.***

1. As a follow up question, can the authors suggest a number of LNs to be analysed, per patient, to confidently determine if the patient has micro/macrometastasis?

***Micro and macrometastasis in LN do not depend of the number of LN assessed. The equivalence between OSNA copy number and metastases detected by conventional histological examination in every LN was proved by Yamamoto and collaborators8,9. The number of LN to be examined is independent of OSNA procedure and must meet current guidelines recommendations.***

1. Could OSNA reduce the number of LN tested per patient for accurate staging, compared to histology “…consensus of 12 LN…” or is this value still insufficient?

***This is not the purpose of OSNA assay. It provides higher sensitivity to detect metastasis in each LN in comparison with conventional histological examination, which allows pathologists to detect occult metastases missed by conventional histological examination. Indeed, current investigations on CRC staging and prognosis are focused on establishing the total tumour burden (TTL) based on OSNA analysis of all LN retrieved in the surgical resected specimen4, similarly with what was proved in breast cancer10,11.***

1. The authors should comment on false negatives associated to OSNA, as a technique, and if this could be a problem, especially considering the low number of OSNA positive LNs per patient.

***In our study none false negative cases of OSNA occurred since, as inclusion criteria, all patients were pN0 (pathological-negative nodes). In accordance with literature, OSNA has a high concordance (>95%) with H&E and low false negative ratio (1.6%1; 0%12; 1.9%13). Existence of most FN are associated to methodological matter due to a bias of metastases allocation within the LN 1,2. Therefore, the examination of the whole LN using OSNA could overcome such issue. Low number of OSNA-positive nodes is not associated to FN ratio. The number of OSNA-positive nodes and the up-staging rate is in line with results reported in other OSNA studies with stage I and II CRC patients.***

1. In the methods the authors describe four hospitals but in the results only three are mentioned, what happened to LNs from patients from University of Coimbra Hospital? This should be clearer in the text.

***Results comprise data from all four hospitals (as figure 2 shows). In paragraph #3 of ‘Results’ section two hospitals were mentioned because of the highest accrual. Another one was also mentioned to denote no up‑staging in this hospital. The omission of University of Coimbra Hospital was a matter of paragraph sense and length. To avoid misunderstandings we have changed the text.***

1. How many LNs were assessed at Sao Joao Hospital? Is there a clear reason why no positives were detected?  Are there technical considerations?  Was the number of lymph nodes tested at this site particularly low? Please comment on this.

***Sao Joao Hospital included 11 patients and analysed 143LN by OSNA so the number of LN examined was in-line with the other hospitals. As far as we are concerned, the absence of up-staging in Sao Joao Hospital is a matter of probability rather than a technical o methodological consideration.***

1. The value 28.8% refers to the number of patients but in the next sentence it is not clear what 47.1 and 38.9% refer to, the percentage of patient upstaged at each hospital? What are the absolute numbers? Please clarify this.

***As suggested by the reviewer, we have included the absolute numbers in order to clarify the text (paragraph #3 in Results section).***

1. Typos:
Page 1 – Abstract Background: Remove “A” in “A 20-30%....”

Page 2 – Abstract Conclusions: Remove “A” in “A 28.8% of patients…”

Page 7 – Introduction (1st paragraph): Should read “Five of them were…” instead “then”

Page 8 – Introduction: Remove “the” before 53.3% in “Among patients with only micrometastases, the 53.3%”

Page 9 – Discussion: Substitute “an” for “a” in “pathological examination (single H&E slide examination) and an molecular staging rate of 20.2% 13.”

Page 10 – Discussion: “follow-up would provide insights about whether there would had been benefit from treatment according the molecular staging with OSNA”. It should probably read “whether they would have benefited”.

Page 10 – Conclusions: substitute “had” for “have” in “…they could had benefited from systemic therapy…”

***All typos indicated by the reviewer have been modified.***

***References used:***

**1. Güller U, Zettl A, Worni M, Langer I, Cabalzar-Wondberg D, Viehl CT, et al. Molecular investigation of lymph nodes in colon cancer patients using one-step nucleic acid amplification (OSNA): A new road to better staging? Cancer. 2012;118(24):6039–45.**

**2. Croner RS, Geppert C-I, Bader FG, Nitsche U, Späth C, Rosenberg R, et al. Molecular staging of lymph node-negative colon carcinomas by one-step nucleic acid amplification (OSNA) results in upstaging of a quarter of patients in a prospective, European, multicentre study. Br J Cancer [Internet]. 2014;110(10):2544–50.**

**3. Yamamoto H, Tomita N, Inomata M, Furuhata T, Miyake Y, Noura S, et al. OSNA-Assisted Molecular Staging in Colorectal Cancer: A Prospective Multicenter Trial in Japan. Ann Surg Oncol [Internet]. 2015;(May):1–6.**

**4. Aldecoa I, Atares B, Tarragona J, Bernet L, Sardon JD, Pereda T, et al. Molecularly determined total tumour load in lymph nodes of stage I–II colon cancer patients correlates with high-risk factors. A multicentre prospective study. Virchows Arch [Internet]. 2016**

**5. Rakislova N, Montironi C, Aldecoa I, Fernandez E, Bombi JA, Jimeno M, et al. Lymph node pooling: a feasible and efficient method of lymph node molecular staging in colorectal carcinoma. J Transl Med [Internet]. 2017;15(1):14.**

**6. Yamamoto H, Tomita N, Inomata M, Furuhata T, Miyake Y, Noura S, et al. OSNA-Assisted Molecular Staging in Colorectal Cancer: A Prospective Multicenter Trial in Japan. Ann Surg Oncol. 2016;23(2):391–6.**

**7. Matsuura N, Tomita N, Inomata M, Murata K, Hayashi S, Miyake Y, et al. 559P - Clinical impact of molecular positive lymph node status in colorectal cancer. POSTER - ESMO. 2017;4(2):559.**

**8. Yamamoto H, Sekimoto M, Oya M, Yamamoto N, Konishi F, Sasaki J, et al. OSNA-based novel molecular testing for lymph node metastases in colorectal cancer patients: results from a multicenter clinical performance study in Japan. Ann Surg Oncol. 2011;18(7):1891–8.**

**9. Yamamoto N, Daito M, Hiyama K, Ding J, Nakabayashi K, Otomo Y, et al. An optimal mRNA marker for OSNA (one-step nucleic acid amplification) based lymph node metastasis detection in colorectal cancer patients. Jpn J Clin Oncol. 2013;43(3):264–70.**

**10. Peg V, Espinosa-Bravo M, Vieites B, Vilardell F, Antúnez JR, De Salas MS, et al. Intraoperative molecular analysis of total tumor load in sentinel lymph node: A new predictor of axillary status in early breast cancer patients. Breast Cancer Res Treat. 2013;139(1):87–93.**

**11. Peg V, Sansano I, Vieites B, Bernet L, Cano R, Córdoba A, et al. Role of total tumour load of sentinel lymph node on survival in early breast cancer patients. The Breast [Internet]. 2017;33:8–13.**

**12. López-Ruiz ME, Diestro MD, Yébenes L, Berjón A, Díaz de la Noval B, Mendiola M, et al. One-step nucleic acid amplification (OSNA) for the detection of sentinel lymph node metastasis in endometrial cancer. Gynecol Oncol [Internet]. 2016;143(1):54–9.**

**13. Del Carmen S, Gatius S, Franch-Arcas G, Baena JA, Gonzalez O, Zafon C, et al. Concordance study between one-step nucleic acid amplification and morphologic techniques to detect lymph node metastasis in papillary carcinoma of the thyroid. Hum Pathol [Internet]. 2016;48:132–41.**

Reviewer F:

Titulo: “Molecular staging of patients with colon cancer. The C-Closer-II study: a multicentre experience in Portugal”

Comentários:

Trata-se de um artigo que descreve os resultados de utilização do método OSNA (One Step Nucleic Acid Amplification) em gânglios isolados de peças cirúrgicas de carcinomas colo-rectais estadiados histologicamente como pN0 (59 pacientes) para detectar metástase oculta nos mesmos. Tratou-se de um estudo prospetivo, em 4 hospitais portugueses no período de 2 anos (2013-2015).  Estudo é interessante na medida em que a utilização do método descrito pode melhorar o estadiamento patológico do cancro colo-rectal e consequentemente o manuseio terapêutico em situações específicas. Assim considero ser apropriado para publicação com correções menores abaixo indicadas. Título adequa-se à descrição do estudo.

1. Resumo está bem estruturado, e é informativo. Sugiro que coloquem por extenso já no resumo o significado de OSNA. Ainda no resumo, para maior precisão, na primeira linha onde diz “neoplasia colo-rectal” sugiro que se coloque neoplasia maligna colo-rectal ou cancro colo-rectal.

***As reviewer suggested we have included ‘OSNA (One Step Nucleic Acid Amplification)’ in the Abstract and replaced “neoplasia colo-rectal” by ‘cancro colo-rectal’.***

1. É necessário rever o texto, incluindo o Resumo em inglês e as Tabelas, onde se identificam erros ortográficos, por exemplo “Disclouser” (no resumo).

***We have checked the spelling in manuscript text and tables.***

1. Ao longo do texto, uniformizar a designação de cancro colo-rectal. Umas vezes é designado de CRC e outras CC apenas. Sugiro a designação sempre de “cancro colo-rectal” (CRC), substituindo por esta sigla, os sítios onde se encontra apenas “cancro do cólon” (CC), uma vez que nos casos estudados também se incluem os cancros do reto-sigmoide.

***We agree with the reviewer. The acronyms have been checked and all replaced by CRC.***

1. Além disso rever a referenciação da revista N. 25 na lista da bibliografia.

***The reference #25 have been corrected.***

1. A Introdução, descrição dos métodos e material, resultados e discussão estão bem apresentados e são percetíveis. Na introdução, colocar os números exatos da incidência e mortalidade, o que é possível na bibliografia consultada.

***The reviewer was right; numbers were incorrect. We have corrected them.***

Os autores estão conscientes das limitações do estudo e estas são descritas no texto.  As conclusões estão percetíveis e refletem os resultados encontrados.

Reviewer G:

I have been invited to review the statistical methods and analyses described in the manuscript "Molecular staging of patients with colon cancer. The C-Closer-II study: a multicentre experience in Portugal". Thus, I have focused my review on the methodological approach and statistical aspects. The statistical analyses performed are limited to summary descriptive statistics and bivariate inferential analyses. The relatively reduced sample size by groups precludes a more detailed statistical analysis.

I have a few comments that I believe would improve the clarity of the paper.

Abstract :

1. Please, check the words “número médio” (mean) and “median” presented in the Portuguese and English versions- It is  stated in the “Results” section that …”The median number of LN assessed with OSNA was 12 ..”, while in the “Resultados” it is referred as: “O número médio de gânglios linfáticos avaliado com OSNA foi de 12…”.

***The reviewer was right. We have corrected the text in the Portuguese version of the Abstract.***

1. Indicate (Portuguese and English versions) the 95% CI for the “positive molecular‑staging rate of 28.8%” presented (and correctly calculated) in the Results section.

***As the reviewer pointed out we have corrected and included the 95%CI in both Abstract versions.***

Material and Methods section:

1. Study design: The authors refer the inclusion and exclusion criteria. Nevertheless, the sample design is not clear. How was sample sized calculated? How were the patients sampled for participation in the study?

***Based in results from previous reported studies, assuming an expected upstaging rate of 20%, we estimated a sample size of 60 CRC patients to reach a 95%CI with a maximum error of 10%. The casuistic in each institution was considered so that a two‑year accrual was established. Subsequently, patients were sequentially included in each hospital till the accrual period was achieved.***

1. Statistics Analysis: The authors have outlined the data analysis procedures. However, there are a few things that the authors might have overlooked: Please correct: CI 95%----should be 95%CI.

***The reviewer was right; we have corrected the spelling.***

1. Furthermore, it is mentioned that “Comparisons between groups were performed using Chi‑Squared test or Fisher test, when required (expected frequency fewer than 5 for categorical variables)”.  Please rephrase and clarify the specific criteria used for these 2 approaches.

***We have rephrased the paragraph #4 of M&M section in order to clarify the criteria. Additionally, we have modified table 4 so that the statistical approaches used for each comparison is now annotated.***

1. The authors also refer “Continuous variables were assessed using Mann‑Whitney U‑test, when normality could not be assumed”. Please clarify that this non-parametric test was used to perform group comparisons.

***The reviewer was right; we have rephrased paragraph #4 of M&M section.***

Results:

1. In the section Molecular staging of pN0 patients - 1st line, it is referred that “….40 LN were OSNA-positive (≥250 copies/μl). Thus, OSNA assay demonstrated 17 patients…”. Please check the values.

***We have checked the paragraph #4 in ‘Results’ section. As far as we are concerned the values are correct. Among all LN assessed (784) only 40 were OSNA-positive, which turned into 28.8% (17/59) patients with at least one positive LN by OSNA (upstaged)***

1. Section Lymph nodes collection and Table 4 - please check the reported values for “Dissection took a median of 45 min…”

***The reviewer was right; there was a mistake in Table 2. We have corrected it.***

1. Minor comment: In the Patient characteristics section--- typo error 1st line “then” should be “them.

***The reviewer was right. We have corrected the spelling.***

1. The statistical analyses performed and presented in the Results section and Tables 1- 4 are limited to summary descriptive statistics and bivariate inferential analyses. Although, it should be recognised and stated in the Discussion section that the reduced sample size by groups precludes a detailed inferential statistical analysis in terms of the clinico-pathological characteristics.

***As the reviewer suggested we have included this matter in ‘Discussion’ section (paragraph #4).***

Eventually, we would like to thank the editor and the reviewers for their consideration of manuscript resubmission.

Sincerely,

Maria José de Brito