Gastroenterology Unit
Hospital of Imola
Imola, Italy

April 16th, 2021

Prof. Monjur Ahmed, Prof. Florin Burada, Prof. Rosa M Jimenez Rodriguez, and Prof. Pashtoon Kasi,
please find enclosed our revised version of the manuscript entitled "**Endoscopic ultrasound (EUS) assessment and tissue acquisition of mediastinal and abdominal lymph nodes**" (no. 64602) for consideration for publication on the World Journal of Gastrointestinal Oncology.

We sincerely thank the Editor and Reviewers for their suggestions and the opportunity of resubmitting a revised version of our manuscript.
Please find enclosed a point-by-point response to reviewers in which are underlined all the changes apported in the original version of the manuscript.

All the authors of this manuscript have made a substantial contribution to the material and information submitted for publication and approved the paper submitted. Neither the manuscript, nor portions of the manuscript, have been already published, nor are they under consideration for publication by another journal.

Thank you for your time in considering this revised paper.
Yours sincerely,
Andrea Lisotti, on behalf of all co-authors

RESPONSE TO EDITORIAL OFFICE

Science editor: Self-cited references: There are 11 self-cited references. The self-referencing rates should be less than 10%. Please keep the reasonable self-citations (i.e. those that are most closely related to the topic of the manuscript) and remove all other improper self-citations. If the authors fail to address the critical issue of self-citation, the editing process of this manuscript will be terminated.

Re: We amended the reference list in order to reduce the self-citation rate.

RESPONSE TO REVIEWERS

Reviewer #1: Scientific Quality: Grade C (Good). Language Quality: Grade B (Minor language polishing). Conclusion: Minor revision. Specific Comments to Authors: The differential diagnosis of benign and malignant lymph nodes is a problem we often encounter in clinical practice, which significantly impacts clinical decision-making. This review provides a detailed summary of the application of EUS in the differential diagnosis of benign and malignant lymph nodes. It is helpful for learners in this field to quickly understand comprehensive technical diagnosis knowledge. The author is excellent at writing. I hope the suggestions given below can help the author in his work.

Re: We sincerely thank the reviewer for the positive global evaluation; we tried to make the suggested changes in order to increase manuscript's overall quality.

Reviewer #1: Can the author add illustrations to illustrate the techniques mentioned in the text for beginners to understand.

Re: Three figures illustrating the described techniques have been added.

Reviewer #1: Is there enough research to infer an optimal number of passes? Obviously, as the number of passes increases, more tissue is obtained, but we cannot increase the number of passes indefinitely. Moreover, the number of passes' requirements are often different in various diseases, so it is not very sensible to vaguely give the optimal number of passes. Uehara H, Sueyoshi H, Takada R, Fukutake N, Katayama K, Ashida R, Ioka T, Takenaka A, Nagata S, Tomita Y. Optimal number of needle passes in endoscopic ultrasound-guided fine needle aspiration for pancreatic lesions. Pancreatology. 2015 Jul-Aug;15(4):392-6. LeBlanc JK, Ciaccia D, Al-Assi MT, et al. Optimal number of EUS-guided fine needle passes needed to obtain a correct diagnosis. Gastrointest Endosc. 2004;59:475–481.

Re: EUS-tissue acquisition is mainly studied in solid pancreatic tumors, that are usually fibrotic and poorly cellulated. Lymph nodes architecture is different to pancreatic one. Malignant lymph nodes are usually poorly fibrotic and highly cellulated. The results observed in solid pancreatic tumors are not 100% reproducible in this field. However, we agree with Reviewer #1 and added a paragraph dealing with needle pass number. We discussed this issue in the text: "There is no robust evidence supporting the choice of different techniques for EUS LN sampling. Indeed, EUS-TA techniques are mainly assessed in solid pancreatic tumors, that are usually fibrotic and poorly cellulated. As described before, lymph nodes architecture is different to pancreatic one: malignant lymph nodes are usually poorly fibrotic and highly cellulated. The results observed in solid pancreatic tumors are not 100% reproducible in this field. Therefore, needle choice, number of needle passes, type and amount of suction could only be deduced from knowledge in the field of solid pancreatic tumors needle aspiration."

Reviewer #1: EUS-FNA false negatives are more common than false positives. This is the main challenge we face. It is recommended to focus on the discussion.

Re: A paragraph dealing with the issue of false positive and false negative EUS-tissue acquisition results in the field of lymph nodes was added.

“False-negative results are mainly related to the characteristics of the lesion (size and nature of the tumor) and to technical aspects of EUS-FNA (sampling errors and interpretative errors). The presence of small lesions or of an interposed vascular structure between the transducer and the biopsy target can lead to inadequate or nonrepresentative samples. Furthermore, the presence of severe inflammation can hide an infiltrating tumor and cause histopathological errors [80,83].

Causes for false-positive results are epithelial cell contamination, EUS sampling error and pathological misinterpretation. Tumor cells can be present in luminal fluid and can enter the FNA needle as it passes through the gut lumen to reach the target lymph node. Main contaminants originate from the duodenal and gastric mucosa, but malignant cells are commonly present even in the luminal fluid of patients with pancreatic cancer and not only with luminal cancers. Furthermore, it is advised not to pass through the primary tumor with the needle when performing EUS-tissue acquisition of LNs, in order to avoid contamination. It is also plausible that false-positive results occur when the interposing mucosa is inflamed or in premalignant/early malignant state (such as in Barrett’s oesophagus with dysplasia or in chronic or autoimmune pancreatitis). In addition, even if uncommon, also the misinterpretation by cytopathologists can drive to wrong diagnosis [42,84,85].

Based on high-quality evidence, the rate of false-negative results is dramatically higher, compared to false-positive ones. This aspect could impact patients’ prognosis since the risk of under-staging with consequent under-treatment could not be excluded. In particular, in patients with early luminal gastrointestinal neoplasms (i.e. Barrett’s adenocarcinoma or early gastric cancer), the incorrect classification of a malignant LN could result in futile endoscopic resection and dramatic delay in curative surgery.”

Reviewer #1: There is a writing error in Table 1 "irregular, sharp???"

Re: The typo was corrected.

Reviewer #2: Scientific Quality: Grade B (Very good). Language Quality: Grade A (Priority publishing). Conclusion: Accept (General priority).

Re: We sincerely thank the reviewer for the positive global evaluation; we tried to make the suggested changes in order to increase manuscript’s overall quality.

Reviewer #2: I think CH-EUS enhancement pattern may include" non enhancement".

Re: We totally agree. Non enhancement pattern was included and described.

“Finally, in case of CH-EUS non enhancement pattern, a colliquative necrosis could be deduced, suggesting the presence of inflammatory LN (i.e., tubercular LN with extensive necrosis).”

Reviewer #2: CH-EUS is not often used in T staging of gastric tumors.

Re: We agree with Reviewer #2. Nomura et al. [Gastrointest Endosc 1999] reported their retrospective experience with CE-EUS using air-filled albumin in patients with upper GI diseases. The authors reported 30 cases with gastric carcinoma in which the accuracy for the assessment of the infiltration depth for CE-EUS was 90% compared with 77% for standard EUS. Iordache et al. [Med Ultrasoun 2012] evaluated the application of CE-EUS for the preoperative assessment of 20 patients with locally advanced gastric cancer. The authors found that CE-EUS assessment of cancer vascularization could be useful for the evaluation of pathologic characteristics (i.e., microvascular density or vascular endothelial growth factor expression). We clarified that CH-EUS for gastric cancer staging is limited to research areas. “other indications, such as staging of gastric tumors and other anomalies are limited to research studies”.

Reviewer #2: The use of EUS-E in differentiating pancreatic tumor and mass forming pancreatitis is limited, However, CH-EUS is often used in our daily practice.

Re: From a personal perspective, we agree with Reviewer #2. However, literature data seem to demonstrate the ability and good performance of EUS-E to discriminate among pancreatic cancer and mass-forming pancreatitis. However, qualitative EUS-E or semi-quantitative EUS-E (strain ratio or Hue histogram) present low reproducibility and standardization. The introduction of quantitative EUS shear-wave measurement (EUS-SWM) failed, to date, to demonstrate any advantage compared to EUS-E [Ohno E, Dig Endosc *in press*]. We included a brief paragraph dealing with this issue.

Reviewer #2: When we do EUS-FNA, 10-20ml syringe is often used.

Re: EUS-tissue acquisition is mainly studied in solid pancreatic tumors, that are usually fibrotic and poorly cellulated. Lymph nodes architecture is extremely different to pancreatic one. Malignant lymph nodes are usually poorly fibrotic and highly cellulated. The results observed in solid pancreatic tumors are not 100% reproducible in this field. The role of syringe suction, type of suction (dry or wet) and amount (5, 10 or 20 mL) has not been assessed in lymph node characterization. We discussed this issue in the text: “There is no robust evidence supporting the choice of different techniques for EUS LN sampling. Indeed, EUS-TA techniques are mainly assessed in solid pancreatic tumors, that are usually fibrotic and poorly cellulated. As described before, lymph nodes architecture is different to pancreatic one: malignant lymph nodes are usually poorly fibrotic and highly cellulated. The results observed in solid pancreatic tumors are not 100% reproducible in this field. Therefore, needle choice, number of needle passes, type and amount of suction could only be deduced from knowledge in the field of solid pancreatic tumors needle aspiration.”

Reviewer #2: Do you think wet suction should be included?

Re: EUS-tissue acquisition is mainly studied in solid pancreatic tumors, that are usually fibrotic and poorly cellulated. Lymph nodes architecture is extremely different to pancreatic one. Malignant lymph nodes are usually poorly fibrotic and highly cellulated. The results observed in solid pancreatic tumors are not 100% reproducible in this field. The role of syringe suction, type of suction (dry or wet) and amount (5, 10 or 20 mL) has not been assessed in lymph node characterization. We discussed this issue in the text: “There is no robust evidence supporting the choice of different techniques for EUS LN sampling. Indeed, EUS-TA techniques are mainly assessed in solid pancreatic tumors, that are usually fibrotic and poorly cellulated. As described before, lymph nodes architecture is different to pancreatic one: malignant lymph nodes are usually poorly fibrotic and highly cellulated. The results observed in solid pancreatic tumors are not 100%

reproducible in this field. Therefore, needle choice, number of needle passes, type and amount of suction could only be deduced from knowledge in the field of solid pancreatic tumors needle aspiration."

Reviewer #2: In the mediastinum and hepatic hilum region, the inflammatory LNs are often irregular strip or flake shaped without demarcation.

Re: We agree with Reviewer #2. We focused on the B-mode appearance of mediastinal inflammatory LNs. "Some typical aspects have been clearly depicted; for example, in the mediastinum and hepatic hilum region, the inflammatory LNs are often irregular strip or flake shaped without demarcation. In these cases, B-mode criteria could be sufficient to establish the benign nature of the LN."

Reviewer #2: Color Doppler hilar vascularity, peripheral signals and spectral analysis are often used in US, but in EUS the use is limited due to small LNs and instrumental ability.

Re: We agree with Reviewer #2. We described this limitation of color-Doppler applications in EUS. "However, color-Doppler hilar vascularization, peripheral signals and spectral analysis present perfect technical results in trans-abdominal ultrasound, while in EUS the use could be limited by scope instability, LNs size and limited probe capacity."

Reviewer #2: EUS-E is a good indicator, but not very stable and less specificity, so it still cannot reduce FNA. In my opinion, the detail from B mode is the most important.

Re: Once again, we agree with Reviewer #2 on a personal perspective. However, as stated even in guidelines, B-mode criteria are able to provide conclusive results in 25% of suspected LNs. A meta-analysis [Xu et al. GIE 2011] demonstrated that EUS-E shows a 88% sensitivity coupled with 85% specificity. We think that EUS-E clinical trials are poorly reproducible; we focused our recent research [Lisotti et al. Endoscopy 2020, abstract presented at ESGE Days 2020] on the combination of EUS-E with B-mode criteria and CH-EUS and find an incremental diagnostic yield based on the utilization of more than one technique. "EUS-E provides diagnostic information to conventional B-mode imaging and can be used for the selection of suspicious LNs worth FNA/FNB thanks to its high PPV; EUS-E alone cannot obviate the need of tissue acquisition in suspected LNs".

Reviewer #2: Usually, there are multiple metastatic LNs and inflammatory LNs at different region in mediastinum, can not be shown in one image, how many times do you inject Sonovue to choose the suspectable LN?

Re: The presence of multiple LNs represents a well-known limitation of CH-EUS; indeed, while the crucial moment in the detection of lesions is the late venous phase, the characterization of LNs should be performed during the arterial and early venous phase. In cases where more than one LN is suspected, repeated UCA injections should be performed; we suggest starting the evaluation with B-mode and even EUS-elastography, and then using CH-EUS to study in detail the LNs with greater evidence of malignancy. Repeated injection of UCAs has been demonstrated to be a safe and reproducible technique; however, no study has used this combined approach to multiple LNs. Of course, this approach leads to an increase in length of procedures and increased costs; on the other hand, in the case of multiple suspected LNs, several EUS-FNAs need to be performed, changing the needle in any station, if possible, to reduce the risk of seeding. We discussed this issue in the text.

Reviewer #2: I cannot find Reference 94

Re: We fixed this issue and updated reference list.