Author's response to reviews

Title: Diagnostic Accuracy of Cyst Fluid Amphiregulin in Pancreatic Cysts

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Author's response to reviews: see over
On behalf of the authors, we thank you for your comments, which provided guidance for an improved manuscript that is now enclosed. We look forward to your response. Our point-by-point response to the reviewers’ and editor’s comments are placed in bold and italicized.

Reviewer: Mike Larvin

Reviewer’s report:
"This study is a potentially useful addition to the thorny issue of pancreatic cyst fluid diagnosis. I enjoyed reading this paper.

Minor essential revisions and queries:

It is self-evident that if 2-8% of the population have a pancreatic cyst and half are potentially malignant, then cystadenocarcinomas would be beginning to dominate the known incidence of pancreas cancers. Was this an error?"

Response: Population based studies on the prevalence of pancreatic cysts do not currently exist. The best studies available are single-center based using consecutive enrollment of over 2000 images to deduce the estimate of 2-8% (2% in the general population over 40 and 8% in the very elderly) [1, 2].

Using these estimates and combining it with surgical series reporting up to 50% of cystic lesions as pre-malignant (mucin-secreting), then cystadenocarcinomas would dominate the distribution of pancreatic cancers. Despite their limitations, these published estimates represent the best available data until better designed studies are performed. It is also possible that these estimates may be, in fact, correct, and what is poorly understood is the disease's natural history - that perhaps many cysts have mucinous characteristics to support malignant potential, but the vast majority never progress to become clinically relevant. An alternative hypothesis is that many cysts may evolve into solid adenocarcinomas. These comments were not included in the discussion section because the intent was to focus the study on the diagnostic potential of this novel biomarker.

"Methods: Could the authors confirm whether 2 freeze-thaw cycles is deleterious to the detection of AREG in their own lab?"

Response: As now noted in the Methods section, paragraph 1, two freeze-thaw cycles does not affect the reproducibility of the ELISA.

"Results: This study adds useful information to existing methods, allowing for sample size as the authors acknowledge. EUS is becoming a gold standard for
pre-operative diagnosis of pancreatic cystic lesions, but in the absence of malignant cells marker elevations are not always helpful. Further studies appear justified and it may be that AREG has a diagnostic role alongside CEA and imaging findings. ”

Response: We agree that this is a small pilot study, but this manuscript reports on the initial discovery of AREG as a biomarker and is needed to stimulate further studies and funding. It is also noted that as an initial proof-of-concept experiment, it was important to use pathology of surgically resected samples as the gold standard to establish the diagnosis. This approach, however, compromised patient recruitment because many patients with presumably benign cyst do not undergo surgical resection. This point is described in paragraph 6 of the Discussion section.

Reviewer: Paula Ghaneh

Reviewer’s report:

"This paper evaluates the use of cyst fluid amphiregulin levels in the diagnosis of pancreatic cysts.

Comments:
1. The question is an important one as the number of patients with cystic lesions in the pancreas is rising and tests which can differentiate benign and malignant cysts are needed."  

Response: We agree.

"2. What were the inclusion criteria for the study – were all patients with cysts considered or were there other criteria such as cyst size or suspicious features? The authors must have had some criteria to ensure an appropriate range of cases."

Response: All adult patients with a pancreatic cyst who presented to our Gastroenterology clinic, Endoscopy unit, or the Surgery clinic were invited to participate. Because pathology and cytopathology were used as the gold standard for diagnosis, only patients who went to surgery were used. While this may limit the generalizability, this was a proof-of-concept study that established whether larger prospective studies are warranted in the future. This is noted in the Methods section under "Diagnosis of pancreatic cysts" and in paragraph 6 of the Discussion.

"3. It is not entirely clear how the cyst fluid samples were obtained – can the authors describe how the cyst fluid was obtained – all EUS or some at surgery? Also how much fluid was required/harvested? "
Response: The fluid could be obtained either at EUS or at the time of surgery. In some cases, fluid was collected in both cases. Given that this study only included samples with associated pathology (vast majority had surgery, small minority with positive cytology) most had their cyst fluid collected at the time of surgery. Those with only cytology had their fluid collected by EUS. As much cyst fluid as possible was reserved after allocating the necessary amount for conventional clinical use. In general, for EUS, 500 ul was aliquoted for CEA, and cytology was obtained only if there was a nodule/mass. At the discretion of individual endosonographers, 500 ul could be used for amylase as well. Any fluid remaining was collected for research. This is now described in paragraph 1 under "Cyst fluid samples" in the Methods section.

4. Was the ELISA fully validated?

Response: The AREG ELISA kit (DY262, R&D systems) has been used for quantifying AREG from patient samples [3, 4] and culture media [5]. For each sample, serial dilutions were used with a minimum of two points within the standard curve. Each dilution was run in duplicate. The data represents the result of two independent experiments. The dynamic range of the ELISA was 5-2,000 pg/ml. Data that the ELISA is specific for the AREG gene product was previously established by our laboratory [8]. This is now described in the description of the "AREG ELISA" in the Methods section.

"5. The main drawback is the size of the patient sample and the mixed patient population – but this is probably acceptable for a pilot feasibility study – did the authors perform a sample size calculation for this study?"

Response: A sample size calculation was not performed for this study as it was a pilot feasibility study.

"6. What was the sensitivity/specificity of CEA for this cohort of patients?"

Response: Of the 33 samples in this study, only 21 had a cyst fluid CEA available for review. The most common reason for not having a CEA available was that the patient proceeded directly to surgery based on meeting clinical criteria for resection. In the discussion section, paragraph 4, we report the median values (with IQR) of the 4 non-mucinous cysts, 11 benign mucinous cysts, and 6 malignant mucinous cysts to be 127 ng/ml (36 – 844), 1294 ng/ml (171 – 8600), and 2400 ng/ml (1245 – 11962), respectively. Mucinous cysts (n=17) had an elevated CEA (median (IQR) 1311 ng/ml (277 – 8600)) compared to non-mucinous cysts (n=4) (126 ng/ml (36 – 844) (p = 0.09). This difference was not statistically significant, which
is likely due to the small sample size, especially with only 4 patients harboring non-mucinous cysts. The sensitivity and specificity using the standard cutoff of 192 ng/ml, is now reported in the Discussion, paragraph 4. The sensitivity and specificity to differentiate non-mucinous (no malignant potential) from mucinous (malignant and pre-malignant) cysts is 76% and 75%, respectively, similar with previously reported studies. CEA does not differentiate cancer (and high grade dysplasia) from non-cancer, which is consistent with our results.

"7. The authors should stress that this test needs further validation in larger prospective series."

Response: This is now emphasized in paragraph 6 of the Discussion.

Reviewer: Christopher Halloran

Reviewer's report:

"This is a retrospective, but small study of analysis of pancreatic cyst fluid using amphiregulin (AREG). The motivation was to use this novel biomarker to differentiate between non-mucinous, benign mucinous and malignant mucinous cysts. This is an important topic and the authors should be congratulated on their endeavours in this field.

Major Revisions / Questions to be answered
"It is unclear whether AREG use was investigated as a biomarker for dysplasia/malignancy or for mucinous/non-mucinous lesions as suggested in the abstract."

Response: Based on the AREG's biology, the initial intent was to explore whether cyst fluid AREG levels possessed clinical value for either scenario, that is differentiating non-mucinous from mucinous cysts or stratifying high grade dysplasia and malignancy from low/moderate grade dysplasia among mucinous cysts. Because CEA already exists, the real clinical need is a biomarker that fulfills the latter goal, which AREG fulfills based on this pilot study. This is stated in the last paragraph of the Discussion.

"It would be helpful to know whether the pre-operative diagnoses differed from the post-operative histology and what the indications for surgery were.

Response: Our group generally follows the international consensus guidelines to determine appropriateness for surgical resection [6]. Pre-operative diagnoses were made and were correct 67% of the time when correlated to the post-operative pathology. This is similar to a previously reported estimate [7]. We did not include this data in the manuscript as we believe it detracts from the focus of the paper- that is cyst fluid AREG's
diagnostic utility using pathology as the gold standard.

"This study attempts to differentiate between benign mucinous lesions and mucinous lesions, which have dysplastic or malignant cells. However the “benign” group contains 9 BD-IPMN, 3 MD-IPMN and 3 MCN. Practically MD-IPMN and MCD should be treated as pre-malignant cysts and should be resected regardless; therefore this use of tumour markers in these cases does help."

Response: In our methods section under the “Diagnosis of pancreatic cysts” subsection, we define benign to include mucinous cysts with low or moderate grade dysplasia. While these lesions have malignant potential, they do not need to be resected immediately and can be watched. High-grade dysplastic lesions and cancerous cysts require clinical action (i.e. surgical resection in surgically fit patients). This is the basis for surveillance recommendations for branch-duct IPMN lesions [6].

The current rationale for removing MD-IPMN lesions is based on data showing a high prevalence of cancer upon resection. The rationale for resecting MCN lesions is based on the demographic factors (young females) with pancreatic tail lesions (less complicated surgical resections), making surgery over surveillance practically favorable. The natural history of MCNs transforming to cancer is poorly understood, and is estimated to be more like branch-duct IPMNs, than MD-IPMNs.

In our series, the 3 MD-IPMN lesions and 3 MCN lesions labeled as benign mucinous showed no histological evidence of high grade dysplasia or cancer. As the biology of these cysts improve and better biomarkers developed to correlate with this biology, the recommendations to remove MD-IPMN and MCNs could change. A biomarker like AREG may represent such a biomarker. Some of these pre-malignant lesions may never progress further.

"Furthermore 3 cases of high-grade dysplasia were found in the MD-IPMN, which were classified as “benign tumours”. Therefore was the analysis performed between the benign cysts and the malignant cyst groups? in which case there was bias or between those that were histologically benign with those of dysplasia/malignancy? Hence I am not sure what the authors point is exactly – clarification is required."

Answer: The 3 cases of MD-IPMN listed in Table 3 (that I believe the reviewer is referring to) are considered cancerous cysts in table 1 and 2. These 3 do not refer to the benign mucinous cysts in table 2 that list 3 MD-IPMN lesions. In the methods section, “cancer” is defined to include high-grade dysplasia lesions. So the 3 high grade MD-IPMN lesions correlate to
3 of the 4 “high grade dysplasia” lesions listed in table 2 line 12, but not those listed in table 2 line 9.

Our analysis looked at whether AREG could differentiate non-mucinous (SCN and Pseudocysts) from mucinous (MCN, IPMN regardless of malignancy). The results were not supportive as mucinous cysts without high grade dysplasia or cancer had similar AREG levels to the non-mucinous cysts.

When only mucinous cysts were evaluated and divided into cancer (defined as high grade dysplasia & cancer) or benign (low grade and moderate grade dysplasia lesions), a significant difference in AREG levels was observed, leading to a conclusion that AREG may serve as a cyst fluid biomarker to help clinicians decide which cysts need surgical resection.

"Looking at these data it is clear that MD-IPMN and MCD have a higher median AREG and should never have been included in the grouping. These data are summarized in table 3 that only have 9 patients not the 12 stated – where are the rest. Overall this is VERY confusing as it is unclear exactly which patients were compared.

As the data pertaining to which patients were used in the accuracy testing and ROC curve are unclear I am hesitant to accept these findings without qualification."

Respond: Table 3 possessed the 12 stated patients. We now realize that in transforming the document to the pdf for submission, that 3 patients may have been cutoff in the submitted copy. We have resubmitted a complete table. Among the benign mucinous cysts in table 2, it does show that MD-IPMN and MCN lesions had high cyst fluid AREG levels. Again, these were grouped in benign by definition that low and moderate grade dysplasia by histology are considered benign mucinous cyst. We do not imply that benign has no malignant potential, just that they are not cancer. The approached utilized pathology as the gold standard. An alternative hypothesis is that AREG is able to detect cysts at high risk for malignant transformation that are currently classified as benign or low risk. Only larger prospective studies will be able to resolve this issue.

"Overall the use of AREG performs no better than CEA in those cases where both results are included. Obviously the small numbers and retrospective approach hinder this study but it would be interesting to see how AREG performs along side CEA and CA 19-9 from cyst aspirates in a prospective group of patients presenting with non-inflammatory pancreatic cysts."

Response: In contrast to CEA, AREG assay does not differentiate non-mucinous from mucinous cysts, but does differentiate cysts with high grade dysplasia and cancer (where CEA does not). As a result, CEA alone
was not compared to AREG alone. Only the combination of the CEA and AREG assays versus AREG alone was evaluated for their ability to detect cancer.

In this smaller sample size of 21 (only 21 of the 33 samples had CEA available) there were only 6 cancerous cysts. The combination of CEA and AREG provided no advantage over AREG alone in diagnosing cancerous cysts. As the reviewer points out, this may be a conclusion limited by a small sample size. This is described in paragraph 5 of the Discussion section.

Using CEA alone to diagnose cancer in this small sample, we observe a sensitivity of 84% with a specificity of 40%. Compared to AREG alone, CEA’s lower specificity is clinically relevant when considering surgical resection.

We agree with the reviewer that larger, prospective studies including AREG would be of further interest.

Editor’s Comments:

The paper is of sufficient interest and describes a novel potential marker that deserves further study. The authors should address all the issues raised by the three reviewers and revise the paper appropriately.

Response: Please see above. We believe we have addressed their comments and questions.

It is important that the authors stress that this is a small pilot study and further work is required; the conclusions should emphasise the limitations of the study.

Response: We believe we have stressed this in the last 2 paragraphs of the discussion.

In table 1 median values should be used.

Response: We will change the average age to median age. All other relevant variables in table 1 are median values.

In table 3; this looks like a print or scan problem with the text; the text must be made clearer.

Answer: We have made modifications and believe it is clearer.
Other Editorial Requirement:

Please make the following formatting change/s during revision of your manuscript. Ensuring that the manuscript meets the journal’s manuscript structure will help to speed the production process if your manuscript is accepted for publication.

1. Tables: (Please make sure that your tables are in .doc format)

Please ensure that the order in which your tables are cited is the same as the order in which they are provided. Every table must be cited in the text, using Arabic numerals. Please do not use ranges when listing tables. Tables must not be subdivided, or contain tables within tables. Please note that we are unable to display vertical lines or text within tables, no display merged cells: please re-layout your table without these elements. Tables should be formatted using the Table tool in your word processor. Please ensure the table title is above the table and the legend is below the table. For more information, see the instructions for authors on the journal website.

Response: Followed

References


