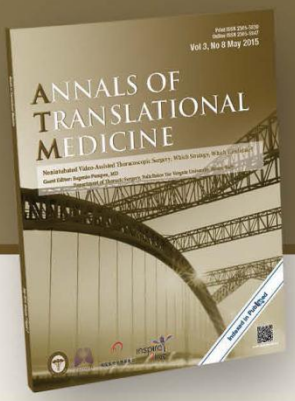
ANNALS OF TRANSLATIONAL MEDICINE



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Comment 1: **********
Reply 1: **********
Changes in the text: **********

Comment 2: **********
Reply 2: **********
Changes in the text: **********

Peer Review File

Article information: http://dx.doi.org/10.21037/atm-20-8053

Reviewer A

• **Comment 1:** Page 2, Abstract: There are no keywords in the abstract. The authors should add keywords.
Reply 1: We have added keywords in the abstract.
Changes in the text: We have added keywords in the abstract (see Page 2, line 10).

• **Comment 2:** Page 3, line 14 to 16: Many authors have done recent extensive works on other medical conditions causing elevated CA125. Also, most of the references in this manuscript are old. The authors can add more recent references to their study. The author may add additional references especially more recent articles that talk about CA125 in malignant and non-malignant conditions, for example " Chronic Medical Conditions and CA125 Levels among Women without Ovarian Cancer. Cancer Epidemiol Biomarkers Prev; 27 (12); 1483–90. _2018 AACR. doi: 10.1158/1055-9965.EPI-18-0203", and some other ones for more detail.
Reply 2: Thank you for your suggestion. We have added recent reference on other medical conditions causing elevated CA125.
Changes in the text: We have added recent reference on other medical conditions causing elevated CA125 (see Page 3, line 16-18).

• **Comment 3:** Page 3, line 18 - 19: The authors can mention where this study was performed by probably saying that "in a study performed at ".....general hospital" or simply say " previous single center hospital study show that.....". These will read better than writing ..."In an investigation conducted in general hospital....."
Reply 3: We have modified the text as advised.
Changes in the text: We have modified the text as advised (see Page 3, line 22).

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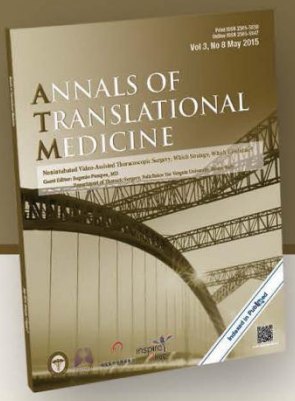

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Comment 2: **********
Reply 2: **********
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• **Comment 4:** Page 5, line 3 (under methods): This statement should be rephrase. Patients don't just present to the hospital with elevated serum CA125 Level, they present with clinical symptoms which may then necessitate doing a CA125 test. Therefore, this statement should be rephrased as "Patients who presented to the hospital with some gynecological symptoms and found to have elevated CA125 during investigations were enrolled in this study....."
Reply 4: We have re-written this part.
Changes in the text: We have re-written this part (see Page 5, line 15 to Page 6, line 12).

• **Comment 5:** Page 3, line 5: Please replace "enrollment were conducted in multiple settings" to "enrollment were conducted at multiple sites or at multiple centers"
Reply 5: We have modified the text as advised.
Changes in the text: We have modified the text as advised (see Page 5, line 15-19).

• **Comment 6:** Page 5, line 12: Complete this by saying "and by clinical examination and investigations"
Reply 6: We have modified the text as advised.
Changes in the text: We have modified the text as advised (see Page 5, line 20 to Page 6, line 1).

• **Comment 7:** Page 5, line 14 - 15: This statement is completely against what is mentioned in line 3 and 4 above under this methods section. Since the authors mentioned above that patients who presented with elevated CA125 before any treatment were enrolled, that means there is no way the authors can have data on histological type, stage, and grade of the disease which were mentioned to be collected at the time of enrollment here. Such detailed information is only gotten after staging laparotomy. Therefore, this should be re-written properly and resolved.
Reply 7: Sorry for the confusion. We have re-written this part.
Changes in the text: We have re-written this part (see Page 5, line 15 to Page 6, line 12).

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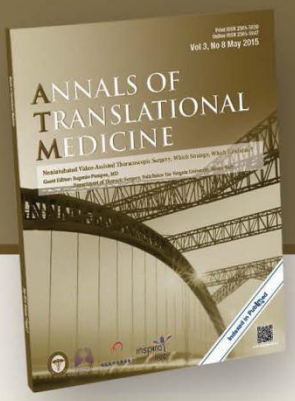

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Reply 1: **********
Changes in the text: **********

Comment 2: **********
Reply 2: **********
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• **Comment 8:** Page 5, line 17 - 19: The authors can elaborate more on this statement, why the written informed consent of participants was not required for participation in the study?
Was there any consent from participants in this study and what kind of consent was obtained?
Reply 8: Since we only used left over serum samples after clinical testing, the written informed consent from participants was not required and was waived by the Institutional Review Board.
Changes in the text: We have re-written this part (see Page 5, line 15 to Page 6, line 12).

• **Comment 9:** Page 6, line 11: Please correct the typos "dipepitide" to "dipeptide"
Reply 9: We have correct the typos "dipepitide" to "dipeptide".
Changes in the text: We have correct the typos "dipepitide" to "dipeptide" (see Page 7, line 7).

• **Comment 10:** On Page 6, Line 21: For completeness, the authors should add some information on the coefficient of variation of the assays used. Inter- or Intra-Assay coefficient of variations should be also added here.
Reply 10: We tested 21 lectins with antibody-lectin assay. Out of them, VVA, which bind to Tn antigen, was selected for further investigation. So, we estimated inter- and intra-assay coefficient of variations only for CA125-Tn ELISA. They were 8%-10% (see Page 10, line 8-9).
Changes in the text: No change**.**

• **Comment 11:** Page 8, line 4: Please correct typos, change "derivetization" to "derivatization"
Reply 11: We have changed "derivetization" to "derivatization".
Changes in the text: We have changed "derivetization" to "derivatization" (see Page 8 ,line 18).

• **Comment 12:** Page 8, line 17: correct the phrase " we asked whether lectins..." to "we sought to find whether lectins....."
Reply 12: We have corrected the phrase " we asked whether lectins..." to "we sought to find whether lectins.....".

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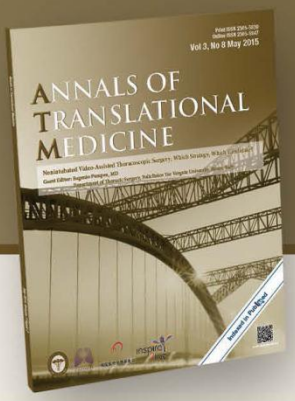

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Reply 1: **********
Changes in the text: **********

Comment 2: **********
Reply 2: **********
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Changes in the text: We have corrected the phrase " we asked whether lectins..." to "we sought to find whether lectins....." (see Page 8 ,line 6-7).

• **Comment 13:** Page 8, line 20: Change "in varies concentration" to " in varying concentration."
Reply 13: We have changed "in varies concentration" to " in varying concentration".
Changes in the text: We have changed "in varies concentration" to " in varying concentration" (see Page 9 ,line 11-13).

• **Comment 14:** Page 11, line 15: correct the typos by changing "carries" to "carry."
Reply 14: We have changed "carries" to "carry."
Changes in the text: We have changed "carries" to "carry" (see Page12 ,line 20).

Reviewer B

The specificity of CA125 is low for ovarian cancer diagnosis, with a high number of false-positive results seen among patients with benign ovarian conditions. The authors proposed that glycosylation of CA125 in cancer patients is different from that in other ovarian conditions. Based on that, they have proposed a new assay (CA125-Tn) for detecting ovarian cancer specific glycosylated CA125 as a diagnostic test for ovarian cancer.

There are several major concerns with the study design as follows,

Comment 1: More information is needed for the sample selection between 2011 and 2018. The total number of patients the CA125 test was done for them, why only such a small number of ovarian cancer patients ended in the study in 8 years?
Reply 1: In this study, we used left over serum sample after CA125 testing. No additional serum sample was collected outside the standard clinical requirement. Some patient did not have

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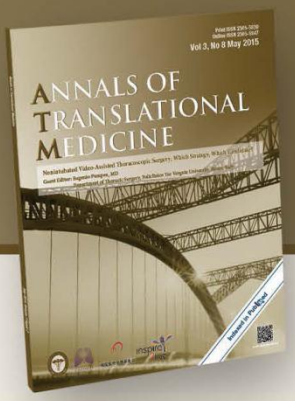

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Reply 1: **********
Changes in the text: **********

Comment 2: **********
Reply 2: **********
Changes in the text: **********

enough serum sample left to be used in this study. Besides, only patients with positive CA125 before treatment and histologically or clinically confirmed disease were included in this study. So, we did not have a lot ovarian cancer patients in this study.
Changes in the text: We have re-written method for patient enrollment to make it more clear (see Page 5, line 3-18).

Comment 2: CA125 is usually measured in plasma, but the authors used serum for their CA125-Tn assay. It is important to know what samples (serum or plasma) used for CA125 measurement and why the authors used serum for their CA125-Tn assay?
Reply 2: We double checked with nurse and lab staff who perform blood sample collecting and CA125 testing in clinic. Blood sample for CA125 testing was collected in tubes without anti-coagulation agent. So, serum sample was used for CA125 testing. In this study, we used left over serum sample after CA125 testing. No additional serum sample was collected outside the standard clinical requirement.
Changes in the text: No change.

Comment 3: It is not clear how the authors did ROC analysis for the CA125 while they only had patients with positive CA125 results in their study. This analysis also needs info on those with CA125 negative results to calculate sensitivity and specificity values. If they have used a creative approach in their analysis without having CA125-negative patients, they have to explain it in their paper.
Reply 3: In this study, only patients with positive CA125 results were included. We have re-written method for patient enrollment to make it more clear. Additionally, we re-emphasized it in ROC analysis results.
Changes in the text: We have re-written method for patient enrollment to make it more clear (see Page 5, line 15 to Page 6, line 12). Additionally, we re-emphasized it in ROC analysis results (see Page 11, line 3).

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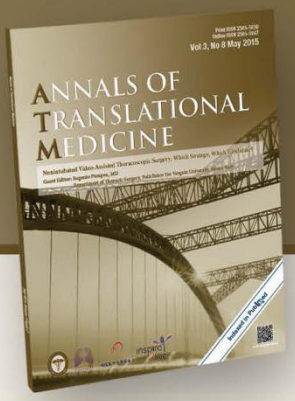

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Changes in the text: **********

Comment 2: **********
Reply 2: **********
Changes in the text: **********

Comment 4: The main problem of CA125 is the low specificity, and the authors should provide info on improving these metrics by using their CA125-Tn. Still, the authors only talked about the improvement of the sensitivity of the test.
Reply 4: Thank you for your suggestion. When sensitivity was fixed at 90%, specificity was improved from 20.1% for CA125 to 58.3% for CA125-Tn.
Changes in the text: We added information on improved specificity by using CA125-Tn (see Page 11, line 9-10).

Comment 5: The way authors designed their study was to select those with positive CA125 test and then did CA125-Tn assay on them. This is sequential testing, and the sensitivity and specificity calculated for their CA125-Tn assay are the metrics for the combination of the two assays rather than the metrics for the CA125-Tn assay alone. In sequential testing, the sensitivity of the combined assays is always worse than each of the assays. In contrast, the specificity of the combined assay is always better than each of the assays. For comparing the sensitivity and specificity of the CA125 and CA125-Tn assays with each other, the authors should test a group of patients for both assays independently.
Reply 5: This study was aimed to investigate whether CA125-Tn will help in improving specificity among patients with positive CA125 results, but not to comparing CA125 and CA125-Tn assays. So, only patients with positive CA125 results were included.
Changes in the text: No change.

Reviewer C

The manuscript titled “CA125-Tn ELISA Assay Improves Specificity of Pre-Operative Diagnosis of Ovarian Cancer Among Patients with Elevated Serum CA125 Levels” represents interesting data on lectin-based glycovariant of OvCa-associated CA125. Based on the changes in carbohydrate structures of CA125, they have reported that a panel of lectins, was screened for

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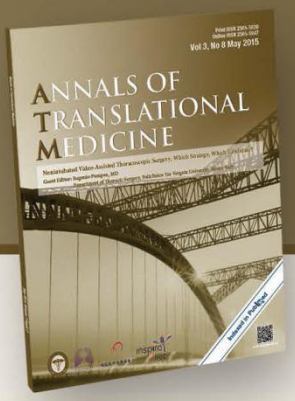

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the detection of the immobilized CA125 based on the ELISA with results showing that Vicia Villosa lectin (VVA) recognize OvCa-associated CA125 glycoforms (CA125-Tn). The development and design of the CA125-Tn assay are quite elegant and reported in good detail. They also showed that serum CA125-Tn measurements better distinguished patients with OvCa from borderline and endometriosis patients compared to the conventional CA125 ELISA. As a result, compared to the conventional CA125 ELISA, they have proposed the CA125-Tn a better assay for clinical uses. I believe this study could be of interest to readers of the journal, and I recommend its publication after a minor revision. Followings are my comments:

Comment 1: In introduction altered glycans (like truncated O-glycans, sialyl Tn antigen (STn), Tn antigen etc) elevated in cancer especially in OvCa associated CA125 which are previously reported should be mentioned (Chen K et al 2013, Akita et al 2012, Gidwani K et al 2016, 2019).
Reply 1: Thank you for your suggestion.
Changes in the text: We have added these references (see Page 5, line 1-2).

Comment 2: Lectins generally have poor affinity against it specific glycans and how author overcome with this issue should be addressed in introduction part
Reply 2: We screened 21 lectins for detection of immobilized CA125 on ELISA. To make sure lectins bind with its specific glycans but not non-specific glycans, we inhibited their binding with its inhibiting monosaccharides. To make sure lectins bind with CA125 associated glycans but not non-specific glycoproteins, we tested liner relationship between antibody-lectin results and CA125 concentrations. As a result, BPL, PNA, SBA, UEA, and VVA were qualified for detecting CA125 associated glycans. These data was described in Results.
Changes in the text: No change. These data was described in Results (see Page 9, line 5-17 and Fig. 2b, 2c).

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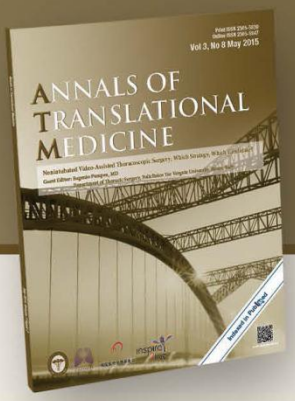

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Comment 3: It seems that author did't used calibration curve for CA125-Tn ELISA. Did the authors exam OvCa cell lines associated CA125 with VVA lectin? Can the authors provide the linearity data and recovery of these assays in human serum?
Reply 3: We tested liner relationship between CA125-Tn results and CA125 concentrations using purified CA125 antigen. This CA125 antigen was purified from human cancer cell line and purchased from Cellsciences, USA. These data was described in Results (see Page 9, line 11-17 and Fig. 2c).
Changes in the text: No change. These data was described in Results (see Page 9, line 11-17 and Fig. 2c).

Comment 4: A patient's group with elevated CA125 (≥ 35 U/ml) was included for evaluation of CA125-Tn ELISA in this study, I would recommend to include the range of CA125 ELISA values. In fig 3a, a dotted line indicating the cut-off of both assays would be more informative for readers. A box plot of conventional CA125 ELISA should also provide where readers can see the range/median of CA125 of each group where the CA125-Tn ELISA already provided in fig 3b.
Reply 4: Thank you for your suggestion, we have added cut-off of CA125-Tn assay in Fig. 3a. In our preliminary study, CA125-Tn ELISA showed low sensitivity when CA125 concentration was low. So, we only included patients with elevated CA125 (≥ 35 U/ml) in this study. In Fig. 3, we added another box plot showing conventional CA125 ELISA (see Fig. 3c) .
Changes in the text: We added cut-off of CA125-Tn assay in Fig. 3a. We also added another box plot showing results of conventional CA125 ELISA in Fig. 3c. Legend for Fig. 3 was modified accordingly (see Page 19, line 12-16).
.
Comment 5: Authors have used a capture Ab X325 and VVA lectin, that specifically recognize a protein epitope and Tn antigen epitope expressed on the CA125 antigen, respectively. Have authors compared their system with anti-Tn Abs, which are commercially available? I think such comparison may help readers to see the usefulness of both approaches.

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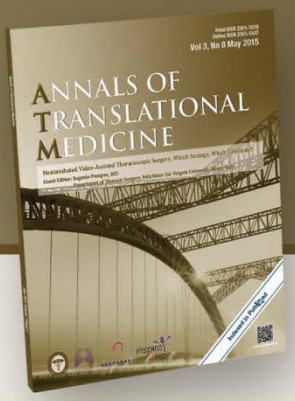

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Reply 5: Thank you for your suggestion. We did not compare anti-Tn Abs with VVA lectin in our system. But, we can try anti-Tn Abs in the future.
Changes in the text: No change.

Comment 6: In results section of page no. 7 author mentioned the name and abbreviation of all lectin used, which may exclude from text as the same already available in table 1 of supplementary.
Reply 6: Thank you for you suggestion. We have moved lectin names and abbreviations to supplementary table 1.
Changes in the text: We have moved lectin names and abbreviations to supplementary table 1 (see Page 8, line 13-14 and supplementary table 1).

Comment 7: In results section of CA125-Tn level was higher in ovarian cancer patients, page no. 8, ‘’Among different histotypes, serous and clear cell ovarian cancer showed higher CA125-Tn levels as compared with mucinous or endometrioid ovarian cancer (Table 1)’’ I can’t see any table for the same, also I would suggest to include the no. of different histotypes of OvCa if you are mentioning it in the results section
Reply 7: Sorry, table 1 was missing in the manuscript. We have added table 1. Also, we have included the No. of different histotypes of OvCa in the Results section.
Changes in the text: We have added table 1(see Page 18). Also, we have included the No. of different histotypes of OvCa in the Results section (see Page 10, line 20-22).

Comment 8: A sensitivity of 72% with 90% specificity is exceptional performance and the weighted population should be clearly stated as a limitation by the authors. It would also be interesting if sensitivities were reported for higher specificities (e.g., 90% and 95%).
Reply 8: For 95% specificity, sensitivity for CA125-Tn to detect ovarian cancer among patients with positive CA125 would be 58.8%.

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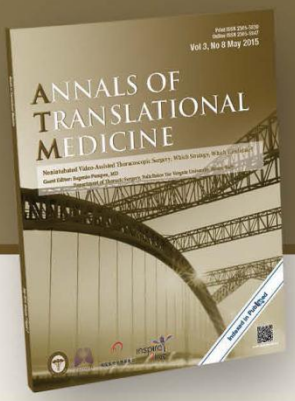

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Changes in the text: We have added a statement about weighted population in the text (see Page 11, line 3-7).

Comment 9: It is not clear that what the LOD and linear range of detection are. This should be clarified and presented both in the abstract and conclusion parts while I could not see a concluding paragraph or section.
Reply 9: According to data we have for now, limit of detection for CA125-Tn assay was 0.07. CA125-Tn was within its linear range when CA125 concentration was between 35-400 mIU/ml. Since this is only an exploratory study, we did not test its linear range with higher concentration of CA125. We will test these two parameter in future validation study with larger population.
Changes in the text: We have added a Conclusion section (see Page 14, line 7-11).

The manuscript would be much more informative if above more data/corrections provided to the reader. The manuscript presents important and novel information regarding research immunoassays for detecting ovarian cancer.

Reviewer D

The authors explore the potential of a lectin based, improved CA125 assay in the diagnosis of ovarian cancer. The prospective study cohort consisted of 68 women with ovarian cancer, 15 women with ovarian borderline tumors and 245 women with benign gynecological diseases. The authors conclude that specifically the CA125-Tn assay (VVA lectin based) is a particularly promising approach to improved diagnostics of ovarian cancer. Although the study is clinically interesting and has novelty value, there are some major concerns that need attention prior to publishing.

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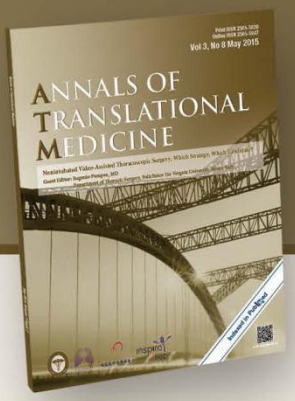

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Comment 1: Introduction, lines 10-11, p 3: “Preoperative diagnosis of ovarian cancer relies mainly on serum CA125 level and ultrasonography.” This statement is outdated. An important part of the standard diagnostic work-up of these patients is a computed tomography scan of the thorax and abdomen.
Reply 1: Thank you for your suggestion. We have modified the text as advised.
Changes in the text: We have modified the text as advised (see Page 3, line 11-13).

Comment 2: Introduction, lines 13-14, p 3: “Elevation of serum CA125 concentration was observed in most of non-mucinous epithelial ovarian cancer patients.” Reference?
Reply 2: We have added reference for this statement.
Changes in the text: We have added reference for this statement (see Page 3, line 15).

Comment 3: A major issue is the lack of written informed consent from the participants, particularly as this is a prospective study and additional serum samples outside of the standard clinical requirements were collected.
Reply 3: In this study, we used left over serum sample after CA125 testing. No additional serum sample was collected outside the standard clinical requirement. Thus, the written informed consent from participants was not required and was waived by the Institutional Review Board.
Changes in the text: We have re-written this part to make it more clear (see Page 6, line 5-12).

Comment 4: Table 1 is missing from the pdf, please fix.
Reply 4: Sorry for that.
Changes in the text: We have added Table 1 at the end of the manuscript (see Page 18).

Comment 5: Another major concern is the expected age bias of the cohort as the majority of the benign diseases included (endometriosis, PID and adenomyosis) are generally present in younger women compared to ovarian cancer. This might influence the serum CA125

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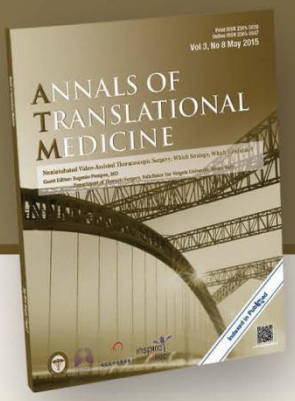

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concentration. Did the authors explore and, if necessary, correct for the age-related trends in the cohort?

Reply 5: Thank you for your suggestion. We found older age was weakly correlated with higher CA125 and CA125-Tn levels. So, we divided the study population into two age subsets, >45y and ≤45y, and re-analyzed the data.

Changes in the text: We re-analyzed the data. Text and Figure were modified accordingly (see Page 11, line 11-21, and Fig. 4b).

Comment 6: Results lines 9-12, p 10: “Since post-menopausal women have higher chance of having ovarian cancer, we calculated CA125-TnM=CA125-Tn/M, and CA125M=CA125/M. M was 1 for postmenopausal women, was 2 for premenopausal women.” What did you base this assumption and calculation on? A better method would be either to correct for the age bias of the cohort with linear regression or include a subset of only postmenopausal patients in the ROC curve analysis.

Reply 6: Thank you for your suggestion. We found older age was weakly correlated with higher CA125 and CA125-Tn levels. So, we thought its better to divide the study population into two age subsets, >45y and ≤45y, and re-analyze the data.

Changes in the text: We re-analyzed the data. Text and Figure were modified accordingly (see Page 11, line 11-21, and Fig. 4b).

Comment 7: Were the patients with borderline tumors included in the diagnostic performance analyses (ROC curves) and if yes, in which of the groups (benign or malignant)? This is important as borderline tumors cannot be put in either category without second thoughts. Please specify.

Reply 7: According to the data we have, CA125-Tn levels in borderline tumor was as low as that in benign conditions (see Table 1 and Fig. 3b). So, we included borderline tumors in non-malignant group in ROC analyses.

Changes in the text: We have specified this point in the text (see Page 11, line 3-7).

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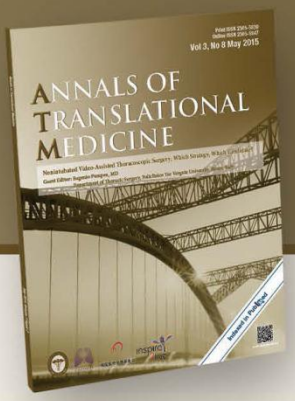

Notice: Please give your response to the comments point-by-point as shown in the following format.
【At the END of each reply/response from you, please DO describe how you responded to the reviewer comment in the text, e.g., "we added some data ....(see Page xx, line xx)" or "we have modified our text as advised (see Page xx, line xx)"】.

Comment 1: **********
Reply 1: **********
Changes in the text: **********

Comment 2: **********
Reply 2: **********
Changes in the text: **********

Comment 8: Discussion, lines 16-17, p 10: “To date, more than 50 serum biomarkers have been developed for pre-operative diagnosis of ovarian cancer” This seems as an excessive statement as only two biomarkers have been validated and approved in the clinical setting (CA125 and HE4).
Reply 8: We have modified the text as advised.
Changes in the text: We have modified the text as advised (see Page 12, line 1-2).

Comment 9: Discussion, lines 7-9, p 11: “According to our results, patients with both elevated serum CA125 level and CA125-Tn level are at high risk for ovarian cancer, and should be referred to a gynecological oncologist, while the remaining who has only elevated CA125 level should be followed up closely.” Too strong statement based on an exploratory study.
Reply 9: We have modified the text as advised.
Changes in the text: We have modified the text as advised (see Page 12, line 13-15).

Comment 10: Some of the references are incomplete with missing journal names and or volumes/issues. Please pay attention to detail. In addition, reference no 1 is from 2012 although there is more recent data on ovarian cancer mortality and survival available. In general, I suggest the critical inspection of the references as a major part of them are published over 15 years ago.
Reply 10: We have updated the references and corrected errors in references.
Changes in the text: We have updated the references and corrected errors in references.

Comment 11: A language check is needed prior to publication.
Reply 11: The manuscript has been sent out for language editing by American Journal experts.
Changes in the text: The language of the manuscript has been edited by American Journal experts.