Author's response to reviews

Title: Pleural Fluid Cell-free DNA Integrity Index to Identify Cytologically Negative Malignant Pleural Effusions Including Mesotheliomas

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Author's response to reviews: see over
10th June 2012

To:

Series Editor (Medicine)
BMC Cancer

From:
Dr K B Sriram
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The Prince Charles Hospital
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Dear Dr Patel,

Re: Pleural Fluid Cell-free DNA Integrity Index to Identify Cytologically Negative Malignant Pleural Effusions Including Mesotheliomas

We thank the reviewers, Drs Siafakas, Holdenrieder and Pinzani, for their time and effort in undertaking a comprehensive appraisal of our manuscript. Their outstanding research work clearly demonstrates that they are leaders in the field of DNA biomarkers in malignancy and their comments have enabled us to improve our manuscript. We have responded to each of the Reviewer’s comments. Major changes that have been made in the manuscript are presented here, with previous statements in red, and revisions in blue.

Reviewer 1: Nikos Siafakas

Major Criticism:
#1.1 From the 72 MPE – 23 had mesothelioma thus 32%. This large number of mesotheliomas may had affected the results and made it questionable if we could extrapolate the results to MPE of other etiologies.

Response 1.1: We agree with Dr Siafakas that the high proportion of mesothelioma subjects in the study may limit the applicability of our findings to other patient settings where mesothelioma may not be a common cause of MPEs. Nonetheless, in our study we also found that median pleural fluid DNA integrity was also higher in non-mesothelioma MPEs compared with benign effusions (1.2 vs. 0.8, p<0.001). We agree that the positive results from the relatively small sample size in our study mandates validation in a larger, preferably multi-center prospective study. We have clarified this by including the following statements:

Pg23, lines 5-11: Thirdly, another limitation of our study relates to the relatively small number of subjects evaluated and the casemix of pleural effusion diagnoses, particularly the relatively high proportion of mesothelioma subjects (31%), derived at a single Australian thoracic tertiary referral centre. It is well recognised that the distribution of aetiologies of pleural effusions (including MPEs) vary considerably according to geographic location [42]. Further validation will be needed to address issues of generalisability and applicability.

#1.2 It is remarkable that in a prospective study like this only 75 out of 100 patients had given serum? Any reason?
Response 1.2: In our study we obtained consecutive pleural fluid samples sent to our pathology department, from where an aliquot of the pleural fluid sample was forwarded to our research laboratory. In 25 patients, the pleural fluid was collected during simple thoracocentesis in the out-patient setting. These patients were discharged before we were able to obtain matched blood samples from them. While these patients provided written consent (which was mailed to them) they were not able to provide matching blood samples. In the planned prospective study we are implementing changes so that matched blood samples can be obtained in all patients. In the revised manuscript we have now also reported sensitivity analyses excluding subjects for whom there were no matched serum samples.

#1.3... it is not clear if the pleural fluid was collected on the first thoracocentesis or subsequent ones. This also may have affected the DNA results.

Response: The pleural fluid samples in our study were collected for diagnostic purposes. This was obtained either during simple thoracocentesis performed for diagnostic purposes or prior to tube thoracostomy or a surgical thoracoscopic procedure. The patients who had the pleural fluid aspirated (for diagnosis) and then underwent tube thoracostomy or surgical thoracoscopic procedure were patients who had a large unilateral pleural effusion, highly suspicious for malignancy. While we have not specifically tested the hypothesis that DNA integrity may be altered by repeated aspiration, we do not consider this likely to have affected our results, since only one patient (eventually diagnosed with congestive cardiac failure) had testing on a second aspirate.

Pg11; lines 3-7: Pleural fluid was collected during simple thoracocentesis, performed during the diagnostic work-up of patients with pleural effusions or prior to tube thoracostomy, or a surgical thoracoscopic procedure. In all but one subject, pleural fluid obtained at the time of the first thoracocentesis was used for analysis.

#1.4 Major limitation is also the final diagnosis of MPE and in particular in those which the diagnosis was based on the disseminated malignancy-exudates and no alternative explanation (page 12, lines 15-18). State how many?

Response 1.4: We recognize that the statements relating to cytology negative MPEs require clarification. Hence we have revised the statements so that the reader now has more information about how the diagnosis of MPE was made in cytology negative subjects.

Methods
Pg 13; lines 19-22: Subjects with negative pleural fluid cytology were also deemed to have MPEs if a tissue biopsy was positive for malignancy or if subjects had disseminated malignancy and an exudative effusion, and there was no alternative explanation for the effusion.

Results
Pg 15; Lines 13-19: Pleural fluid cytology analysis identified malignant cells in twenty-nine patients (56%). Among the remaining 23 patients with MPEs, the diagnosis was established by pleural biopsy in 16 patients. For the remaining seven patients, the treating clinician determined the pleural effusion to be malignant in the context of disseminated malignancy.

# 1.5 What was the explanation of transudateds (11%) in the MPE group? (page 14, line 1-
Response 1.5: In the revised cohort including only those subjects with matched pleural fluid and serum, there were only seven transudates of which five were malignant (1 cytology positive, 4 with disseminated malignancy) and two subjects had benign effusions. The reduced numbers mean that no meaningful comparison can be conducted. Previous studies have found that 1%-10.7% of MPEs are transudates (Porcel et al, Chest 1999, Assi et al, Chest 1998, Moltyaner et al, Chest 2000 and Gonlugur et al, Ann Acad Med Singapore 2008). Reasons for this include early stages of mediastinal involvement, or concomitant atelectasis or congestive cardiac failure (Light et al, Ann Intern Med, 1972).

#1.6 Page 16, lines 24-25: Serum mesothelin was measured in ONLY 37 subjects. Thus, this is very small number for comparisons and solid conclusions.

Response 1.6: In ten subjects (including nine with cytology negative effusions) the volume of pleural fluid available was sufficient for DNA isolation but insufficient for mesothelin testing. We acknowledge that this is a weakness of our study and have added appropriate statements in the revised manuscript.

Pg 21; lines 23-24: However a weakness of our findings is that serum mesothelin results could not be obtained in 13% (10/75) of study subjects.

Minor Comments

#1.7 Table 1: A column of the p values should be added (page 13, line 20) 2.

Response 1.7: We calculated p-values for the significance testing between subjects with MPE and benign effusions in relation to the characteristics listed in Table 1, and included them in the revised table, now Table 2.

#1.8 The definition of “necrotic DNA” (page 14, line 11) is not clear.

Response 1.8: In order to clarify this statement and ensure consistency (text and Tables) throughout the manuscript we have now replaced “necrotic DNA” with “ALU 247” and “total DNA” with “ALU 115”.

# 1.9 The major finding of the integrity DNA index been higher in benign vs MPE, although had been validated by ROC analysis, the overlap figures are not shown.

Response 1.9: We have now included these figures (Figure 3 and Figure 4) in the revised manuscript.

# 1.10 It is not clear when pleural fluid measurements were compared with serum if only the matched subjects (74) were used.5 Page 15, lines 12-16. It is not clear what numbers referred to what comparison? The whole paragraph has to be rewritten.

Response 1.10: Following the suggestions of Dr Siafakas and the other reviewers, in the revised manuscript we have only included subjects where both pleural fluid and matched serum was available for testing. The rewritten paragraph is as follows:-
Both pleural fluid (median 38nM vs. 4.7nM, \(p<0.001\)) (Figure 1d) and serum mesothelin (median, 2.0nM vs. 0.8nM, \(p<0.001\)) (Supplementary Figure 3d) were significantly higher in the mesothelioma subjects compared to lung cancer subjects (median, 38.0nM vs. 6.0nM; \(p<0.001\); 2.0nM vs. 0.7nM; \(p=0.001\)) and subjects with benign effusions.

Pg 17; lines 2-8: Mesothelin was measured on 75 pleural fluid and 65 serum samples. (There was insufficient serum for mesothelin testing in 10 subjects.) Subjects with mesothelioma, in comparison to subjects with benign effusions, had significantly higher pleural fluid mesothelin (median 38 nM vs. 4.6 nM, \(p<0.001\)) (Figure 3d) and serum mesothelin levels (median, 2.0 nM vs. 1.0 nM, \(p<0.001\)) (Figure 4d). In the subjects with mesothelioma, there was a significant correlation between the serum and pleural fluid mesothelin levels (Spearman’s rho = 0.632, \(p<0.001\)) (Figure 5).

Response 1.12: We have now amended these statements to provide more clarity as to how pleural fluid DNA integrity adds to the clinical utility of cytology in achieving a diagnosis of MPE.

Cytology remains the diagnostic standard for evaluating pleural effusion samples. However it is can be difficult to establish a diagnosis of MPE in subjects with cytology-negative effusions, since in such circumstances diagnosis requires invasive pleural biopsy tests, such as thoracoscopy. We found that when combined with cytology, pleural fluid DNA integrity index substantially increased the ability to distinguish benign effusions from MPE (sensitivity 81% vs. 55%) and mesothelioma (sensitivity, 81% vs. 31%). Importantly, almost a third of false positive pleural fluid DNA integrity index results were due to parapneumonic effusions. Additionally, we showed that in cytology negative effusions, elevated pleural fluid DNA integrity index had 81% PPV. Hence pleural fluid DNA integrity index provides valuable additional information to pleural fluid cytology, particularly in subjects with either inconclusive or “suspicious” cytology results. This may have clinical implications since an elevated pleural fluid DNA integrity index in a cytology-negative subjects should be prioritized to undergo thoracoscopy.

References: Although the list of References is long few important ones that use other DNA marker are missing such as Economidou et al “Microsatellite DNA analysis....” Oncology Reports 2007;18:1507-12.

Response 1.13: We agree that the study by Economidou et al is relevant to the discussion of DNA biomarkers in pleural fluid. In the revised manuscript, we have acknowledged the contributions of their paper to the understanding of the limitation of DNA based diagnostic biomarkers for malignant pleural effusions.

An alternative approach to distinguish malignant from benign effusions has been to evaluate the utility of quantitative and qualitative tumour-specific alterations, such as microsatellite alterations in effusion DNA [24-28]. Economidou et al studied patients with malignant (n=26) and benign (n=22) effusions and found that microsatellite instability and loss of heterozygosity in DNA from pleural fluid and blood were not diagnostically useful [27].
Reviewer 2: Stefan Holdenrieder

Major compulsory points for revision:
# 2.1 For a fair comparison, only those results with markers measurements in both materials should be included.

Response 2.1: As suggested by Dr Holdenrieder we have now excluded patients in whom serum DNA was not available. Accordingly, the Results text, Tables (1,2,3,4 and 6) and Figures (3,4 and 6) have been revised to only include the data for 75 subjects with matched pleural fluid and serum samples. Pleural fluid DNA integrity index still has diagnostic utility in identification of MPEs.

#2.2 Best criteria for comparison of the markers is AUC of ROC curves plus sensitivity at a defined specificity...

Response 2.2: We appreciate this guidance in improving the data analysis and have revised the manuscript accordingly. The AUC of ROC curves and sensitivity were evaluated at a defined specificity of 90%. We found that the pleural fluid DNA integrity index (cut-off level of 1.02) pleural fluid mesothelin (cut-off: 12.72 nM) and serum mesothelin (cut-off: 1.32 nM) distinguished MPE from benign effusions with a sensitivity of 57%, 37% and 44% respectively. We have included this information in the revised manuscript.

Pg 17; lines 13-18: We used a specificity threshold of 90% to compare the utility of pleural fluid DNA integrity index, pleural fluid mesothelin, and serum mesothelin in differentiating MPE from benign effusions. Pleural fluid DNA integrity index (cut-off level of 1.02) pleural fluid mesothelin (cut-off: 12.72 nM) and serum mesothelin (cut-off: 1.32 nM) distinguished MPE from benign effusions with sensitivity of 57%, 37% and 44% respectively.

# 2.3 It should be clearly indicated how the DNA integrity index is calculated. If it is the ratio of the long to the short fragments, and the short fragments are a part of the long ones (as indicated in the Umetani work), then ratios higher than 1 are unlikely. However, this seems not to be a single event as the medians of several groups are higher than 1. This should be explained and discussed.

Response 2.3: In our study we determined DNA integrity to be a ratio of long fragments to short fragments (ALU 247/115). We performed gel electrophoresis to determine the size of the amplicons that were produced by the ALU 115 and 247 primer sets.
As these figures demonstrate, the ALU 115 and 247 primer sets produce amplicons of the expected sizes, i.e. 115bp and 247bp respectively. In addition, we also determined the melting profiles of the ALU 115 and 247 amplicons and found that they were consistent with those reported by Deligezer et al, Clin Chem Lab Med 2008.

In this study we determined DNA integrity to be a ratio of the ALU long fragments to short fragments, i.e. ALU 247/115. Interestingly, Agostini et al Ann Surg Oncol 2011, found that DNA integrity (ALU 247/115) in the plasma of patients with rectal adenocarcinoma (n=67, median 1.1, IQR25-75 0.7-1.9) compared to healthy control subjects (n=35, median 0.1, IQR25-75 0.0 – 0.4). The high DNA integrity index identified in patients with advanced colorectal cancer is consistent with our findings of high DNA integrity index in patients with advanced pleural malignancies.
Additionally, the serum DNA integrity index in our cohort of subjects with benign effusions was also relatively high and not significantly different from subjects with MPEs (median 0.9, IQR 25-75; 0.5-1.0). This is not completely unexpected since our ‘control’ population were not normal healthy volunteers but subjects with non-malignant pulmonary diseases. It is possible that the different results across laboratories maybe explained given that each laboratory has different equipment and methodologies for pre- and post-PCR processing steps. In the revised manuscript we have now included statements that better reflect the results of the methods that were achieved in our laboratory.

Pg 10; lines 23-25: The ALU 115 primer set amplifies both shorter (115bp) and longer (247bp) fragments, while the ALU 247 primer set only amplifies the longer fragments. The ALU 115 results reflect the total amount of cfDNA (‘total DNA’) and the ALU 247 results reflect cfDNA released from necrotic cells (‘necrotic DNA’).

Pg 12; Lines 1-13: The ALU 115 primer set amplifies both short 115bp product while the ALU 247 primer set amplified a longer 247bp fragments. The amplicon sizes were confirmed on gel electrophoresis. Quantification of DNA in each sample was determined by a standard curve with serial dilutions (10ng-0.01pg) of commercially available human female genomic DNA (Promega, Sydney, Australia) (Figure 1). Standard curves were determined for both ALU 115 and 247 primer sets respectively and the curves were obtained by putting the concentration log of the standard in x-axis and the values of the threshold cycles (Ct) in the y-axis. The quantity of DNA (ng/ml) present in the sample was extrapolated from the standard curve according to the Ct value. The DNA integrity index, represented by the ratio of the longer to shorter (ALU 247bp/ALU 115bp) fragments, was calculated for each individual sample (pleural effusion and serum) by dividing the ALU247 DNA quantity (ng/mL) by the ALU 115 DNA quantity (ng/mL).

Pg12; lines 21-22: The PCR protocol was optimised to yield optimal results to suit the equipment, reagents and conditions of our laboratory.

# 2.4 As there are several formula for calculation of DNA integrity index it would be interesting whether there would be different results using the formula of Wang 2003? At the moment the DNA integrity assay is only a research tool. It has to be questioned how well it will be suited to be used in clinical routine.

Response 2.4: In the revised manuscript we have now included information about the different methods available for measuring DNA integrity. To the best of our knowledge, there are no studies that have directly compared the different methods or standardized the protocols for measuring DNA integrity. While it would be interesting to compare the results of our study by using different published methods for measuring DNA integrity, we feel it is beyond the scope of our pilot study. In the revised manuscript, we have also attempted to clarify that DNA integrity and cfDNA are still only research tools and that studies such as these will help determine if they will have a role in clinical practice.

Pg22; lines 5-9: …it should be noted the method used for measuring DNA integrity in our study is one of several reported in the literature [12]. In this study, we have used the ratio of the longer DNA fragments to the shorter DNA fragments to measure DNA integrity (ALU 247/115), as described by Umetani et al [13].

Pg 22; lines 20-25; pg 23; lines 1-5: Secondly the different studies have not only used other
methods to measure DNA integrity, they have also used other protocols to process and extract DNA from blood [12]. Recently Fleischhacker et al found that significantly different amounts of absolute DNA values were obtained from plasma using different DNA isolation methods [41]. Indeed Jung et al, note that the considerable heterogeneity in preanalytical and analytical factors considerably determine the interpretation of cfDNA and DNA integrity studies in malignancy, thereby limiting the translation of this test into clinical practice [12]. Hence, despite extensive research into the utility of cfDNA and DNA integrity index as diagnostic and prognostic biomarkers in malignancy, the methodological discrepancies have dictated that the tests have remained as research tools.

*Minor points:*

# 2.5 The table of patients characteristics could be more informative with age, gender etc-

**Response 2.5:** As suggested, we have expanded Table 2 which details the characteristics of the study subjects. Below is the revised Table 2.
2.6 The quality of the figures is poor in the submitted version. Therefore the figures cannot be reviewed.

Response 2.6: All of the figures have now been modified and uploaded as pdf files, as per the BMC Cancer Figure Preparation Guidelines (http://www.biomedcentral.com/ifora/figures).

2.7 Supplemental data could be included in parts in the original paper.

Response 2.7: We have now incorporated Supplementary Tables and Figures into the body of the main manuscript.

Table 2 - Clinicopathological characteristics of study subjects

<table>
<thead>
<tr>
<th></th>
<th>Malignant pleural effusion</th>
<th>Benign pleural effusion</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 52</td>
<td>N = 23</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>68</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>30 – 94</td>
<td>41 – 92</td>
<td></td>
</tr>
<tr>
<td>&lt; 60 years of age</td>
<td>12 (23)</td>
<td>4 (17)</td>
<td>0.762</td>
</tr>
<tr>
<td>&gt; 60 years of age</td>
<td>40 (77)</td>
<td>19 (83)</td>
<td></td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31 (60)</td>
<td>17 (74)</td>
<td>0.301</td>
</tr>
<tr>
<td>Female</td>
<td>21 (40)</td>
<td>6 (26)</td>
<td></td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current or Former</td>
<td>33 (63)</td>
<td>17 (84)</td>
<td>0.435</td>
</tr>
<tr>
<td>Never</td>
<td>19 (37)</td>
<td>6 (26)</td>
<td></td>
</tr>
<tr>
<td>Pleural fluid, median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein, g/L</td>
<td>43.5 (37.0 – 48.0)</td>
<td>40 (31.8 – 45.8)</td>
<td></td>
</tr>
<tr>
<td>LDH, IU/L</td>
<td>390 (198.5 – 926.5)</td>
<td>222 (168 – 322)</td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>6 (4 -7)</td>
<td>6 (4 – 8)</td>
<td></td>
</tr>
<tr>
<td>Light’s criteria classification, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transudate</td>
<td>5 (10)</td>
<td>2 (9)</td>
<td>1.00</td>
</tr>
<tr>
<td>Exudate</td>
<td>47 (90)</td>
<td>21 (91)</td>
<td></td>
</tr>
<tr>
<td>Pleural fluid cytology, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive for malignancy</td>
<td>29 (56)</td>
<td>0 (0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Negative for malignancy</td>
<td>23 (44)</td>
<td>23 (100)</td>
<td></td>
</tr>
</tbody>
</table>
Reviewer 3: Pamela Pinzani

# 3.1 Pag 6 line 4 “integrity index has been reported to be a marker of malignancy”:
Authors should stress the concept that this topic is far away to be defined and conflicting results have been published.....

Response 3.1: In the revised manuscript we have now included information about the different methods available for measuring DNA integrity. We have also attempted to clarify that DNA integrity and cfDNA are still only research tools. Please see Response 2.4.

# 3.2...eventually change “pleural fluid cfDNA” in “pleural fluid DNA”.

Response 3.2: As suggested, in the revised manuscript we have replaced ‘pleural fluid cfDNA’ with ‘pleural fluid DNA’.

# 3.3 About sample processing, the use of serum instead of plasma must be explained.
Response 3.3: In the revised manuscript we have provided the rationale for using serum instead of plasma for our study.

Pg 19; lines 14-18: Previous reports have demonstrated that cfDNA is 4-6 fold higher in serum compared to plasma [23] and maybe a better biological specimen to screen for cfDNA in malignancy [13]. Hence we used matched serum as a reference comparator to pleural fluid in evaluating the utility of cfDNA and DNA integrity index in diagnosing malignancy.

# 3.4 Are the centrifugation steps used sufficient to get free of cell related DNA? In my opinion only one centrifugation step and a storage to -20°C can definitely determine an incorrect sample recruitment. Can it be that this fraction of circulating DNA coming from cell lysis masks the difference between normal and disease subjects? Or how long serum samples have been stored at -20°C? cfDNA can be degraded very easily. Did you test for this possibility?

Response 3.4: Dr Pinzani has raised very interesting methodological issues pertaining to our study. To the best of our knowledge, no agreed standards exist and many publications fail to specify how samples were obtained, processed and analyzed. In regards to serum processing we used the method of serum processing used by Ellinger et al International Journal of Cancer 2008 to measure DNA integrity in serum. In relation to DNA isolation from serum samples, it has been demonstrated that repeated freeze-thaw cycles have a negative effect on specimen quality (Rai AJ, Proteomics 2005). Consequently we took care to ensure that the serum samples in our study were not subject to this effect. In our study, DNA was isolated from serum samples within 1 week of collection and stored at -80°C. During storage, the samples did not undergo freeze-thaw cycles before DNA isolation. By using this approach we minimized the possibility of DNA degradation.

# 3.5 page 11 line 12 “Individual samples were analyzed in triplicate and the result accepted only if the standard deviation of the Ct values was less than 1 Ct.” The criteria for good reproducibility should rely on a cut-off value based on intra and inter assay CV% value (as reported in the MIQE guidelines).
Response 3.5: We appreciate this guidance to improve our reporting so that we are in concordance with the MIQE guidelines and we have revised the statement accordingly.

Pg 11; lines 11-15: Individual samples were analyzed in triplicate and the result accepted only if the standard deviation of the Ct values was less than 1 Ct. Samples triplicate standard deviation greater than 1 Ct were re-analyzed. 10ng of female genomic DNA (Promega, Sydney, Australia) was used as a positive control in each plate run.

Pg12; lines 24-25; pg 13; lines 1-3: …the median intra and inter-assay coefficient of variation (CV) for 115bp were 4% (IQR, 3%-6%) and 4% (IQR, 3%-4%). Similarly, the median intra and inter assay CV of the female genomic DNA for the 247bp primers were 4% (IQR 3%-7%) and 6% (IQR, 5%-7%) respectively.

# 3.6 page 11 line 16 “The median coefficient of variation (CV) of 17 replicate DNA concentrations for 115bp and 247bp primers were both 4%” Is the sentence referred to the female genomic DNA used as the positive controls or to the standard curve points. Please clarify.

Response 3.6: We used commercially available human female genomic DNA as the positive control and as the reference for determining the inter- and intra assay CV. The methods section has been revised accordingly.

Pg 11; lines 16-18: The median coefficient of variation (CV) of replicate DNA concentrations for 115bp and 247bp primers were both 4% (interquartile range (IQR, 3%-6% and 3%-7%) respectively.

Pg12; lines 24-25; pg 13; lines 1-3: Using the human female genomic DNA (positive control) we found that the median intra- and inter-assay coefficient of variation (CV) for 115bp were 4% (IQR, 3%-6%) and 4% (IQR, 3%-4%) respectively. Similarly, the median intra- and inter- assay CV of human female genomic DNA for the 247bp primers were 4% (IQR 3%-7%) and 6% (IQR, 5%-7%) respectively.

# 3.7 Page 12, lines 6-10. Was the coefficient of variation evaluated using the absorbance value or using sample concentration? 15% on the basis of absorbance will result a huge error on the concentration of samples. Please specify! Moreover, use the previously reported abbreviation for coefficient of variation.

Response 3.7: We evaluated CV of sample concentrations, not their absorbance values. We agree with Dr Pinzani that readers not familiar with the Fujireibo Mesomark kit may appreciate a more detailed methods description. We have now followed a similar pattern of reporting to previous publications based on this kit to assay mesothelin, including seminal publications relating to serum and pleural fluid mesothelin (Robinson et al, Lancer 2003, Creaney et al Thorax 2008). In performing the mesothelin assay we strictly followed the manufacturer’s instructions.

Pg12; lines 6-10: Sample data with mean absorbance readings ≥0.2 absorbance units (a.u.) was only accepted if the coefficient of variation was ≤ 15%, and data from samples with mean absorbance readings ≤0.2 a.u. were only accepted if individual replicates were within ±0.03 a.u. of the mean as recommended by the manufacturer.
The inter-assay and intra-assay CV of mesothelin concentration of the positive control samples were 3% (IQ 2%-4%) and 4% (2%-5%) respectively.

# 3.8 Page 18 line 9-11 Along the whole manuscript the authors deal with “serum DNA integrity”, but the reference citations are relative to both plasma and serum. I suggest to rename as “serum/plasma cfDNA integrity index” where it is necessary.

**Response 3.8:** As suggested, we have now replaced the term ‘serum DNA integrity’ with ‘serum/plasma DNA integrity’.

# 3.9 Figure 1 and Figure 2 are not printable to a readable quality, thus it is also impossible to me to evaluate them. Replace this suitable figures.

**Response 3.9:** All of the figures have now been modified and uploaded as pdf files, as per the BMC Cancer Figure Preparation Guidelines (http://www.biomedcentral.com/ifora/figures).

**Minor comments:**

# 3.10 pag 10 line 18 e segg. Please look for space between numbers and unit of measurements.

**Response 3.10:** As suggested, we have now inserted spaces between numbers and unit of measurements.

# 3.11 pag 11 line 6 modify adding “by” before “35 cycles”

**Response 3.11:** As suggested, we have modified the sentence accordingly.

We are grateful for the time and effort that the reviewers and editor allocated to improving our paper, and hope that with the recommended modifications our manuscript is now of sufficient merit to be accepted for publication.

Thank you,

Yours sincerely,

Krishna Bajee Sriram
MBBS, FRACP