Author's response to reviews

Title: Matrix-metalloproteinases-1, -2, -3, -9, their inhibitors TIMP-1, -2, and the MMP1/TIMP1-complex in blood plasma as markers for transitional cell carcinoma of the bladder

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Author's response to reviews: see over
RE: Resubmission of original work manuscript:
No. 1736728878568775

„Matrix-metalloproteinases-1, -2, -3, -9, their inhibitors TIMP-1, -2, and the
MMP1/TIMP1-complex in blood plasma as markers for transitional cell
carcinoma of the bladder”
Andrea Staack, Steffen Badendieck, Dietmar Schnorr, Stefan A. Loening,
Klaus Jung
From the Department of Urology, University Hospital Charité, Humboldt
University of Berlin, Germany

New Title:
“Plasma MMP2 in combination with MMP9 and TIMP1 improves non-
invasive detection of transitional cell carcinoma of the bladder”

Dear Members of the Editorial Board,
Dear Dr. Eissa,
Dear Dr. Kanayama,
Dear Dr. Roddam,
Dear Dr. Sier,

Thank you for your detailed review of the manuscript named above. I
appreciate your comments, ideas, and suggestions and I incorporated them in
the revision. I provided two documents. One document shows all corrections we
have made in marked color and the second document is plane to give a better overview. We have numbered all lines on each page in the second document and will refer in this letter to them.

Thank you very much for giving me the opportunity for resubmission of our manuscript to the Journal BMC Urology.

I would like to describe the changes we have made in regards to the reviewers’ comments.

Sincerely,

Dr. med. Andrea Staack
**Reviewer’s report 1: Dr. Sanaa Eissa**

**Comments**

1. The reviewer demands a better presentation of the results, e.g. with tables, graphs, and figures.

   a) Non-parametric analysis of MMPs, TIMPs, and MMP1/TIMP1 should be tabulated illustrating median values, mean ranks, range in two tables:
      1. Comparison of normal vs malignant groups
      2. Comparison of the different malignant subgroups (different stages and grades) to each other and the normal group

   We have changed Table I and present now the medians and ranges for all groups as also suggested by another reviewer (Dr. Roddam). Statistical differences among the groups (normal vs malignant; different malignant groups with different stages and grades) were calculated by the Mann-Whitney-test (Table I). However, we decided to summarize all results in one table for a better overview and comparison among the groups and gave explanations in the text: page 12, lines 9-18.

   b) Why were the cutoff values selected for the investigated parameters as 95th percentile value? Why were the cutoff values not calculated from the ROC curves, or not used?

   In regards to the reviewer’s suggestion we calculated the cutoff values at the point with the highest diagnostic accuracy of the ROC curves (Fig. 1, 2, Table IV). Additionally, we have used the cutoff values at the 90% and 95% limits of sensitivity and specificity, respectively (Table IV) and made calculations (see sensitivity and specificity data in Table IV.

   c) The reviewer requests a plotting of the ROC curves denoting the calculated cutoff points (best cutoff value which discriminates between non-malignant and malignant groups) and an identification of the area under the curve (AUC), confidence limits, and significance.

   We have added the figures, which show the ROC curves for MMPs, TIMPs, and the MMP1/TIMP1-complex for patients with bladder cancer (patients with non-metastasized and metastasized bladder cancer were combined) in comparison to the healthy control group (Fig. 1). The values with the highest diagnostic accuracy are symbolized by the black dots in each curve. In a newly created table (Table III) we also present AUCs with the confidence intervals and the
accordant significances as suggested (page 13-14).

d) The reviewer demands an analysis (Chi square test) of positivity rates of different investigated parameters (MMPs, TIMPs, and MMP1/TIMP1-complex) in different groups and in different malignant subgroups.

We analyzed the positivity rates of the investigated parameters in different groups based on the cutoff values with the highest diagnostic accuracy (minimal false-negative and false-positive results) for all analytes (see the first row of the respective analyte in Table IV). Based on these calculated cutoff values, test results were classified as either negative or positive. These dichotomous variables were analyzed using the chi-square test for the association of sensitivity in relation to stage and grade categories, non-metastasized and metastasized bladder cancer. Only the sensitivity of TIMP1 was associated with the tumour grading (P=0.038) as we expected from the correlation coefficients results as presented in table II. We included these results into the text (page 16, lines 6-14).

eye The reviewer asks for calculation of the ratio between MMPs/TIMPs and cutoff values, sensitivities and specificities (better than using absolute values).

We calculated the ratio of the two best single markers (MMP2, TIMP1) and we have made new calculations with the special mROC statistics program [Kramar A, Faraggi D, Fortune A, Reiser B. mROC: a computer program for combining tumour markers in predicting diseases states. Comput. Methods Programs Biomed. 2001;66:199-207]. Using that software we have analyzed all MMPs, TIMPs, and the MMP1/TIMP1-complex alone and in different combinations to determine the best markers and marker combinations by means of the AUC values. We calculated the best combinations of two and of three markers, since it is not the goal of this study and not practical relevant to include all seven MMPs and TIMPs for such a combination. In addition, there is also the potential risk of over-fitting. We therefore combined only the best two- and three-marker combinations as listed in tables III and IV only. The results show that the best single indicators with the highest sensitivity and specificity in combination among each other reach not necessarily the highest sensitivities and specificities. Our results present that different indicators in specific combinations could result in an improved sensitivity and specificity in comparison to the best single indicator (Table IV).

All these points are included into the text of our revised version at various places:
page 15, line 13-page 16, line 5;
page 20, line 3-page 21, line 16;
page 22, line 8-20.
2. Why did not the authors include a group with benign urological diseases as non-malignant control group?

Our control group consists of patients without any history of cancer, immunodeficiency diseases or inflammation because of evidence in the literature for interference with MMPs and TIMPs expression. We think it is essential for the value of this study to rule out any patients with suspicion of having an inflammation (page 9, line 5-8). However, to make the reader aware of the limitations of our study we have been mentioned them in the Discussion section (page 19, line 19).

<table>
<thead>
<tr>
<th>Spelling errors</th>
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<tbody>
<tr>
<td><em>The reviewer listed spelling errors, which have been changed in the text as mentioned.</em></td>
</tr>
<tr>
<td>1. Page 2</td>
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<tr>
<td>We changed the abstract completely.</td>
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<td>2. Page 7, line 14</td>
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<tr>
<td>I.V.-pyelography</td>
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<tr>
<td>3. Page 7, line 18</td>
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<td>G3, n=14</td>
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<td>4. Page 12, line 17</td>
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<tr>
<td>We deleted this.</td>
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<td>5. Page 12, line 16-18</td>
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<tr>
<td>Changes have been made:</td>
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<tr>
<td>Plasma concentrations of MMPs, TIMPs, and MTC1 except for TIMP2 were higher in the group of metastasized tumours compared with the patients of non-metastasized TCC (Table I).</td>
</tr>
</tbody>
</table>
**Reviewer' report 2: Dr. Hiro-omi Kanayama**

**Comments**

1. *The reviewer requests a better title.*

   We changed the title to:
   “Plasma MMP2 in combination with MMP9 and TIMP1 improves the non-invasive detection of transitional cell carcinoma of the bladder”

2. *The reviewer requests to add the ROC-curves in the Results.*

   The new figures (Fig. 1, 2) presenting the ROC curves are included in the Results section and also new tables (Tables III-IV).

   *The reviewer requests to show how MMP9, TIMP1, TIMP2 increase the diagnostic value, compared with MMP2 alone.*

   We have added table IV for a better presentation of the sensitivities and specificities of each MMP, TIMP, and the MTC1. The first value of each category resembles the cutoff point at the value with the highest accuracy. Please refer to Figure 1, where the ROC curves with individually marked cutoff points are presented. In addition to the diagnostic values of the single markers, we used the mROC program (Kramar et al., see reference number 29) to calculate the sensitivities and specificities for marker combinations. We could demonstrate that MMP2 in combination with MMP9 and TIMP1 gives the highest sensitivity and specificity than using those proteases by itself. We could also show that MMP2, MMP9, or TIMP1, which give the highest sensitivity and specificity in their combination, do not necessarily have a high sensitivity or specificity tested by itself (page 13, line 13-page 15, line 14).

3. *An expression change in the Abstract and Conclusion is required in regards to MMP2 as a statistically significant marker in blood plasma.*

   We have shown (please refer to Table I) that MMP2 is a statistically significant marker (p<0.001) in comparison between healthy controls and patients with non-metastasized bladder cancer. However, MMP2 does not reach a high sensitivity when tested alone. At the cutoff point with the highest diagnostic accuracy (please refer to Table IV) it reaches only 75% sensitivity and specificity than when tested in combination with MMP9 and TIMP1 (97% sensitivity, 94% specificity). Changes are made in the abstract and text (page 3, line 1-6; page 13, line 13-page 15, line 14; page
4. More explanation is needed in the Discussion on the relationship between the expression levels of MMP2, 9 and TIMP1, 2 in blood plasma from a viewpoint of tumour marker.

Changes are included in the text (Discussion page 20, line 17-page 21, line 7).
**Reviewer’ report 3: Dr. Andrew Roddam**

1. *It is suggested that the authors should chose either the 5th to 95th percentile or the IQR to display and be consistent for cases and controls.*

   We have changed Table I and present now only medians and ranges for all values.

2. *Why are tests for normality mentioned? Wouldn’t it be simpler to state that non-parametric tests were used throughout due to small sample size?*

   In regards to the reviewer’s suggestion we only included non-parametric tests (please refer to table I). We have added the remark in the text that we only used non-parametric tests (page 11, line 20-page 12, line 1).

3. *Which samples are paired, which are unpaired? Clarify the analysis section to describe statistical calculation (Mann Whitney U test/ Wilcoxon test?).*

   The reviewer is right since all samples were unpaired we only used the Mann-Whitney-U-test. We corrected this mistake (page 11, line 20).

4. *The reviewer asks for to include the correlations between the MMPs and their inhibitors prior to investigate the diagnostic accuracy.*

   We have included a new table, which presents the correlations between all analytes (Table II). Nevertheless, we also included the correlations for tumour stage and grade in the table for a better presentation and made few additional remarks in the text (page 12/13, lines 20-23/1-9).

5. *Display the logistic regression model in the paper.*

   Please refer to 7.); we eliminated the logistic regression model calculations as suggested by the reviewer and used the approach of the mROC program-test instead to combine markers. Changes have been made in the text: Page 14, line 1-12.

6. *Discuss the limitation in the discussion with reference to the analyses of diagnostic prediction. In an ideal situation you would split the data into two, estimate the cut-off in one half and test it in the other.*
We did not primarily split the data into a training and a test sample. However, we have changed the method to evaluate the data using the mROC approach of Kramar et al. instead of the logistic regression to calculate the improvement of diagnostic performance by the use of marker combinations. After that calculation we have been surprised about the high diagnostic performance data of the two- and three-marker combinations with MMP2, MMP9, and TIMP1 values (see Table III, IV), which made us verifying them. Therefore, we split the data of the normal and disease group (2/3 for training and 1/3 for test; randomization in the SPPS program): Using the equations for the training data calculated by the mROC program, we estimated comparable results (AUCs, sensitivities, specificities) for the test group as shown in Table III, IV and figure 2 without splitting the data. However, to avoid overemphasizing that point, we have included only one sentence about this additional calculation that confirmed the distinct improvement of diagnostic performance by the use of marker combinations (see page 14/15, lines 22-23/1-2).

7. The reviewer is concerned that the logistic regression analysis could be misleading especially if the markers are highly correlated as there could be a great deal of overfitting. He suggests either deleting the results or playing down their interpretation both in the summary of the paper and in the abstract. Or the author is able to clearly demonstrate that the results do not occur due to overfitting or excess internal correlations between the MMPs and their inhibitors.

As suggested we have eliminated the logistic regression analysis. As explained in the replies to the preceding remarks of the reviewer we have used the mROC program to find the best marker combinations.

Changes are made in the text and the abstract (page 3, line 14-18; page 13, lines 14-page 15, line 14).
Reviewer’s report 4: Dr. Cornelis Sier

- **General**

*Title-change is requested:*

We have changed the title:

“Plasma MMP2 in combination with MMP9 and TIMP1 improves the non-invasive detection of transitional cell carcinoma of the bladder”
Aim

Why did the authors decide to evaluate MMP1, 2, 3, 9, TIMP1, 2, but not for instance MMP7, 10, or 14, which are also important considering the literature?

The reviewer is right, in regards to the literature the MMPs 7, 10, and 14 are also important markers for bladder cancer. In our study we decided to focus on the analysis of MMP1, 2, 3, 9, TIMP1, 2, and the MTC1. Our laboratory has a special interest in those MMPs and TIMPs and we have gained a lot of experiences over the last time. We have focused in those MMPs and TIMPs in different cancer types, e.g. prostate cancer (Jung et al. International Journal of Cancer, 1997), in rheumatic arthritis (Keyszer et al., Z Rheumatol, 1998), and in renal cell carcinoma (Lein et al., Int J Cancer, 2000). Our group has developed a special curiosity in looking at the impact of those MMPs and TIMPs in patients with bladder cancer (page 6, line 9-16).

...for the MMPs/TIMPs that were selected for this study, ELISA’s are commercially available from EG/Amersham? The choice for the extra MMP1/TIMP1 complex ELISA is not explained, nor are the results discussed with respect to the single ELISAs for MMP1 and TIMP1.

It is correct that we used only commercially available tests as indicated in the Method section since we have good experiences with these tests (see above) that allow long-term reliable measurements. The analysis of all concentrations of MMP1, 2, 3, 9, TIMP1, 2, and the MMP1/TIMP1-complex in blood plasma was performed by the commercially available sandwich-ELISA test-kit (Amersham International, Little Chalford, England). We have gained promising preliminary results with those tests including the MMP1/TIMP1-complex as mentioned above and were interested in performing further analyses with them.

For EARLY diagnosis of TCC are not a lot of patients in this study. The extra and valuable information from advanced and metastasized stages has not been discussed sufficiently.

The reviewer is right, the goal of this study is not the evaluation of MMPs for EARLY diagnosis of TCC of the bladder since we also included patients with metastasized bladder cancer in this study. The goal is to find a non-invasive tumour marker (like in blood) for initial detection and follow-up and explain the advantages of blood plasma in comparison to blood serum. Rigorous changes have been made in the text (page 18, line 14-page 21, line 7).

Since our study group for patients with metastasized bladder cancer is limited (n=11), we decided to combine both groups, non-metastasized and metastasized bladder cancers, for the final evaluation. We are not able to draw
a conclusion on a special impact of the MMPs, TIMPs, or the MTC1 in more advanced or metastasized bladder cancers. Only TIMP1 reached statistical significance in comparison of non-metastasized with metastasized bladder cancer (p=0.012).

The presentation of the data in a single table is not effective.

We included further tables (Tables II-IV) and figures (Figures 1 and 2) for better presentation of data.

The discussion is superficial with little indications of the impact of the study.

The Discussion chapter has been revised. All additional information become further discussed in the Discussion (page 16-page 21).

- Major compulsory revision

1. Although TCC is found primarily in males, as is the case in this cohort (45 vs 12), the patients and controls group are NOT matched for gender and age in this study. A sentence like 'There was also no statistical difference in age or sex between the healthy volunteers and the patients with TCC' is not enough to validate the approach. Especially not when a simple X2-test for gender shows that these groups are by no means comparable (P<0.001)! The fact that there were apparently no significant differences for all 7 MMP-related parameters between female and male controls (P<0.05 ?) makes an impression of statistical coincidence and could easily have been prevented by a presentation in a more controllable way.

The reviewer is right, this statement is misleading in regards to the age. Changes have been made (page 12, line 2-8).

2. Anyway, the way the results are presented, in a table with the controls as means plus 5th and 95th percentiles and the patient groups as median values with min./max., is uncommon and makes it impossible for the reader to compare the groups for him/her self.

We have changed table I to present medians and ranges for all groups. We summarized all results in one table for a better overview, consistence, and comparison among the groups and included additional tables for a better explanation of the study.
3. It would be nice to compare this study to another, more or less established marker to estimate the potential for its performance. A more clinical relevant conclusion is sought for ‘a new clinical marker’-study.

We focused on the diagnostic value of different MMPs, TIMPs or the MTC1 alone and in combination with each other. In the revision we changed the Discussion and discussed this different aspect more thoroughly. To our knowledge there are no studies in the literature, which combine those different MMPs and TIMPs for an improved diagnostic statement in regards to the sensitivity and specificity in bladder cancer (page 18, line 14-page 21, line 7).

Is the plasma MMP2 determination indeed an easy and economical option for detection of early TCC for the future? Or are additional (MMP) measurements always necessary?

In this study we have shown that MMP2 tested alone did not reveal the same results for sensitivity and specificity than tested in a two-marker and three-marker combination. This marker combination in plasma is a non-invasive option for diagnosis of TCC with a promising sensitivity and specificity (page 20, line 17-page 21, line 7).

There should have been at least some discussion about the relevance of the use of MMPs, next to their inhibitors, and the comparison with the complexes.

We included in more detail the relevance of the use of MMPs and their inhibitors in the discussion (p. 19). We concluded that the two- and three-marker combinations have a higher sensitivity and specificity than testing the individual MMPs.

4. What is the explanation for the relatively high MMP1 and TIMP1 levels in metastasized patients?

In the literature several studies show an increased TIMP1 level in tumours of different organs: Baker et al. (Br J Surg, 2000) showed with the ELISA technique that TIMP-1 was significantly greater in tumour tissue than in normal colon. 

Increased expression of TIMP1 in renal cell carcinoma (RCC) correlates with poor prognostic variable including shortened patient survival. The paradoxical poor prognostic implication of TIMP overexpression documented in the literature complements the dual function of TIMPs and warrants further investigation (Kallakury et al., Clin Cancer Research, 2001).
We included this discussion in the manuscript (page 17, line 14-page 18, line 6).

In regards to the high level of MMP1 mentioned by the reviewer; we find that MMP1 is not relevantly elevated in our study. We could not find a statistical significant difference for MMP1 between the controls and the metastasized bladder cancer group (p=0.208) or between the non-metastasized bladder cancer group and the metastasized group (p=0.163). Only MMP2 revealed significantly elevated values between the controls and the metastasized bladder cancer group (p<0.001).

• **The reviewer listed minor essential revisions.**

  1. *What was actually the dilution of the plasma for MMP3?*

  The probes were diluted with component-specific assay buffers: MMP1 1:5, MMP2 1:51, MMP3 1:1, MMP9 1:11, TIMP1 1:101, TIMP2 1:4, MMP1/TIMP1-complex 1:11. We have added corresponding remarks in the Method section (page 10, lines 5-7).

  2. *What is the median MMP9 level for G3 patients?*

  The median MMP9 level is 22.9 µg/l for the bladder cancer group without metastasis and 17.7 µg/l for the group with metastasis as shown in Table I.

  3. *Which were the paired samples, as mentioned in the M&M section (Statistical analyses)?*

  A correction has been made in the text (p. 11, line 20-page 12, line 7). The Mann-Whitney test was used for the unpaired samples.

  4. *Reference the specific IFCC guidelines, which are not in the cited two references.*

  Since we used the cutoff values from the ROC curves we are now not using the reference intervals anymore. Those references (the first reference explains the theory of using reference values and the second one describes the procedure to calculate the reference intervals) became deleted in the text.

  5. *The conclusions are drawn out of the group of 57 non-metastasized patients. State more clearly.*
The conclusions are drawn out of the total group of 68 patients suffering from TCC of the bladder with and without metastasis. We combined the group with non-metastasized and with metastasized bladder cancer.