Author's response to reviews

Title: Circulating cell death products predict clinical outcome of colorectal cancer patients

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Author's response to reviews: see over
Dear Diana Marshall, PhD
Assistant Editor BMC Cancer,

Please find enclosed the revised version of our manuscript (MS: 4464876262428287) entitled “Circulating products of cell death predict clinical outcome of colorectal cancer patients” by Koelink et al. The manuscript has been revised according to the comments and suggestions made by you and the reviewers. A detailed response to the comments made is given below.

**Reviewer 1: Hendrik Jan Ankersmit  Reviewer’s report:** Major Compulsory Revision.

The aim of this study was to describe the impact of circulating death products on predicting clinical outcome in colorectal patients. This paper was very interesting to read, however, I have one major reservation:

**Comment 1:** The authors must provide information about pre- or post-operative chemo-schemata. Since circulating death products are increased (as stated correctly in the discussion section) during chemotherapy, neo-adjuvant treatment will influence M30 levels. If M30 levels are independent of chemotherapy administered then indeed M30/CK18 can be seen as prognostic parameters. If not, measured parameters are most likely epi-phenomena due to more aggressive treatment strategies in advanced tumor stages (Duke C/D).

*Reply:* We included post-operative treatment details of the patients (page 10) as asked by reviewer 1 (Hendrik Jan Ankersmit) and added a file (additional file 1) which shows that post-operative plasma CK18-Asp396, total CK18 and CK18-Asp396/CK18 ratios are not related to the treatment the patients received. None of the patients received pre-operative therapy (page 6). These results are also mentioned/discussed in the discussion (page 13/14).

**Comment 2:** Minor Essential Revision page 5: spelling of "assessed" is incorrect.

*Reply:* The spelling of “assessed” is corrected (page 5).

**Reviewer 2: Stig Linder  Reviewer’s report:** Major Compulsory Revision.

This is a well conducted study addressing the possible use of serum caspase-cleaved cytokeratin/total cytokeratin as a prognostic biomarker for colon carcinoma. A weakness of the study is the relatively small size of the patient material (49 patients).

**Comment 1:** The paper describes the usefulness of calculating CK18-Asp396/CK18 ratios. This is potentially a very interesting concept, but the underlying biology is complex. On page 8 it is stated that a ratio of 0.20 means “20% apoptosis” and “80% necrosis”. This is not correct. CK18 is not always cleaved to completion during apoptosis, and ratios of 0.20 can be observed after treatment of a cancer cell line with an apoptotic agent (differs between cell lines). The CK18-Asp396/CK18 ratio can be used to determine whether a particular compound induces apoptosis/necrosis in vitro using standard compounds as a
reference, but it is not possible to say whether a 0.20 ratio means pure apoptosis or a mix between apoptosis and necrosis. However, and importantly, the decrease in the ratio observed in Fig. 2 is very interesting (and does indicate an increasing necrotic component during progression), and the possible prognostic information is also very interesting. The writing of the paper needs to be modified to clarify this issue.

Reply 1: The meaning of CK18-Asp396/CK18 in relation to apoptosis and necrosis has been corrected by stating that if the ratio decreases this indicates more necrosis over apoptosis (page 9).

Minor Essential Revisions

Comment 2: On page 11 it is written that "the CK18 ELISA also recognizes the M30 antigen". I understand what the authors mean, but the statement is not correct. The term "M30 antigen" is usually used to describe the CK18-Asp396 neo-epitope created by caspase cleavage. This is not recognized by the M65 ELISA. However, the M65 ELISA recognizes the soluble CK18 fragments that are detected in the M30-Apoptosense ELISA (as well as other soluble CK18 C-terminal fragments that are not caspase-cleaved).

Reply 2: We altered the statement that the CK18 ELISA also detects the M30 antigen (page 12) to: because the CK18 ELISA recognizes the soluble fragments of CK18 that are detected in the M30 ELISA, as well as other soluble non-caspase cleaved CK18 fragments.

Comment 3: Different authors have used different nomenclatures to described the CK18 protein complexes detected in the M30 and M65 ELISAs. One possibility is to use "CK18-Asp396" and "total CK18", or "M30 antigen". These designations are not completely correct (the molecules detected are complexes that need to expose to distinct epitopes, for the M30 Apoptosense both the caspase-cleaved epitope and an epitope in the 300-330 region). In this manuscript the term "M30 levels" is often used, which is not good. "M30" is an antibody; this should be changed through-out.

Reply 3: We have changed M30 levels to CK18-Asp396 levels throughout the manuscript, as suggested by the reviewer.

Comments made by the Editorial Office.

Comment 1: In addition to the comments raised by the reviewers, please also discuss limitations to the power of the study, given that the cohort is only 49 patients.

Reply: We included in the discussion on page 15: In order to be conclusive, however, these interesting preliminary observations, due to the limited power of our study with only 49 colorectal cancer patients, merit further evaluation in larger patient groups. This was also added to the general conclusions on page 15: which should be confirmed in larger prospective studies.

Comment 2: We also need you to clarify ethical approval for the study. Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Research carried out on humans must be in compliance with the Helsinki Declaration. A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval, with a reference number where appropriate.
Reply: The study was approved by the MEC of our hospital. We added to the M&M section: The study was performed according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Centre in compliance with the Helsinki Declaration, on page 6.

Comment 3: Please also ensure that your revised manuscript conforms to the journal style. It is important that your files are correctly formatted.

Reply: The manuscript has been revised conform the journal style.

On behalf of all co-authors,

Yours sincerely,

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