Author’s response to reviews

Title: Eosinophil-cationic protein - a novel liquid prognostic biomarker in melanoma

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Dear Dr. Gummlich,

Thank you for reviewing our manuscript and your interest in our work on ECP serum levels in melanoma patients and their association with prognosis. We really appreciate your thoughtful comments and think that they have substantially improved the manuscript.

Enclosed we submit our revised manuscript addressing the concerns of the editors of BMC Cancer point-by-point:

Reviewer reports:

Ernest Ramsay Camp (Reviewer 1): The authors make an interesting association between ECP and melanoma. However, more details are need.
1. Cutaneous and mucosal/uveal melanomas should be analyzed separately as the biology is very different.

We agree with the reviewer on the appropriateness of studying cutaneous and mucosal/uveal melanoma separately. We have inserted the data in the Methods section:

“Due to biological differences between mucosal/uveal and cutaneous melanoma, overall survival (OS) of patients with cutaneous melanoma was analysed separately. Melanoma of unknown primary (MUP) was subsumed under cutaneous melanoma.”

“Event-time distributions were estimated with the Kaplan-Meier method. Log–Rank (Mantel–Cox) test was performed to determine the p-value. […] Graphing was created using GraphPad Prism and R.”

Results:

“In a subsequent analysis the subgroup of uveal/mucosal melanoma with their different biology was excluded. The remaining patients with cutaneous melanoma (n = 45) were analysed separately. Using a cut-off of 16.0 ng/ml, patients with elevated ECP levels (n = 24) had a median OS of 12 months, compared with 28 months for patients below this threshold (n = 21; p = 0.0597; Fig. 2A). Dichotomizing at 12.2 ng/ml, patients with lower ECP levels (n = 16) showed a statistically significant longer OS (median OS not reached) than patients with higher ECP levels (median OS 12 months; n = 29; p = 0.0393; Fig. 2B). Uveal and mucosal melanoma patients were not analysed separately due to the small number of subjects, with 4 and 7 cases, respectively.”

Discussion:

“Remarkably, survival difference was even more distinct in the subgroup of patients with cutaneous melanoma or MUP, excluding uveal and mucosal melanoma with their different biology. Furthermore, nearly all patients with uveal and mucosal melanoma showed elevated ECP levels.”
The groups of patients with uveal and mucosal melanoma were too small to come to a meaningful conclusion. However, the majority showed high ECP levels which we have added in the Results section:

“Interestingly, 3 out of 4 uveal melanoma patients and all mucosal melanoma patients (7/7) were ECP high (cut-off: 16.0 ng/ml).”

2. Detailed treatments should be noted in the first table and analyzed.

We have added detailed information about treatments and analysed differences in Table 1 (see Table 1).

An analysis of treatments has been incorporated into the revised version of the manuscript in the Methods section:

“The patient cohort incorporated patients independently of their subsequent therapy.”

Results:

“Subgroups of ECP low and ECP high were balanced with respect to age and gender. Treatments of the two cohorts were comparable (radiotherapy, surgery, interferon-alpha) whereas differences were found in the frequency of chemotherapy, immunotherapy and signal transduction therapy (Table 1).”

Discussion:

“Importantly, all ECP values were measured at diagnosis of stage IV metastatic disease. It is unclear why the ECP low group was subsequently treated with immunotherapy more often. They were however treated less often with targeted therapy.”
3. Univariate and multi-variable analysis should be performed based on ECP levels to understand potentially confounding variables.

We agree with the reviewer on the appropriateness of univariate and multi-variable analysis based on ECP. In order to identify potentially confounding variables, Cox-proportional hazard models were performed using R. We have added the data into the revised version of our manuscript into the Methods section:

“Univariate and multivariate analysis were performed with Cox proportional hazard models. Graphing was created using GraphPad Prism and R.”

Results:

“In order to identify potentially confounding variables, Cox proportional hazard models were used for univariate and multivariate analysis. Age and gender had no influence on effect (Table 2). High ECP (cut-off: 16 ng/ml) increases the risk for a death, whereas targeted therapy as well as immunotherapy decrease this risk. The effect size of the influence of high ECP on survival is diminished by subsequent treatment with targeted or immunotherapy, but remains above 1 across all models and thus represents a marker for a decrease of OS.”

Discussion:

“Both, age and gender did not influence ECP’s effect size on OS.”

“Targeted as well as immunotherapy, as life prolonging therapies, diminish ECP’s effect size on OS, but high ECP levels still increase the risk of death when adjusting for those variables. Possibly, there is a latent variable that is connected to both, ECP and therapy, which has to be further investigated.”
Mary E. Aronow, MD (Reviewer 2): The authors investigate eosinophil-cationic protein (ECP) as a prognostic biomarker in metastatic melanoma. The work is interesting and the manuscript is well written. Several areas should be addressed.

1. It is difficult to know what to make of the results as there is no multivariate analysis here. There are basically 2 groups (high ECP and low ECP) but we do not know about other potential contributing variables, including whether one group underwent a substantially different therapy, or one group had more advanced disease to start with, etc.

We agree with the reviewer on the importance of a multivariate analysis. In order to identify potentially confounding variables, Cox-proportional hazard models were performed using R. We have added the data into the revised version of our manuscript into the Methods, Results and Discussion section (see above).

We had stated in the Methods section that

“Sera from the time at initial diagnosis of metastatic melanoma were taken defining the initial diagnosis as 0-6 months from the date of stage IV diagnosis.”

Furthermore, we have revised Table 1, which now compares the two subgroups of our cohort, patients with high ECP on the one hand and patients with low ECP on the other, including brain metastases (M1d) as a variable (see Table 1).

2. A cut-off value of 16 ng/ml for serum ECP is used. The 2 groups are then dichotomized at 10 ng/ml. How did the authors arrive at these cut-off values? Generally, there is statistical analysis that goes into determining cut-off values.

We have used 16 ng/ml as cut-off of since it is the normal range for 95% of individuals and had stated and referenced the explanation in the Methods section:
“Serum levels of at least 16.0 ng/ml were defined as elevated, because healthy individuals have a 95% range from 2.3-15.9 ng/ml in the serum [28].”

To further clarify we have added this information in the Results section:

“A cut-off value of 16.0 ng/ml was used since 95% of healthy individuals have ECP values in the range from 2.3-15.9 ng/ml in the serum [28].”

We statistically determined a further cut-off value for comparison. For traditional ROC analysis a dichotomous outcome is needed. The outcome of our study however, is the survival time per patient, which is continuous. So we have decided to perform recursive partitioning (tree-based procedure) and added the results into the Methods section:

“Additionally, a cut-off of 12.2 ng/ml, as determined by recursive partitioning, was analysed and correlated with survival.”

“Cut-off values were determined with recursive partitioning.”

We have re-analysed the overall survival of our cohort based on both cut-off values, 16.0 ng/ml as well as 12.2 ng/ml. The data have been inserted into the Results section:

“With a cut-off at 16.0 ng/ml serum ECP, the median OS for patients with ECP levels of at least 16.0 ng/ml (n = 34) was 12 months, compared with 28 months for patients with levels below this threshold (n = 22; p = 0.0916; Fig. 1A).”

“Dichotomizing at 12.2 ng/ml, patients with higher serum levels (n = 39) had a median OS of 12 months, compared with 28 months for patients below this threshold (n = 17; p = 0.0642; Fig. 1B).”
3. Page 7, It is stated that patients who received to immunotherapy were compared to those who did not, and no difference was found. Then it is stated that these data were not shown. Why not?

We agree with the reviewer on the relevance of a separate analysis of immunotherapy patients which we are going to address in a further manuscript. Therefore, we deleted this statement.

4. Table 1. Rather than show the overall characteristics of the group, there should be a comparison table of high ECP and low ECP and the demographic/clinical characteristics of each group, with p-values included. We want to see if the groups were similar in terms of age, gender, treatment, etc.

We have revised Table 1. Instead of showing the overall characteristics of our cohort, the table now compares two groups of patients with high ECP on the one hand and patients with low ECP on the other, with p-values included (see Table 1). Information about how we arrived at these p-values has been added to the Methods section:

“The patient characteristics are depicted in Table 1, with p-values included.”

“Log–Rank (Mantel–Cox) test was performed to determine the p-value. […] For contingency analyses Chi-square test and Fisher’s exact test were utilised. Mann-Whitney test was used for nonparametric tests.“

5. How do the authors explain the unexpected result- high ECP correlates with poorer overall survival?

We had stated in the Background section that

“[…] ECP might […] promote tumour infiltration through muscle fiber corrosion [7].” and “[…] in vitro studies showed that ECP inhibits immune functions such as the production of immunoglobulins as well as T cell proliferation [7,27].”
We have further elaborated that

“According to in vitro experiments, ECP was involved in degradation of membrane-associated cytoskeletal proteins and myofibrillar proteins, such as myosin heavy chain as well as α-actin [7].”

In the Discussion section we have added the following

“Given this unexpected finding of correlation of ECP high with poorer survival further studies in in vitro models and patient cohorts are required to characterize the role of ECP.”

Manoj Kumar Pandey, Ph.D. (Reviewer 3): Study entitled "Eosinophil-cationic protein- a novel liquid prognostic biomarker in melanoma" by Drs. Krickel et al, estimated the levels of ECP in serum of 56 patients and correlated with the OS. The study may contribute significantly to literature regarding the prognostic significance of ECP in melanoma. Overall, study was performed in optimal number of patients and manuscript is well written. However needs minor modification.

Authors should elaborate more about ECP in Introduction or Discussion section, with emphasis on the outcome of studies.
Additional information about ECP has been incorporated into the revised version of the manuscript in the Background section:

“It was suggested that ECP might, aside from harming various microorganisms [7,12,22,23], also have cytotoxic activity against cancer cells, such as Hodgkin lymphoma and colorectal tumor cells [7,12,24-26], however, its definite role in human cancer is yet to be investigated. In studies on oral squamous carcinoma cell lines, ECP did not just limit cell survival, but induced morphological transformation, including vacuolation, formation of blebs and disabled cell adhesion [24]. On the contrary, ECP was suggested to promote tumour infiltration through muscle fiber corrosion [7]. According to in vitro experiments, ECP was involved in degradation of membrane-associated cytoskeletal proteins and myofibrillar proteins, such as myosin heavy chain as well as α-actin [7]. Moreover, in vitro studies showed that ECP inhibits immune functions such as the production of immunoglobulins as well as T cell proliferation [7,27].“

We hope that the revised and substantially improved version of our manuscript will meet with your approval.

Sincerely,

Lucie Heinzerling, MD, PhD, MPH