Author's response to reviews

Title: Complement activation in astrocytomas: deposition of C4d and patient outcome.

Authors:

  Katri S Mäkelä (katri.s.makela@uta.fi)
  Pauli Helén (pauli.helen@pshp.fi)
  Hannu K Haapasalo (hannu.haapasalo@fimlab.fi)
  Timo Paavonen (timo.paavonen@uta.fi)

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Author's response to reviews: see over
Dear editor,

Here are point-by-point responses to the questions and concerns that the referees have presented. The corrections made in the manuscript have been highlighted in yellow color. We gratefully thank for the attention that the manuscript has received so far. We would also like to thank the referees for revising the manuscript and for the valuable comments they have given to improve the quality of the manuscript.

Sincerely,

Katri Mäkelä, corresponding author

Point-by-point response to the concerns of referee 1

1. Were the recurrent gliomas previously exposed to adjuvant radiochemotherapy, which might alter their C4d expression? It seems that analysis of primary and recurrent tumors should also be split apart, to get a clearer picture of what primary C4d expression patterns are like.

In the period of the study (until 2001) adjuvant radiochemotherapy including temodal was not used in our hospital. As presented in the manuscript, most grade III-IV patients received then only postoperative radiotherapy. We can’t exclude that radiotherapy might alter the C4d expression, but we think that most probably the progression of the tumors to higher grades is the reason for the pattern of C4d expression.

Added to M&M (page 5, paragraph 2, row 3): Adjuvant radiochemotherapy was not used in the period of the study.

In statistical analysis, when the correlations were tested, primary and recurrent tumors were tested separately. As presented in the results, there were more C4d positive cases in recurrent tumors than in primary resections. In the appearance of C4d no other significant differences between recurrent tumors and primary resections were found. This information has also been added to the manuscript text. Added to results (page 9, paragraph 4, line 4): Comparing the other C4d variables between primary and recurrent tumors, no statistically significant differences were found.
2. While increased C4d might correlate with worse outcome, it is not clear that it could be an independent prognosticator since multivariate analysis was not done, taking into account WHO grade. Thus, the significance of Figure 5, and its related conclusions in the Discussion, are diminished.

We agree with the referee that WHO grade would be more important prognosticator than C4d in the multivariate analysis if that could be performed. In most studies of diffusely infiltrating astrocytomas only histological grade and patient age appear as independent prognostic factors. However, we think that our material of 67 primary grade II-IV astrocytomas is too small for the reliable multivariate analysis and therefore we used only univariate survival analysis. When we tested the prognostic value of WHO grade in the material of the present study by univariate analysis, WHO grade was a very significant prognosticator (\( p < 0.001, \log\text{-rank test} \)). Thus WHO grade seems to have more prognostic power than C4d also in our material. Added to results (page 10, paragraph 1, line 7): WHO grade was the most significant prognosticator in the grade II-IV astrocytomas of the present study (\( p < 0.001, \log\text{-rank test} \)).

3. Much of the Discussion is highly speculative, drawing causative conclusions from their correlative data. For example, “diffusely infiltrating astrocytomas form probably more inflammatory mediators in a particular period of time, since they have faster proliferation and cell death rates” should be deleted, since it is purely speculative and cannot be supported by the data. Many slow-growing brain tumors, including PAs, can also elicit brisk inflammatory responses. Likewise, “Thus the pathological microenvironment that inflammation provides is the most likely aspect to explain the survival study results, which showed that a worsened outcome is associated with intensified complement activation in diffusely infiltrating astrocytomas” is not proven by this correlative study, especially since no multivariate analyses were done to adjust for WHO grade. “C4d positivity in diffusely infiltrating astrocytomas could predict the recurrence after resection and also the malignant progression of these tumors” is also not justified by the data. All these things might be true, but such unequivocal conclusions as are presented in the Discussion need to be toned down.

To tone down the discussion all the sentences criticized by the referee have been deleted. Also the paragraphs, in which these sentences have originally appeared, have been toned down. Also according to the concerns of the other referee, the discussion part has been shortened and edited to be more focused.

4. Along these lines, have the authors considered the possibility that C4d accumulation is merely a secondary event to other better-characterized phenomena, such as robust microvascular proliferation causing poorly-formed, leaky blood vessels that might elicit some inflammatory changes? For example, are the authors aware that microvascular proliferation is a feature of both grade I PAs and grade IV GBMs, which is why both tumors routinely show contrast enhancement on MRI?
The authors are aware that the microvascular proliferation is a feature of both grade I and grade IV astrocytomas. We would like to thank the referee for this reminder. This issue has been now added to the manuscript as the fact has been presented in the background section and also considered in the discussion. 

**Added to background** (page 3, paragraph 1, line 11): Glioblastomas have a vivid microvascular proliferation rate and this results often in abnormal, even glomeruloid microvascular growth patterns. This can sometimes be seen in pilocytic astrocytomas, as well. **Added to discussion** (page 10, paragraph 3, line 1): Vivid microvascular proliferation can be seen in pilocytic astrocytomas and glioblastomas [1]. One simple reason to the high C4d expression in these tumors could be that the abnormally proliferative, leaky blood vessels elicit inflammatory changes resulting in complement activation.

**Minor essential revisions:**

1. **Cutoffs for C4d in the glioma cells seem to be rather low. Is this typical for C4d expression in other cancers and tissue types?**

In the articles of our references, the study materials have been presented as either negative or positive for C4d. In these studies the positivity has not been measured quantitatively and no cutoff points have been used.

2. **How can the authors be certain that “moderate diffuse cytoplasmic” staining (e.g. figure 1) isn’t just nonspecific background staining?**

We had samples of normal, non-neoplastic brain tissue to serve as negative controls. The negative controls were stained simultaneously with the study material by an automated immunostainer. There is no background staining in the negative controls and therefore the staining of the astrocytomas can be considered truly positive. **Added to M&M** (page 5, paragraph 1, line 10): Also samples representing normal, non-neoplastic brain were included into blocks to serve as controls.

3. **In Figure 4, use Roman numerals when referring to WHO grade (i.e. I-IV not 1-4).**

This has been corrected to the Figure.

4. **The authors should cite Gousias et al. (J Neuroimmunol. 2010 Sep 14;226(1-2):136-42), as this group showed reduced serum C4 in patients with glioma and an inverse correlation between serum C4 and glioma grade. Perhaps this is because all the complement is being “consumed” by hypervascularized gliomas?**

**Added to discussion** (page 11, paragraph 1, line 1): Furthermore, the extensive activation of the complement in abnormal blood vessels may consume the serum C4 component and this way explain the study finding of
Gousias et al., in which they showed a reduced serum C4 in patients with grade II-IV glioma and an inverse correlation between serum C4 and glioma grade [10].

Point-by-point response to the concerns of referee 2

ABSTRACT.

(1) Is there any previous study which described the significance of C4d expression as a potential prognostic marker in patients with high-grade astrocytomas,

Not to our knowledge.

(2) Of the 93 cases of grades II-IV astrocytomas, 26 consisted of recurrent which was not all mentioned in this section. Instead, it was revealed in the materials & methods section. Presenting the data in such a manner resulted in a serious flaw, and

*Added to abstract* (line 5): The material consisted of 9 pilocytic astrocytomas and 93 grade II-IV astrocytomas, of which 67 were primary resections and 26 recurrent tumors.

(3) Missing from the text are the defining criteria of determining the intensity or extent of immunohistochemical(IHC)staining. This section should be self-explanatory, without having to turn to the method section to find them.

*Added to abstract* (line 6): The intensity of C4d staining as well as extent of C4d and CD34 staining were evaluated. The intensity of C4d staining was scored semiquantitatively. The extent of the staining was counted morphometrically with a point counting grid yielding a percent of C4d and CD34 positive area of the sample.

BACKGROUND.

(1) The text in this section is unfocussed and poorly written. For example, paragraphs 01 to 03 could be condensed, focusing on to the aim of the study. Again, text in the last paragraph which begins with the current study quickly morphed back into what reads much like an introduction again.

The background has been rearranged and partly rewritten to make it more focused and shorter. According to the comments of the referee, the first two paragraphs have been condensed into one. Also the rest of the text is more focused and the aim of the study is presented more clearly.
RESULTS.

(1) The percentage of C4d-positive tumor lesions was significantly higher in pilocytic tumor than those of the high-grade tumors. To compare their findings, they have not provided any data of C4d-positive and/or CD34-positive cells in morphological normal area, away from the tumor lesions. Moreover, they have not included any normal (uninvolved) brain tissue as control in their study. In the absence of such data, comparison of C4d-positive and/or CD 34-positive non-tumor versus tumor microenvironment-associated cells can not be made to understand the biological significance of the finding.

There were samples of normal brain tissue in the multitissue blocks of the study material. The samples of the normal brain tissue were noted to be C4d negative. They can also be seen as controls since they have been immunostained in the same batch with samples of astrocytic tumor tissue used as study material. We understand the concern of the referee, and this issue has now been added to the manuscript text. Added to M&M (page 5, paragraph 1, line 10): Also samples representing normal, non-neoplastic brain were included into blocks to serve as controls. Added to results (page 8, paragraph 3, line 3): C4d negative normal brain tissue is demonstrated in Figure 4.

(2) The background staining in Figures 1 and 2 is well beyond what is considered acceptable.

We have added a new Figure (Figure 4) demonstrating normal brain tissue. The tissue sample, which is photographed in this Figure, has been immunostained with the Ventana BenchMark LT Automated IHC Stainer in the same batch as the tissue samples photographed in Figures 1 and 2. Using an automated device in immunostaining the staining should be of uniform quality. Taking into account the absence of background staining of normal brain in the new Figure, we consider the immunostaining in Figures 1 and 2 to be true C4d positive staining. Added to Figure legends (page 16, paragraph 4): Fig 4. Normal brain tissue which served as a control in C4d staining. Brain tissue and endothelium in the capillary are C4d negative. Magnification x 200.

DISCUSSION.

(1) Again, as in the background section, this section is unnecessarily long and unfocused,

The discussion has been shortened and also toned down according to the comments and instructions of the other referee.

(2) In summary, a major revision with IHC data on normal brain tissue may improve the quality of the manuscript.

As explained above, this issue has been now included to the manuscript.