Author's response to reviews

Title: Evaluation of CXCL9 and CXCL10 as circulating biomarkers of human cardiac allograft rejection.

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Version: 2 Date: 3 May 2006

Author's response to reviews: see over
Reviewer's report
Title: A search for potential biomarkers of cardiac allograft rejection using expression profiling of human endomyocardial biopsies
Version: 1 Date: 3 April 2006
Reviewer: Luciano Adorini
Reviewer's report:
General
This study searches for potential biomarkers of acute heart rejection. Three candidate biomarkers, identified by expression profiling are analyzed in detail, but none is representative of changes detectable in the serum. Some points in this study should be addressed further:

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)
1. Serum chemokine levels have been determined in 10 patients only. A more extensive analysis could have lead to significant differences. It would be important to extend the number patients tested.

Answer: The reviewer's remark is valid and we agree that the study has low power due to a small sample size. However, considering the large inter- and intra-individual variation observed in levels of CXCL9 and 10 before, during and after rejection (figure 3 revised) we feel quite confident that a larger sample size would not affect the present results. In our opinion, an optimal circulating biomarker should display homogeneous responses during rejection and show low intra-individual variability. None of the studied biomarkers fulfilled these criteria. If the reviewer still feels that a larger sample size would be informative, we would be willing to consider further analysis.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
2. It would be more informative to show individual values in Fig. 3A and B.

Answer: This is a very good comment. The individual values of the serum analysis clearly show the heterogeneity of CXCL9 and 10 serum levels and support our conclusion that CXCL9 and CXCL10 are unlikely to be useful as biomarkers of cardiac rejection.

In the revised version of the manuscript we have chosen to present the data as a line graph on a logarithmic scale since we feel that such a figure explains the data in the best way (figure 3 revised).

3. The discussion should address possible reasons for the failure to confirm data published in ref.16.

Answer: Our finding, with respect to myocardial up-regulation of CXCL9 during rejection, is in line with observations reported by Zhao et al (previous ref 16) In contrast to Zhao et al, activation of CXCL10 in our study did not reach statistical significance, which, is likely due to a smaller sample size (type II statistical error). Zhao et al came to the conclusion that chemokines could serve as useful biomarkers of the cardiac rejection process based on the analysis of myocardial transcription data. Since, in our opinion, a useful biomarker of the rejection process should be
A section discussing these issues has been included in the revised manuscript (page X, section y).

4. The lack of correlation with CRP levels could be shown.
Answer: This has been included in the article as revised figure 4 B and C

Reviewer’s report
Title: A search for potential biomarkers of cardiac allograft rejection using expression profiling of human endomyocardial biopsies
Version: 1 Date: 6 April 2006
Reviewer: Marja Steenman
Reviewer’s report:
General
The goal of the study by Karason et al. was to find serum biomarkers for diagnosis of cardiac allograft rejection. They used Affymetrix DNA microarrays to look for genes differentially expressed in cardiac biopsy material during rejection in 3 patients. They identified a list of 16 genes with an interesting gene expression profile and selected 2 genes for analysis by ELISA in blood samples of a larger group of patients. Since these 2 genes had already been described in the literature as being preferentially expressed in cardiac allografts during rejection, an analysis of the literature would have provided the same result as the microarray analysis. The subject of the manuscript is important and interesting, even the negative result. However, in my opinion the data have not been explored to their full potential, and positive results can not be excluded at this point.
Answer to the general comments: Thank you for an insightful review of our manuscript, which has helped us improve the manuscript. As you state a literature search would have aimed us at investigating CXCL9 and 10 as biomarkers.
However, our microarray experiment was designed as an unbiased approach to search for circulation biomarkers of cardiac rejection. We could not prior to the microarray experiment know that the analysis would point us to CXCL9 and 10 as the most promising candidates. We have been working with the Affymetrix microarray system since year 2000 and performed over 40 projects using human samples. In none of these projects we have observed an upregulation of the same magnitude as CXCL9 and CXCL10 displayed during rejection. Therefore these genes were our most promising candidates.
More in depth analysis of the microarray data than the clustering have been preformed (using a combination of the Affymetrix change call algorithm and a fold change criteria). However, also these analyses indicate that CXCL9 and 10 were the

non-invasive we developed the concept further by analyzing serum levels of CXCL9 and 10 (which to our knowledge has not been performed previously). The heterogenicity of CXCL9 and 10 serum levels led us, therefore to the opposite conclusion that these chemokines are not optimal biomarkers of cardiac rejection.
most promising candidates. ----------------------------------------

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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

1. The main message of the authors is that CXCL9 and CXCL10 are not serum biomarkers for cardiac allograft rejection. This is not at all reflected by the title, therefore the title should be changed.

   Answer: We agree that the title should be more specific and describing the primary findings in the study. The title of the revised version of the manuscript is now “Evaluation of CXCL9 and CXCL10 as circulating biomarkers of human cardiac allograft rejection.”

2. ‘Data analysis’ paragraph in the ‘Methods’ section: I was not able to reproduce their analysis since the description is too succinct. “Genes were classified as detectable”: This means that they were classified as P(resent), M(arginal) or A(bsent). What do the authors do with this information? Do they keep all genes for their analysis of the 9 arrays? Probably not, otherwise there would be no need to classify the genes. Do they delete Absent genes in at least one array on all arrays? Do they keep genes that are Present or Marginal on at least one of the arrays? There are many ways to use this information and without it I have no way of knowing on how many (and what) genes they performed their search for differential genes. The inclusion filters are also not clear: The minimal signal of 200: It is not specified if this concerns before, during or after rejection, or in all 3 situations. Based on Table 2 I am assuming that this only concerns during rejection. They also mention a minimal fold change of 1.6 without specifying between what situations: Between ‘during rejection’ and ‘before rejection’, between ‘after rejection’ and ‘during rejection’, for both comparisons or for at least one? It is also not explained how they redefine the 9 clusters. And finally, I think they may have missed genes: Why not look for genes that are Present during rejection but Absent before and after rejection? In this way I identified HLA-G in patients 12 and 8, CCL14-CCL15 in patient 1 and AIF1 (allograft inflammatory protein 1) in patient 8.

   Answer: We apologize for the unclearness of the description of the DNA microarray analysis. A more comprehensive and detailed description has been added to the revised version of the manuscript (page 7, section 3). The use of absent/present calls has also been clarified more in detail. However, for cluster 3, no genes were omitted due to this criterion.

Several different parameters and numbers of clusters in the clustering analysis were evaluated. The final parameters used for the clustering yielded in our opinion the best results in terms of number of genes included and distribution of different non-redundant patterns. To further clarify the clustering procedure a table containing the exact clustering parameters used has been included in the revised manuscript as an additional file (additional table 1).

The use of change from absent before rejection to present during rejection was a criteria that we have not tested on this dataset. HLA-G is to the best of our knowledge a plasma membrane bound molecule. CCL14-CCL15 and AIF1 both have low expression levels and only one of the subjects fulfilled the Absent/Present criteria in one subject. We agree that an approach based on this criterion is interesting but we feel that addition of further genes and further selection criteria would only confuse the reader.
3. ‘Serum analysis of CXCL9 and CXCL10 concentrations’, page 11: Why didn’t the authors test BNP as a positive control (Hammerer-Lercher et al. J Heart Lung Transplant. 2005 Sep;24(9):1444)?

Answer: In our microarray experiment BNP was only marginally increased (1.6-fold) during rejection and displayed a large individual variability. Hammerer-Lercher et al state in the abstract of the article that “BNP yielded only a moderate diagnostic accuracy” “which was too low to replace endomyocardial biopsies”. They also state that “there was a considerable scatter in BNP concentrations in individuals as well as in patients”. Based on this, we do not think that BNP is an optimal positive control for this purpose.

However, the article by Hammerer-Lercher et al and this group of molecules are of high relevance. Therefore, we have included the by Hammerer-Lercher et al reference in the introduction (page 3 section 3) and included a section in the discussion about this topic (page 13 section 3).

4. In the Discussion, the authors mention microarray studies in animal models. They should compare the results from those studies to their results. What happened to those genes in the patients in this study? Where they differentially expressed or not at all. Differences and similarities should be discussed.

Answer: In the study by Stegall et al, more than 50 up regulated and 50 down regulated genes are presented (table 3 and table 4 in their article). We therefore feel that a complete comparison of the result from this study is beyond the scope of this article. We choose to compare only the expression of allograft inflammatory protein 1 (AIF1) in the discussion because we feel that this is the most relevant molecule in a biomarker context. This has been added to the revised manuscript (page 12, section 2). Saiura et al have used have specifically investigated cardiac rejection in IFN-[-gamma]-/- mice. From this study we have chosen to discuss the expression of CXCL9 (Mig), CXC10 (IP-10) and macrophage inflammatory protein 1 alpha. (Page 12, section 2).

5. Although the negative result (CXCL9 and CXCL10 are not serum biomarkers for cardiac allograft rejection) is in itself important, it would have been interesting to now the identities of the genes in clusters 1, 2, 8 and 9.

Answer: We agree with the reviewer that clusters 1, 2, 8 and 9 have patterns that could contain potential biomarkers. These clusters have also been carefully analyzed but they contained relatively few secreted products (Classified as extracellular by gene ontology) and none of these displayed a larger magnitude of activation/repression during the rejection phase. To get a more complete view for the reader clusters 1, 2, 8 and 9 have been included in the manuscript as an additional file (additional table 2). A reference to additional table 2 and additional text describing these clusters has also been included in the revised manuscript (page 10 section 3).

It is not impossible to think that the absence of a marker might be linked to the diagnosis of rejection. This should have been further explored, especially since no marker was identified.

Answer: This is a valid comment and data were also explored with respect to genes that appeared to be down regulated during rejection. As a matter of fact, the
Microarray experiment did indicate a down-regulation of several enzymes involved in fatty acid oxidation. However, these observations could not be verified with real time RT-PCR. We decided not to report on this explorative procedure since we felt that it was beyond the scope of the paper.

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
1. In the ‘Background’ section, the authors cite several studies that used DNA microarrays to search for genes with altered expression in human diseases. Since their study is on heart tissue, they should cite at least 1 DNA microarray study on cardiac disease.
   Answer: This has been done (page 4, section 4)

2. The name of PPIA should be written in full.
   Answer: peptidylprolyl isomerase A (cyclophilin A) has been written out in the text (page 8, section 2, and in figure legend 2). Also, the name of the other potential reference genes was written out in full.

3. ‘Serum analysis’ paragraph, page 8: “CXC9 and CXC10” should read “CXCL9 and CXCL10”.
   Answer: This has been corrected (page 9 line 1)

4. On page 10 and in Table 1 the authors mention “cardiomyopathy” when referring to DCM patients. I am assuming this should be “dilated cardiomyopathy”.
   Answer: This has been corrected (page 10 line 4 and table 1)

5. Last sentence on page 12: “only 3 of the subjects” should read: “only 3 of the biopsies”, since from the cited paper it is not clear whether these biopsies are from 1, 2 or 3 patients.
   Answer: This entire section has been rewritten as a response to other comments raised by the reviewers.

6. Legend to Figure 2: “The gene expression of” should read: “Gene expression levels of”. “Bars indicate the analysed time-points in the histopathological sequence” should be deleted since the function of the bars is not to indicate the time-points but the actual gene expression levels. (This comment also goes for the legend to Figure 3.) “The gene expression was related to” should read: “Gene expression levels were relative to”. “*<0.05” should read “*: p<0.05”.
   Answer: This has been corrected

7. (Legend to) Table 1: “HCMP” should read “HCM”
   Answer: This has been corrected

8. Table 1 (and not Tabel 1): “Diagnose” should read “Diagnosis”.
   Answer: This has been corrected
9. In Figure 2 commas should be replaced by dots.
Answer: This has been corrected

10. In Figure 3 the correlation coefficient should be given.
Answer: The correlation coefficient is given in the figure. In response to the other reviewer, illustrations for the correlation between CRP/CXCL9 and CRP/CXCL10 have also been included (revised figure 4 A-C).

Discretionary Revisions (which the author can choose to ignore)
1. In the ‘Methods’ section, ‘DNA microarray analysis’ paragraph the authors cite the Affymetrix manual and one of their own papers. It is not clear to me what information is in their paper that is not in the Affymetrix manual.
Answer: We agree that this double citation is redundant. Our reference does not substantially contribute to the clarity of the procedure and have therefore been removed.

2. Why weren’t all patients analyzed by ELISA?
Answer: For the first two patients (1 and 4) no serum samples were taken.