Author's response to reviews

Title: Altered miRNA expression in pulmonary sarcoidosis

Authors:

Justyna Kiszalkiewicz (justyna.kiszalkiewicz@gmail.com)
Wojciech J Piotrowski (wojciech.piotrowski@umed.lodz.pl)
Dorota Pastuszak-Lewandoska (dorota.pastuszak-lewandoska@umed.lodz.pl)
Pawel Gorski (pawel.gorski@umed.lodz.pl)
Adam Antczak (adam.antczak@umed.lodz.pl)
Witold Gorski (mcwitek@tlen.pl)
Daria Domanska (daria.domanska@umed.lodz.pl)
Monika Migdalska-Sek (monika.migdalska-sek@umed.lodz.pl)
Karolina H Czarnecka (karolina.czannecka@umed.lodz.pl)
Ewa Nawrot (ewa.nawrot@umed.lodz.pl)
Ewa Brzezianska-Lasota (ewa.brzezianska@umed.lodz.pl)

Version: 3
Date: 30 October 2015

Author's response to reviews: see over
We thank the reviewers for their valuable comments, we hope in a comprehensive way we have responded to their questions.

Answer for reviewer's reports

Nobuhisa Ishikawa

1. However, the author selected only some selected miRNAs which have previously been evaluated in patients with several lung diseases. The rationale for examining selected miRNAs could be make more clear. What is novelty of this study?

   It should be stressed that in our study we selected miRNA important in different lung diseases, but mainly important in idiopathic pulmonary fibrosis, cystic fibrosis asthma or chronic obstructive pulmonary disease. Until now, there was no fully confirmed if chosen by us miRNAs would be important in etiopathology of sarcoidosis.

   As so far in case of sarcoidosis there are published only thee original papers (Crouser ED et al 2012, Maertzdorf J et al 2012, Jazwa A et al 2015) focus on miRNA expression status in sarcoidosis patients compared to heathy control or tuberculosis., Moreover, not all of our chosen miRNAs have been tested in sarcoidosis. In earlier published studies, using a similar to ours technique the miR 15 b -16,-192 were analyzed. In Crouser ED et al and in Maertzdorf J et al studies different molecular technic was used. In case of mentioned studies, unique miRNA microarray assay on a CodeLink platform was performed, where about 4 000 mature miRNAs probes and their corresponding precursors from human and mouse miRNA genes has been analyzed. In our approach we chose only a few RNAs that regulate genes involved in two important signaling pathways in the pathogenesis of sarcoidosis in TGF-/ Smad pathway and HIF-1 / VEGF, key signaling in hypoxia-angiostasis and angiogenesis regulation. Additionally, our research objectives were not to study the miRNA expression profile is sarcoidosis. We conducted the search for markers which may correlated with the stages of disease development and/or may be associated with clinical features of the disease and its severity. According to our knowledge this study is the first focus on analysis of miRNA expression depending on the clinical course.

2. The expression signatures of miRNA vary depending on the tissues and cell types examined. Did the author evaluate the correlation between expression levels of studied miRNAs BALF cells and PB lymphocytes?

Yes we evaluate the correlation between expression levels of studied miRNAs in BALF and PB lymphocytes in result section “Relative expression levels of the
studied miRNAs: BALF cells vs PB lymphocytes in sarcoidosis patients” in the article. The most important results are: "Expression patterns of two of the studied miRNAs, miR-16 and miR-128a, overlapped in BALF cells and PB lymphocytes, while the expression patterns of the rest of studied miRNAs were not non-overlapping (see Figure 4). However, only for three non-overlapping miRNAs (miR-15b, miR-130a and miR-let7f) statistically significant differences between BALF cells and PB lymphocytes were observed: for miRNA-15b (P = 0.00001, Mann-Whitney U- test), and miRNA-130a (P = 0.000012, Mann-Whitney U- test) with higher expression in PB lymphocytes and for miRNA-let7f with higher expression levels in BALF cells (P = 0.000003, Mann-Whitney U- test)."

3. Discussion: Too hypothetical and diffused. This section should be reduced by 25%

The authors change the discussion, and also discussion was reduced, but not 25%, because the other reviewer's want to evaluate and add some issues.
Answer for reviewer's reports

Isaac K Sundar.

1. Include the graphs for total number of macrophages, lymphocytes, neutrophils and eosinophils including total cell counts for all the subjects analyzed in this study.

The graphs for total number of cells is included as a Figure 1 in major text.

![Percentage of total number of cells in BALF](image)

2. The results section should include all the graphs for both miRNA expression data from BALF and PB lymphocytes whether they are statistically significant or not in the Figures in an order as per the list of miRNA signatures tested in this study.

We add all the 98 graphs and included in the and at the answers. We gently asked the reviewers to indicate which graphs are interested and which graph should be included in article.

3. It would be important if the authors can conduct additional experiments to confirm if the mRNA expression correlates with protein expression of the
significantly altered miRNAs from BALF cells and peripheral blood lymphocytes.

And

4. Provide additional data on the expression levels of specific miRNA expression in control and pulmonary sarcoidosis primary cells or lung tissues by immunoblotting and or immunohistochemistry along with quantification data to support the observed findings.

It is commonly known that miRNAs are important regulators of many genes expression in most biological processes. They act by managing the RNAi-induced silencing complex (RISC) to partially complementary sequences in target mRNAs. In this way miRNAs may suppress gene expression by a combination of translation inhibition and mRNA decay. There is not possible to assessed the miRNAs expression by immunoblotting and/ or immunohistochemistry in relation to individual miRNA: mRNA interactions. Experimental validation of specific miRNA targets as a downstream effects of differential miRNA expression may be observed at the protein level, although these measures will not distinguish between direct and secondary miRNA targets.

In our study we assessed the protein level of some genes with are under the selected by us miRNAs. The data was published in separate publication:


5. Include the figure 4 with statistics and SD or SE along with symbols for significance between different sample tested.

The figure 4 is corrected and included.
6. Explain in detail the heterogeneity of altered miRNA expression observed in BALF cells vs. lymphocytes

And

7. Why certain miRNAs co-relates well in both BALF cells and PB lymphocytes (miR-192, miR-221) but not the others (miR-15b, miR-let7f etc…)

In many study has been documented that miRNA expression level varies in different biological material. It is linked with different biological role and effects of particular miRNAs in different cells or tissue. The global expression pattern could be dependent of the cell-specific/tissue-specific selection of target genes. Therefore it is commonly known that expression levels of miRNAs will fluctuate between tissues in particular stages of diseases. Some of miRNAs appear to be down-regulated in patients while the others may be up-regulated. They have different regulation points, therefore they not overlap. Thus, in order to search for specific markers for disease, it seems to be valuable to evaluate the miRNAs expression level at the system level and at local level— in our study in blood and in BALF. The expression patterns of miRNAs was heterogenic, as we expected. Non-overlapping of the same miRNAs in various biological materials also was observed by other authors in different diseases in human (Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM, Harris CC. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell. 2006 Mar;9(3):189-98. Rabinowits G1, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer. 2009 Jan;10(1):42-6. doi: 10.3816/CLC.2009.n.006.
8. Include in the references and discuss some of the important findings from prior studies including other recent report in the context of the present study:

Suggested references was consider by authors and added in the discussion.

9. Article has been read and corrected in terms of language

All the graphs for both miRNA expression data from BALF and PB lymphocytes

**Figure 1.** Box and whisker plots, presenting miR-15b expression mean values in BALF cells of patients and controls ($P = 0.04$, Mann-Whitney U-test)
Figure 2. Box and whisker plots, presenting miR-16 expression mean values in BALF cells of patients and controls ($P > 0.05$, Mann-Whitney U-test)
Figure 3. Box and whisker plots, presenting miR-20a expression mean values in BALF cells of patients and controls ($P > 0.05$, Mann-Whitney U-test)
Figure 4. Box and whisker plots, presenting miR-27b expression mean values in BALF cells of patients and controls \((P > 0.05, \text{Mann-Whitney U-test})\)
Figure 5. Box and whisker plots, presenting miR-128a expression mean values in BALF cells of patients and controls ($P > 0.05$, Mann-Whitney U-test)
Figure 5. Box and whisker plots, presenting miR-130a expression mean values in BALF cells of patients and controls ($P > 0.05$, Mann-Whitney U-test)
Figure 7. Box and whisker plots, presenting miR-222 expression mean values in BALF cells of patients and controls ($P > 0.05$, Mann-Whitney U-test)
Figure 8. Box and whisker plots, presenting miR-let7f expression mean values in BALF cells of patients and controls ($P > 0.05$, Mann-Whitney U-test)
Figure 9. Box and whisker plots, presenting miR-15b expression mean values in PB lymphocytes of patients and controls (P= 0.00009, Mann-Whitney U-test)
**Figure 10.** Box and whisker plots, presenting miR-16 expression mean values in PB lymphocytes of patients and controls (P>0.05, Mann-Whitney U-test)
Figure 11. Box and whisker plots, presenting miR-20a expression mean values in PB lymphocytes of patients and controls (P>0.05, Mann-Whitney U-test)
Figure 12. Box and whisker plots, presenting miR-27b expression mean values in PB lymphocytes of patients and controls (P>0.05, Mann-Whitney U-test)
Figure 13. Box and whisker plots, presenting miR-128 expression mean values in PB lymphocytes of patients and controls (P>0.05, Mann-Whitney U-test)
Figure 14. Box and whisker plots, presenting miR-130a expression mean values in PB lymphocytes of patients and controls (P = 0.02, Mann-Whitney U-test)
Figure 15. Box and whisker plots, presenting miR-192 expression mean values in PB lymphocytes of patients and controls (P = 0.005, Mann-Whitney U-test)
Figure 16. Box and whisker plots, presenting miR-221 expression mean values in PB lymphocytes of patients and controls (P = 0.000005, Mann-Whitney U-test)
Figure 17. Box and whisker plots, presenting miR-222 expression mean values in PB lymphocytes of patients and controls (P = 0.0061, Mann-Whitney U-test)
Figure 18. Box and whisker plots, presenting miR-let7f expression mean values in PB lymphocytes of patients and controls (P = 0.00004, Mann-Whitney U-test)
Figure 19. Box and whisker plots, presenting miR-15b expression mean values in BALF cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 20. Box and whisker plots, presenting miR-16 expression mean values in BALF cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 21. Box and whisker plots, presenting miR-20a expression mean values in BALF cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 22. Box and whisker plots, presenting miR-27b expression mean values in BALF cells of patients without and with parenchymal involvement (P = 0.04, Mann-Whitney U-test)
Figure 23. Box and whisker plots, presenting miR-128 expression mean values in BALF cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 24. Box and whisker plots, presenting miR-130 expression mean values in BALF cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 25. Box and whisker plots, presenting miR-192 expression mean values in BALF cells of patients without and with parenchymal involvement (P = 0.02, Mann-Whitney U-test)
**Figure 26.** Box and whisker plots, presenting miR-221 expression mean values in BALF cells of patients without and with parenchymal involvement (P = 0.03, Mann-Whitney U-test)
Figure 27. Box and whisker plots, presenting miR-222 expression mean values in BALF cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 28. Box and whisker plots, presenting miR-let7f expression mean values in BALF cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 29. Box and whisker plots, presenting miR-15b expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 30. Box and whisker plots, presenting miR-16 expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 31. Box and whisker plots, presenting miR-20a expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U test)
Figure 32. Box and whisker plots, presenting miR-27b expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P = 0.002, Mann-Whitney U-test)
Figure 33. Box and whisker plots, presenting miR-128 expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 34. Box and whisker plots, presenting miR-130a expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 35. Box and whisker plots, presenting miR-192 expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P = 0.01, Mann-Whitney U-test)
Figure 36. Box and whisker plots, presenting miR-221 expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P = 0.02, Mann-Whitney U- test)
Figure 37. Box and whisker plots, presenting miR-222 expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 38. Box and whisker plots, presenting miR-let7f expression mean values in BALF cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 39. Box and whisker plots, presenting miR-15b expression mean values in BALF cells of patients with abnormal vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 40. Box and whisker plots, presenting miR-16 expression mean values in BALF cells of patients with abnormal vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 41. Box and whisker plots, presenting miR-20a expression mean values in BALF cells of patients with abnormal vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
**Figure 42.** Box and whisker plots, presenting miR-27b expression mean values in BALF cells of patients with abnormal vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 43. Box and whisker plots, presenting miR-128a expression mean values in BALF cells of patients with abnormal vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 44. Box and whisker plots, presenting miR-130a expression mean values in BALF cells of patients with abnormal vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 45. Box and whisker plots, presenting miR-192 expression mean values in BALF cells of patients with abnormal vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 46. Box and whisker plots, presenting miR-221 expression mean values in BALF cells of patients with abnormal vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 47. Box and whisker plots, presenting miR-222 expression mean values in BALF cells of patients with abnormal vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 48. Box and whisker plots, presenting miR-let7f expression mean values in BALF cells of patients with abnormal vs. normal spirometry ($P > 0.05$, Mann-Whitney U-test)
Figure 49. Box and whisker plots, presenting miR-15b expression mean values in BALF cells of patients with restriction vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 50. Box and whisker plots, presenting miR-15b expression mean values in BALF cells of patients with restriction vs. normal spirometry (P = 0.009, Mann-Whitney U-test)
Figure 51. Box and whisker plots, presenting miR-20a expression mean values in BALF cells of patients with restriction vs. normal spirometry (P = 0.04, Mann-Whitney U-test)
Figure 52. Box and whisker plots, presenting miR-20a expression mean values in BALF cells of patients with restriction vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 53. Box and whisker plots, presenting miR-128a expression mean values in BALF cells of patients with restriction vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
**Figure 54.** Box and whisker plots, presenting miR-130a expression mean values in BALF cells of patients with restriction vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
**Figure 55.** Box and whisker plots, presenting miR-192 expression mean values in BALF cells of patients with restriction vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 56. Box and whisker plots, presenting miR-221 expression mean values in BALF cells of patients with restriction vs. normal spirometry ($P > 0.05$, Mann-Whitney U-test)
Figure 57. Box and whisker plots, presenting miR-222 expression mean values in BALF cells of patients with restriction vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 58. Box and whisker plots, presenting miR-let7f expression mean values in BALF cells of patients with restriction vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 59. Box and whisker plots, presenting miR-15b expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P = 0.001, Mann-Whitney U-test)
Figure 60. Box and whisker plots, presenting miR-16 expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P = 0.05, Mann-Whitney U-test)
Figure 61. Box and whisker plots, presenting miR-20a expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 62. Box and whisker plots, presenting miR-27b expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 63. Box and whisker plots, presenting miR-128a expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 64. Box and whisker plots, presenting miR-130a expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 65. Box and whisker plots, presenting miR-192 expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 66. Box and whisker plots, presenting miR-221 expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 67. Box and whisker plots, presenting miR-222 expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 68. Box and whisker plots, presenting miR-let7f expression mean values in PB lymphocytes cells of patients without and with parenchymal involvement (P > 0.05, Mann-Whitney U-test)
Figure 69. Box and whisker plots, presenting miR-15b expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P = 0.004, Mann-Whitney U-test)
Figure 70. Box and whisker plots, presenting miR-16 expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 71. Box and whisker plots, presenting miR-20a expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 72. Box and whisker plots, presenting miR-27b expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 73. Box and whisker plots, presenting miR-128a expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 74. Box and whisker plots, presenting miR-130a expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P = 0.04, Mann-Whitney U- test)
Figure 75. Box and whisker plots, presenting miR-192 expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 76. Box and whisker plots, presenting miR-221 expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U- test)
Figure 77. Box and whisker plots, presenting miR-222 expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 78. Box and whisker plots, presenting miR-let7f expression mean values in PB lymphocytes cells of patients with acute vs. insidious onset of disease (P > 0.05, Mann-Whitney U-test)
Figure 79. Box and whisker plots, presenting miR-15b expression mean values in PB lymphocytes cells of patients with with obturation vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 80. Box and whisker plots, presenting miR-16 expression mean values in PB lymphocytes cells of patients with obturation vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 81. Box and whisker plots, presenting miR-20a expression mean values in PB lymphocytes cells of patients with with obturation vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 82. Box and whisker plots, presenting miR-27b expression mean values in PB lymphocytes cells of patients with with obturation vs. normal spirometry (P > 0.05, Mann-Whitney U- test)
Figure 83. Box and whisker plots, presenting miR-128a expression mean values in PB lymphocytes cells of patients with obturation vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
**Figure 84.** Box and whisker plots, presenting miR-130a expression mean values in PB lymphocytes cells of patients with with obturation vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 85. Box and whisker plots, presenting miR-192 expression mean values in PB lymphocytes cells of patients with with obturation vs. normal spirometry (P > 0.05, Mann-Whitney U- test)
Figure 86. Box and whisker plots, presenting miR-221 expression mean values in PB lymphocytes cells of patients with with obturation vs. normal spirometry (P > 0.05, Mann-Whitney U-test)
Figure 87. Box and whisker plots, presenting miR-222 expression mean values in PB lymphocytes cells of patients with with obturation vs. normal spirometry (P > 0.05, Mann-Whitney U- test)
Figure 88. Box and whisker plots, presenting miR-let7f expression mean values in PB lymphocytes cells of patients with obturation vs. normal spirometry (P = 0.001, Mann-Whitney U- test)
Figure 89 Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-15b (P = 0.0001, Mann-Whitney U-test)
Figure 90. Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-16 (P > 0.05 Mann-Whitney U-test)
Figure 91. Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-20a (P > 0.05 Mann-Whitney U-test)
Figure 92. Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-15b (P >0.05 Mann-Whitney U-test)
**Figure 93.** Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-128a (P >0.05 Mann-Whitney U-test)
Figure 94. Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-130a (P = 0.000012 Mann-Whitney U-test)
Figure 95. Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-192 (P >0.05 Mann-Whitney U-test)
Figure 96. Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-221 (P >0.05 Mann-Whitney U-test)
Figure 97. Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-222 (P >0.05 Mann-Whitney U- test)
Figure 98. Box and whisker plots, presenting expression mean values in BALF cells and PB lymphocytes cells for miR-let7f (P =0.000003 Mann-Whitney U-test)