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Mycolic acids as diagnostic markers for tuberculosis case detection in humans and drug efficacy in mice

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 June 2011

Thank you for the submission of your manuscript " Mycolic acids as diagnostic markers for tuberculosis case detection in humans and drug efficacy in mice " to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, reviewer #1 feels strongly about the lack of detailed descriptions of the technological aspect but also regarding the form of the paper. This last concern was somehow shared by reviewers #2 and #3 who found the manuscript rather unstructured in parts, with missing or inadequate information. Importantly, reviewer #1 questions the choice of the BCG strain while reviewer #2 is unsure about how quantitative the measure of MAs is.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address all issues that have been raised in a detailed point-by-point letter and modify your manuscript accordingly, within the time constraint outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #1:

This paper is strong on basic science, but light on detail, background and choice of literature references. There are insufficient data supplied to allow proper assessment and reproduction of the results. For example, on Page 3, for sputum profiles it is stated that "four representative samples are shown in Fig. S4" but there are no such profiles at that location. There are a number of problems with the references; for example, the following are inappropriate in certain contexts: Tonge 2000, Butler 2001, Erht and Schnappinger 2007, Layre et al. 2009. It is not appropriate to provide detailed criticisms of minor points, but the following matters are important.

The claimed sensitivity and specificity of 94 and 93%, respectively, is excellent and there is no need to doubt its authenticity. However, to allow verification of the procedure, it is necessary to provide precise coherent data to enable others to repeat the work.

Figure 1 is not ideal, as BCG (presumably Pasteur) (Fig. 1A) is a poor example, in that it lacks methoxymycolates; use a representative *M. tuberculosis* strain. Again, in Fig. 1B, C76 mycolic acid is a bad example; it would be better to show the C80 component, ensuring that the mycolic acid structure is correct. As noted above, no sputa mass spectra are included for inspection; ideally Fig. 1 should have included a positive and negative sputum example.

It is claimed that the procedure allows differentiation of a range of mycobacterial strains, using cluster analysis. However, it is difficult to clearly understand the profiles on which these results are based (Fig. S4). All these figures must be explained much more clearly, particularly pointing out which signals correspond to the different mycolic acid classes.

Referee #2 (Comments on Novelty/Model System):

The authors convincingly demonstrate the utility of using a sub-class of mycolic acids that can be extracted directly from tubercle bacilli in the sputum of smear-positive TB patients and in the lungs of acutely infected mice as a marker of TB infection. Therefore, this novel Mtb-derived biomarker has potential applicability both in pre-clinical and in clinical studies of anti-tubercular drug efficacy.

Referee #2 (Other Remarks):

There is an urgent need for new tools for detecting active TB infection and for use as biomarkers to monitor responses to new TB drugs in human disease and in animal models of TB infection. This manuscript makes some progress towards achieving these goals by establishing the potential utility of mycolic acids (MAs) as biomarkers for detecting TB in humans and for monitoring the effects of chemotherapy in infected mice. In this study, the authors carried out an exhaustive lipidomic analysis of MAs in the CMN family of Actinomycetes thereby producing an extremely valuable resource of information on this important class of lipids. They then apply this information to identify a subclass of MAs that can be extracted directly from TB patient sputum and mouse lung and used as a highly specific signature MA marker to detect acute TB infection in humans and mice.

This is a well written manuscript that reports interesting and potentially significant findings. However, the authors should address the following points:

1. Pg. 4, paragraph 3, and Fig. 3B. What was the lung bacillary load in the "MTB-infected" animals

shown in Fig. 3B? What was the limit of detection in terms of colony-forming units per lung? Moreover, how does the sensitivity of the MA-based assay compare in detecting *M. tuberculosis* in sputum vs. mouse lung tissue vs. *in vitro* culture?

2. MA analysis may provide a potentially useful bacterial biomarker for monitoring TB drug efficacy in human disease or animal models, thereby providing an alternative to the gold- standard CFU count currently used in pre-clinical and clinical studies. Although the authors convincingly demonstrate that the MA signal can be used to differentiate active TB from non-active disease in humans and acute infection from no infection or drug-cured infection in mice, it is not clear how quantitative of bacillary load this measure actually is. What is the limit of detection in mouse lung or human sputum? Moreover, is there a linear relationship between MA level and CFU count?

3. The sensitivity and specificity of an MA-based assay was shown to approach that of smear microscopy. However, the smear microscopy method for TB detection has been surpassed by other methods with significantly superior sensitivity, such as GeneXpert. These newer methods should be mentioned since any new diagnostic method will have to compare with these rather than smear microscopy in order to have an impact on TB diagnosis.

4. Many acronyms are used which are buried in the manuscript and not easy to find in the text when first defined. Acronyms such as MA, ESI/MS, MS/MS, ROC, NTM, MRM, CMN, etc. are confusing to the non-expert reader and the abbreviations should be more readily accessible.

Referee #3 (Comments on Novelty/Model System):

1. The analysis of MA with this sensitivity, from a complex matrix is an achievement. The published spectra are of excellent quality.

2. Classification of bacteria my MS is not novel. MALDI analysis of bacteria is commercially available, as are peak databases for identification.

3. This method will typically lead to faster diagnosis and start of treatment several days earlier than achievable with conventional culturing. Both from a perspective of patient care and cost, this is an important improvement.

4. The authors use HIV co-infection and geography/ethnicity to present an adequate model system.

Referee #3 (Other Remarks):

The manuscript by Shui *et al*. describes the analysis of mycolic acids in sputum as a diagnostic tool for the detection of tuberculosis in humans. The authors also show the use of the same technique in the classification of bacterial strains, which is crucial information when deciding on a therapeutic strategy. The fact that there is no need to culture bacteria makes this diagnostic tool fast, with advantageous effects on patient health and/or hospitalization costs. The characterization of nearly 2000 mycolic acids is a significant contribution to the current mycolic acid database. The work is well performed and is a valuable contribution to the field of molecular medicine. I have only minor comments on the manuscript, listed below.

1. Introduction, third paragraph, first line. Fig S1 does not contain molecular structures. Table S1 would probably be a good place to include this information.

2. Supplementary table 1 is missing. This table should definitely be included in the final version, and should at least contain Q1 and Q3 masses, (optimal) collision energies, used dwell times and corresponding (tentative) structures. Quantifying 1942 mycolic acids simultaneously is not a standard procedure, and requires a steady, stable signal for some time. Have the authors verified this by multiple cycling of the MRM transitions? This should be included.

3. Color coding of Fig S6 is not according to legend. NTM infected patients are in orange, not blue, at least in the version available to referees. This should be corrected.

4. Figures and legends need some attention. In Fig 2C, numbered dots correspond to patient IDs in table 1, but this doesn't become clear until the discussion. I suggest to include this information in the legend.

5. In Fig 2B/C the meaning of "(24)" and "(26)" should be included in the legend.

6. Legends of supplementary figures are difficult to understand, in particular Fig S3. FigS5: ")" missing.

1st Revision - Authors' Response 09 September 2011

Referee #1:

This paper is strong on basic science, but light on detail, background and choice of literature references. There are insufficient data supplied to allow proper assessment and reproduction of the results. For example, on Page 3, for sputum profiles it is stated that "four representative samples are shown in Fig. S4" but there are no such profiles at that location.

Single stage mass spectra of 5 different strains of *Corynebacteria* (Supplementary Fig. 1), 4 *Nocardia* (Supplementary Fig. 2), 5 MTB (Supplementary Fig. 3) and 4 non-tuberculous *Mycobacteria* (Supplementary Fig. 4) are now included.

A sputum profile is now shown in Fig. 1D.

There are a number of problems with the references; for example, the following are inappropriate in certain contexts: Tonge 2000, Butler 2001, Erht and Schnappinger 2007, Layre et al.2009.

We have carefully checked all referencing and have made adjustments to the cases mentioned above.

It is not appropriate to provide detailed criticisms of minor points, but the following matters are important.

The claimed sensitivity and specificity of 94 and 93%, respectively, is excellent and there is no need to doubt its authenticity. However, to allow verification of the procedure, it is necessary to provide precise coherent data to enable others to repeat the work.

We have added definitions for sensitivity and specificity. Page 3, end of second last paragraph now reads:

> "Using these data, we calculated a statistical sensitivity of 94% and a specificity of 93% (sensitivity: number of true positives divided by the sum of true positives and false negatives; specificity: number of true negatives divided by the sum of true negatives and false positives) and even slightly better values for the HIV positive individuals alone (Fig. 2F)."

Our cutoff values used to calculate these values are now indicated in Fig. 2C and additional technical information for others to repeat these results are presented in Table S1.

Figure 1 is not ideal, as BCG (presumably Pasteur) (Fig. 1A) is a poor example, in that it lacks methoxymycolates; use a representative M. tuberculosis *strain.*

We agree with the reviewer and have replaced the *M. bovis* BCG Pasteur strain profile with that obtained from *M. tuberculosis* (Fig. 1A).

Again, in Fig. 1B, C76 mycolic acid is a bad example; it would be better to show the C80 component, ensuring that the mycolic acid structure is correct.

Modified as suggested. A C80 mycolic acid species from *M. tuberculosis* (m/z 1,164; C80H156O3) is now presented in Fig. 1B.

As noted above, no sputa mass spectra are included for inspection; ideally Fig. 1 should have included a positive and negative sputum example.

Modified as suggested. A mass spectrum of sputum from a TB patient is shown in Figure 1D together with a negative control condition.

It is claimed that the procedure allows differentiation of a range of mycobacterial strains, using cluster analysis. However, it is difficult to clearly understand the profiles on which these results are based (Fig. S4). All these figures must be explained much more clearly, particularly pointing out which signals correspond to the different mycolic acid classes.

We have added more explanations to the figure legend as requested. Major MA signals are now labeled in this figure (Supplementary Fig. 6).

Referee #2 (Comments on Novelty/Model System):

The authors convincingly demonstrate the utility of using a sub-class of mycolic acids that can be extracted directly from tubercle bacilli in the sputum of smear-positive TB patients and in the lungs of acutely infected mice as a marker of TB infection. Therefore, this novel Mtb-derived biomarker has potential applicability both in pre-clinical and in clinical studies of anti-tubercular drug efficacy.

Referee #2 (Other Remarks):

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This is a well written manuscript that reports interesting and potentially significant findings. However, the authors should address the following points:

1. Pg. 4, paragraph 3, and Fig. 3B. What was the lung bacillary load in the "MTB-infected" animals shown in Fig. 3B? What was the limit of detection in terms of colony-forming units per lung?

Bacillary load for the experiment shown in Fig. 3 was measured in 5 out of the 10 animals used in this experiment. The average value was 15.2E6 cfu/lung. We have included this information in the legend to Figure 3.

Moreover, how does the sensitivity of the MA-based assay compare in detecting M. tuberculosis *in sputum vs. mouse lung tissue vs.* in vitro *culture?*

We thank the reviewer for this valuable comment, which we have addressed in two ways:

1. An additional spiking experiment was performed to determine the minimum number of bacteria needed for MA detection in sputum and culture medium.

Increasing numbers (0 - 2.5E6 cfu) of bacilli (*M. tuberculosis* H37Rv) were added to negative sputum as well as culture medium, which was used as an *in vitro* control for comparison. The results from this approximation experiment have been included as a new panel (Fig. 1E):

- A linear response is observed over 3 orders of magnitude (R2>0.998).

- The minimum number of bacteria needed for detection by our mass spectrometry method was approximately 10,000 cfu for a signal to noise ratio of 3 (S/N=3) in medium and sputum.

- The complexity of the body fluid sputum results in matrix effects leading with slightly lowered sensitivities (see also below). This is mentioned in text on page 3, 3rd paragraph, which now reads:

> " We further determined the minimum number of bacterial cells necessary for our MA detection approach, by performing a spiking experiment with a serial dilution of *M. tuberculosis* cells added to non-TB sputum as well as culture medium. In sputum, around 10,000 cfu were sufficient to detect bacteria based on their MA signal (signal to noise ratio, S/N=3). A linear increase in MA levels was observed with increasing numbers of bacterial cells, for both, sputum (R2=0.998) and medium (R2=0.999). In extracts from medium, MA signals were slightly higher than in extracts from sputum, which was probably due to MA extraction efficiency or ion suppression effects caused by the complex matrix of this body fluid (Fig. 1E)."

2. Comparison of sensitivities between sputum, lung tissue and *in vitro* cultures

- medium: 2.5xE6 cells give a signal corresponding to 0.84xE-6 g of MA (Fig. 1E). Thus, the ratio of 0.84xE-6/2.5xE6 = 0.33 can be used a measure for the sensitivity under these conditions where MA are detected from cells extracted from medium.

- sputum: in the case of sputum this value is reduced to approximately 0.2 (compare slopes in Fig. 1E).

- lung tissue: If we assume comparable linearity also in the case of lung tissue, this value is approximately 0.13 (2 ug MA/lung and approx. 15.2xE6 cfu/lung, Fig. 3B).

Thus, sensitivity for MA extracted and analyzed from culture medium is more than double that of extracts from lung tissue; not surprising given the much more complex nature of tissue as opposed to culture medium with respect to extraction and matrix effects.

2. MA analysis may provide a potentially useful bacterial biomarker for monitoring TB drug efficacy in human disease or animal models, thereby providing an alternative to the gold- standard CFU count currently used in pre-clinical and clinical studies. Although the authors convincingly demonstrate that the MA signal can be used to differentiate active TB from non-active disease in humans and acute infection from no infection or drug-cured infection in mice, it is not clear how quantitative of bacillary load this measure actually is. What is the limit of detection in mouse lung or human sputum? Moreover, is there a linear relationship between MA level and CFU count?

Yes, there is a linear relationship between MA levels and CFU counts (see above and Fig. 1E).

At this stage, we do not have a time course that would allow us to draw any conclusions on mycolic acid clearance (e.g. from the mouse lungs over the duration of treatment). Therefore, we can only say that upon successful treatment (as judged by zero cfu in the treated animals), the mycolic acids signal is strongly reduced, almost to baseline levels (Fig. 3).

We agree with the reviewer that understanding clearance of MA will be an exciting avenue for future investigations.

3. The sensitivity and specificity of an MA-based assay was shown to approach that of smear microscopy. However, the smear microscopy method for TB detection has been surpassed by other methods with significantly superior sensitivity, such as GeneXpert. These newer methods should be mentioned since any new diagnostic method will have to compare with these rather than smear microscopy in order to have an impact on TB diagnosis.

The GenXpert method is now mentioned and referenced in the text (p. 5, 5th paragraph, 3rd line from bottom) which now reads:

> "It will be particularly interesting to combine existing and other novel diagnostics (e.g., culture or molecular test such as the novel GeneXpert system, Boehme *et al.*, 2010) with MA profiles in a longitudinal manner across different body fluids (i.e., sputum, urine, and blood), as well as in connection with latent TB infections".

4. Many acronyms are used which are buried in the manuscript and not easy to find in the text when first defined. Acronyms such as MA, ESI/MS, MS/MS, ROC, NTM, MRM, CMN, etc. are confusing to the non-expert reader and the abbreviations should be more readily accessible.

We have replaced the abbreviations ROC, NTM and CMN by the entire word whenever applicable.

Referee #3 (Comments on Novelty/Model System):

1. The analysis of MA with this sensitivity, from a complex matrix is an achievement. The published spectra are of excellent quality.

2. Classification of bacteria my MS is not novel. MALDI analysis of bacteria is commercially available, as are peak databases for identification.

3. This method will typically lead to faster diagnosis and start of treatment several days earlier than achievable with conventional culturing. Both from a perspective of patient care and cost, this is an important improvement.

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The manuscript by Shui et al. describes the analysis of mycolic acids in sputum as a diagnostic tool for the detection of tuberculosis in humans. The authors also show the use of the same technique in the classification of bacterial strains, which is crucial information when deciding on a therapeutic strategy. The fact that there is no need to culture bacteria makes this diagnostic tool fast, with advantageous effects on patient health and/or hospitalization costs. The characterization of nearly 2000 mycolic acids is a significant contribution to the current mycolic acid database. The work is

well performed and is a valuable contribution to the field of molecular medicine. I have only minor comments on the manuscript, listed below.

1. Introduction, third paragraph, first line. Fig S1 does not contain molecular structures. Table S1 would probably be a good place to include this information.

We have included elemental compositions and information on the alpha branch chain in Table S1.

2. Supplementary table 1 is missing. This table should definitely be included in the final version, and should at least contain Q1 and Q3 masses, (optimal) collision energies, used dwell times and corresponding (tentative) structures.

This information is now included in Table S1.

Quantifying 1942 mycolic acids simultaneously is not a standard procedure, and requires a steady, stable signal for some time. Have the authors verified this by multiple cycling of the MRM transitions? This should be included.

Indeed, and we have clarified this aspect in the legend to Table S1. Mycolic acids were separated into groups depending on collision energies and strains: 2 lists of approximately 340 MRM transitions for mycobacterial MAs); 3 lists of nocardial MRM transitions (350 each) and 1 single list of and corynomycolate MRMs (247). Dwell time was 10msec for each MRM pairs.

This technical information should now be sufficient for others interested in repeating our results.

3. Color coding of Fig S6 is not according to legend. NTM infected patients are in orange, not blue, at least in the version available to referees. This should be corrected.

Modified as suggested.

4. Figures and legends need some attention. In Fig2C, numbered dots correspond to patient IDs in table 1, but this doesn't become clear until the discussion. I suggest to include this information in the legend.

Modified as suggested.

5. In Fig2B/C the meaning of "(24)" and "(26)" should be included in the legend.

Modified as suggested.

6. Legends of supplementary figures are difficult to understand, in particular Fig S3. FigS5: ")" missing.

Modified and we hope this is clearer now.

2nd Editorial Decision 30 September 2011

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

- Referee #2's recommendation of using a non-linear regression analysis to determine correlation

coefficients in Figure 1E should be followed, and Figure 1E changed accordingly.

- Make sure that entry of sample 18 is correct.

- In the Material and Methods section p7, some text/embedded image is missing under "Isotopic Correction", please change accordingly.

- Please add a 5th key word.

Please submit your revised manuscript within two weeks.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #1:

The authors have made the necessary changes.

Referee #3 (Comments on Novelty/Model System):

The reason not to qualify the novelty as "high" lies in the fact that bacterial typing on MS profiles is not novel. However, using MA for this purpose, is.

Referee #3 (Other Remarks):

I have only two minor comments to the revised manuscript.

The first concerns Fig 1E. The authors appear to have performed a linear regression, but this gives too much weight to the data point at high(-er) 'number of cells'. A non-parametric regression analysis would be a better way to determine the correlation coefficient.

As a second remark, sample number 18 is listed as coming from a patient with AIDS, but a negative HIV status (table 1). Is this correct?

2nd Revision - Authors' Response 11 October 2011

We have addressed the points you list below (see my comments in this email in CAPS) and I have submitted the modified version via the online system.

Please let know if you have additional questions or if you need more information. I hope this revised version will now be acceptable for publication in this final form.

Thank you once again.

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

- Referee #2's recommendation of using a non-linear regression analysis to determine correlation coefficients in Figure 1E should followed, and Figure 1E changed accordingly.
WE HAVE REPLACED LINEAR REGRESSION BY NON-LINEAR POLYNOMIAL REGRESSION AS SUGGESTED AND HAVE CHANGED THE TEXT AND FIGURE LEGEND ACCORDINGLY.

- Make sure that entry of sample 18 is correct.
WE HAVE CHECKED THE INFORMATION OF SAMPLE 18 AND HAVE CHANGED THE LISTED HIV STATUS OF THIS INDIVIDUAL FROM NEGATIVE TO POSITIVE.

- In the Material and Methods section p7, some text/embedded image is missing under "Isotopic Correction", please change accordingly.
THE EMBEDDED FORMULA IS NOW VISIBLE IN THE MERGED DOCUMENT.

- Please add a 5th key word.
AN ADDITIONAL KEY WORD ("LIPIDOMICS") HAS BEEN ADDED.