Author's response to reviews

Title: Fragile X protein in newborn dried blood spots: implications for newborn screening

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Author's response to reviews: see over
1. We are especially grateful to the reviewers for pointing out the poorly written and misleading parts of the abstract which has been substantially rewritten and clarified according to their suggestions. (The revisions are shown italicized and footnotes explain their relationship to the reviewers’ concerns.)

Background: The fragile X syndrome (FXS) results from mutation of the FMR1 gene that prevents expression of its gene product, FMRP. We previously characterized 215 dried blood spots (DBS) representing different FMR1 genotypes and ages with a Luminex-based immunoassay (qFMRP). We found variable FMRP levels in the normal samples and identified affected males by the drastic reduction of FMRP.

Methods: Here, to establish the variability of expression of FMRP in a larger random population we quantified FMRP in 2000 anonymous fresh newborn DBS. We also evaluated the effect of long term storage on qFMRP by retrospectively assaying 74 aged newborn DBS that had been stored for 7-84 months that included normal and full mutation individuals. These analyses were performed on 3 mm DBS disks. To identify the alleles associated with the lowest FMRP levels in the fresh DBS, we analyzed the DNA in the samples that were more than two standard deviations below the mean.

Results: Analysis of the fresh DBS revealed a broad distribution of FMRP with a mean approximately 7-fold higher than that we previously reported for fresh DBS in normal adults and no samples whose FMRP level indicated FXS. DNA analysis of the lowest FMRP DBS showed that this was the low extreme of the normal range and included a female carrying a 165 CGG repeat premutation. In the retrospective study of aged DBS, the FMRP mean of the normal samples was less than 30% of the mean of the fresh DBS. Despite the degraded signal from these aged DBS, qFMRP identified the FXS individuals.

2. List of modifications of the manuscript Adayev et al.

1 In response to M. Mila’s concern for sample size we revised this section to correct the impression that this study was intended as a pilot screening (for which, we agree, it has an inadequate number of samples) and made it clear that this study is actually focused on the variability of FMRP expression in an unselected newborn population. This revision was also intended to clarify the purpose of including the “76 newborn retrospective DBS” which are not intended to be controls, but rather a separate group of “aged” DBS that are included to show how DBS storage time affects the assay. This section was also revised to satisfy F. Tassone’s similar concern about distinguishing between the two groups of samples. Note that we now use “fresh” and “aged” to distinguish between the two groups throughout the manuscript. This section was also revised to address her concern about the enumeration of the normal and full mutation samples in the “aged” group. Note that the total number of samples was corrected to 74.

2 This section was modified in response to F. Tassone’s concern, to clarify which normal adult samples were being referred to. These alterations were also to satisfy M. Mila’s concerns about potential confusion of the “fresh” and “aged” sample groups (“...two groups of samples with different technical characteristics. These samples should not be used as control group.”)
The changes in the Abstract are explained in detail above. Some of the reviewers’ comments prompted multiple changes in the manuscript. For example, the potential confusion of the NY State and Australian newborn DBS analyses was addressed by insertion or substitution of “fresh” and “aged” to distinguish the “fresh” NY State DBS from the “aged” Australian DBS. Clarification of the different objectives for analyzing “fresh” and “aged” DBS was similarly addressed at different places.

1. Title was changed to: “Fragile X protein in newborn dried blood spots”.
2. Line 19: the running head was changed to “FMRP in fresh and aged newborn DBS”.
3. Line 91: Three sentences were inserted after “…genotypes”: “In samples from normal individuals we found a broad distribution of FMRP. The level of the protein declined with age from infants to preteens. It leveled off in teenage years and remained unchanged through adulthood, with no difference between males and females”.
4. Line 93: “affected (full mutation) males” was inserted to replace “males carrying the full mutation”.
5. Line 95: “mandate” was corrected to “mandated”.
6. Lines 97-98: Deleted and replaced with “…use 2000 randomly selected anonymous fresh newborn DBS to characterize the variability of FMRP expression in the newborn population which is a potential target for screening with this assay. Considering the estimated prevalence of fragile X, it was relatively unlikely that we would find any affected individuals (i.e. those with virtually no FMRP) among the 1000 male and 1000 females sampled. We were primarily interested, however, in the variability of FMRP expression, especially the low extreme of normal expression.
7. Lines 99-102: Deleted and replaced with: “To evaluate the effect of long term storage of newborn DBS on the qFMRP assay, we conducted a retrospective study of 74 “aged” newborn DBS that had been stored for up to 7 years. These DBS were from a different newborn screening program and included samples from 6 affected (full mutation) males.
8. Lines 117-118: Replaced with “The seventy-four disks from DBS that had been stored for 7 to 84 months included samples from 6 males diagnosed with the fragile X syndrome and 68 normal individuals.”
9. Line 120: Because two full mutation female DBS that had been excluded from the analysis had been inadvertently included in the total, “70” was corrected to “68”.
10. Line 134-135: This sentence was deleted and replaced with: “FMRP assays were performed with the anti-FMRP mouse monoclonal antibody mAb6B8 (MMS-5231, Covance Inc., Dedham, MA) and the anti-FMRP rabbit polyclonal antibody R477 [12]. These antibodies are highly specific and each recognizes a different epitope of the protein [12]. A GST fusion protein, GST-SR7 carrying an abbreviated sequence of FMRP that includes the epitopes of mAb6B8 and R477 was used as standard. The immunoassays were performed as previously described [12]”.
11. Line 136: We deleted “(MMS-5231, Covance Inc., Dedham, MA)”.
12. Line 164: We replaced “for five weeks” with “for only five weeks (fresh DBS)”. 

13. Line 165: We deleted “an abbreviated FMRP standard (GST-SR7)” and inserted “GST-SR7 as previously described [12]”.
14. Line 187: The number “76” was corrected to “74”.
15. Line 188: The number “6” was inserted before “full mutation males”; and “68” was inserted in front of “normal individuals”.
16. Line 189: “archived” was replaced with “aged”.
17. Line 193: “aged” was inserted in front of “DBS”.
18. Line 195: “aged” inserted in front of “sample set”.
19. Line 199: “affected (full mutation)” was inserted to replace “full mutation”.
20. Line 200: We inserted “that could lead to false positive” after “DBS”.
21. Line 232: We inserted “aged” in front of “DBS” and “fresh” in front of “2,000 DBS”.
22. Line 233: “These newborn” was changed to “The former”.
23. Line 241: “affected (full mutation)” was inserted and “with full mutation” was deleted.
24. Line 242: We inserted three sentences after “…affected (full mutation) males: “However, prolonged storage of newborn DBS (more than 47 months) could lead to an increase in false positive samples. Thus, the qFMRP assay will have limited utility for DBS stored for four years or more. Future retrospective studies with aged newborn DBS should be performed with samples that have been stored less than 47 months, and the levels of FMRP should be compared to those of aged normal DBS having the same prolonged storage time”.
25. Line 270: “archived” was replaced with “aged”.
26. Line 272: Three sentences were inserted after “screening program”: “In a second screening step, characterization of CGG size and/or methylation status of the FMR1 alleles associated with DBS at the low extreme of the FMRP distribution would indicate the presence of the fragile X syndrome. The correlation between highly reduced or absent FMRP expression and the fragile X syndrome is firmly established for males. This correlation is likely to apply to females as well but further studies will be necessary to firmly establish this link”.
27. Line 272: “It” was replaced with “Fragile X screening by qFMRP”.
28. Line 411: In Table 2, we inserted line: “nl: normal, full: full mutation”

3. Response to the Reviewers’ comments:
(Changes are shown in bold-faced type.)

Flora Tassone:

**Reviewer's report**
**Title:** Fragile X protein in newborn dried blood spots: implications for newborn screening
**Version:** 1  **Date:** 30 August 2014
**Reviewer:** Flora Tassone
Reviewer's report:
This is a follow up study of the development of a simple and inexpensive immunoassay capable of measuring FMRP levels in dried blood spots. A larger set of samples from the general population was used to establish the variability in FMRP expression detected by the assay. The feasibility of the assay was tested in fresh and archived spots showing that a potential FMRP degradation may have occurred in archived spots and suggesting that its utility would be most effective if applied to fresh samples. Overall it is a good and reliable technique and the feasibility of the analysis is appropriate. The study is important as a quantitative methodology able to reliably measuring FMRP expression levels is lacking.

This reviewer has the following comments:

In the abstract:
Methods:
1. From the abstract it is not possible to distinguish whether the only difference between the “2000 fresh random DBS” and the “set of 76 newborn DBS that had been stored…” is the age of the spots. May want to also explain that the 2000 samples were anonymous and that you retrospectively analyzed the 76 samples from controls and 1 FXS case.

In the new version of the abstract we specified that the 2,000 fresh DBS were anonymous, and that the two sets of DBS differed in the age of the spots.

“Methods: “Here, to establish the variability of expression of FMRP in a larger random population we quantified FMRP in 2000 anonymous fresh newborn DBS. We also evaluated the effect of long term storage on qFMRP by retrospectively assaying 74 aged newborn DBS that had been stored for 7-84 months that included normal and full mutation individuals”.

Results:
2. It is somewhat confusing when the author refers to the first and second set, I may suggest to use a descriptive adjective to distinguish the samples, ie “fresh DBS” and “aged DBS” instead or being more specific.

As suggested, we used the adjectives “fresh” for the 2,000 DBS samples and “aged” for the 74 archived DBS. These adjectives were used in the title page (running head), the abstract and in the text.

3. The author reports a mean distribution about 7 fold higher than that of normal adults in the first set. Which normal adult samples were used for comparison? If the author refers to FMRP levels obtained from fresh DBS versus FMRP levels from older DBS, this should be stated instead. Or was from a previous study?

The comparison was with the levels found in the normal adult population described in our previous paper. This is now stated in the abstract:

“Results: “Analysis of the fresh newborn DBS revealed a broad distribution of FMRP with a mean approximately 7-fold higher than that we previously reported for fresh DBS in normal adults and no samples whose FMRP level indicated FXS”.

4. From the sentence: “…”Despite the degraded signal from these samples, it was
possible to recognize all the affected males”…. There were affected males out of the 76 from the general population? It is clear only later on in the Result section maybe just mention in the abstract that the 76 blood spots included samples from full mutation males as well as normal individuals.

(See 1 above.)

In the Results section

5. line 136-138- Please describe further: is the Covance anti FMRP against a different species than rabbit? Are the two antibodies selecting different epitopes?
To address the concerns of the reviewer, we modified the Methods. qFMRP Assay Procedure as follow:
“FMRP assays were performed with the anti-FMRP mouse monoclonal antibody mAb6B8 (MMS-5231, Covance Inc., Dedham, MA) and the anti-FMRP rabbit polyclonal antibody R477 [12]. These antibodies are highly specific and each recognizes a different epitope of the protein [12].”

6. line 165- The author state that “FMRP concentration (pM) in each sample (3-mm-diameter disk eluate) was calculated by comparison to dilutions of an abbreviated FMRP standard (GST-SR7).” The quantification of FMRP is very important for the conclusions in this paper and there is no detailed description of the GST-SR7 FMRP standard used and how exactly were the dilutions compared in order to quantify FMRP concentration for the DBS. Some specifics should be added or reference the authors’ previous study.
To address the concerns of the reviewer, we modified the Methods. qFMRP Assay Procedure as follow:
“A GST fusion protein, GST-SR7 carrying an abbreviated sequence of FMRP that includes the epitopes of mAb6B8 and R477 was used as standard. The immunoassays were performed as previously described[12].”

7. line 188- n=? for full mutation and normal
As requested we specified the numbers:
“We also applied the qFMRP assay in a retrospective study of 74 newborn DBS that had been stored for an extended period and included 6 full mutation males as well as 68 normal individuals”.

8. ……”Despite the loss of detectable FMRP with DBS storage time, the qFMRP assay identified all of the full mutation males”...
I believe that the problem could be that the loss of FMRP the author shown to be due to prolonged storage could lead to false positives.
We modified the text to as follow:
“Despite the loss of detectable FMRP with DBS storage time, the qFMRP assay identified all of the affected (full mutation) males. Because of the decline of detectable FMRP in normal control DBS that could lead to false positives, the Mann-Whitney analysis…”.

9. Based on their observations, how would the authors propose the finding of a low
FMRF sample with this test could be handled? Follow up with CGG sizing test?

As suggested, we added few sentences in the Conclusion to deal with handling samples with low FMRP levels:

“Our data suggests that the qFMRP assay could serve as the initial step in a fragile X newborn screening program. In a second screening step, characterization of CGG size and/or methylation status of the FMR1 alleles associated with DBS at the low extreme of the FMRP distribution would indicate the presence of the fragile X syndrome. The correlation between highly reduced or absent FMRP expression and the fragile X syndrome is firmly established for males. This correlation is likely to apply to females as well but further studies will be necessary to firmly establish this link. Fragile X screening by qFMRP has distinct advantages over techniques that detect a CGG expansion...”.

10. Also based on the presented results with aged blood spots, what are the authors’ recommendations for the age range of the spots that should be used to obtain reliable data?

We added to the Discussion:

“However, prolonged storage of newborn DBS (more than 47 months) could lead to an increase in false positive samples. Thus, the qFMRP assay will have limited utility for DBS stored for four years or more. Future retrospective studies with aged newborn DBS should be performed with samples that have been stored less than 47 months, and the levels of FMRP should be compared to those of aged normal DBS having the same prolonged storage time”.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician

Montserrat Mila

Reviewer’s report
Title: Fragile X protein in newborn dried blood spots: implications for newborn screening
Version: 1 Date: 12 August 2014
Reviewer: Montserrat Mila
Reviewer’s report:
Major Compulsory Revisions
The authors present a study about the variability of FMRP expression in 2000 samples as well as 76 retrospective samples, showing the usefulness of this method as screening method for FXS in dry blood spots. They conclude that this method is feasible, economical and can be readily adapted to high-throughput application.

1. There is a previous work from the same group (LaFauci et al., 2013) where they clearly demonstrated the utility of the method in males, and with some limitations in females. It is well known that FMRP expression study is different for females and males, since FMR1 is an X-
linked gene. Based on these and previous results, the application of this methodology for a newborn screening can be questionable.

We agree with the reviewer that the phenotypic characterization of FMRP levels is complicated by the fact that the FMR1 locus is X-linked and that in females there is incomplete penetrance. We modified the Conclusions segment of the Abstract: “The assay showed that newborn DBS contain high levels of FMRP that will allow identification of males and potentially females, affected by FXS. The assay is also an effective screening tool for aged DBS stored for up to four years.”

We also added in the final Conclusion section: “The correlation between highly reduced or absent FMRP expression and the fragile X syndrome is firmly established for males. This correlation is likely to apply to females as well but further studies will be necessary to firmly establish this link.”

2. One major problem of this work is the sample size. Taking into account the prevalence of full mutation in the general population, it could be expected not to find any full mutation.

We agree with the reviewer. To clarify that this study was not intended as a screening but as an examination of the variability of FMRP. We changed the title to: “Fragile X protein in newborn dried blood spots”.

We also stated the aim of the study more clearly in the Background section:
Line 91: We inserted three sentences: …” In samples from normal individuals we found a broad distribution of FMRP. The level of the protein declined with age from infants to preteens. It leveled off in teenage years and remained unchanged through adulthood, with no difference between males and females”.

Lines 97 to 98 were replaced by:
[...we decided to] use 2000 randomly selected anonymous fresh newborn DBS to characterize the variability of FMRP expression in the newborn population which is a potential target for screening with this assay. Considering the estimated prevalence of fragile X, it was relatively unlikely that we would find any affected individuals (i.e. those with virtually no FMRP) among the 1000 male and 1000 females sampled. We were primarily interested, however, in the variability of FMRP expression, especially the low extreme of normal expression.”

3. Why are the 76 newborn retrospective DBS included in the study? They could just have included FXS samples as positive controls. The data presented in table 2 show 6 full mutation samples plus 3 “nl” from Australian newborns. Which is the meaning of “nl”? To address the reviewer’s concern that the purpose of the analysis of the 76 samples was not clear we modified the Background as follow: “To evaluate the effect of long term storage of newborn DBS on the qFMRP assay, we conducted a retrospective study of 74 “aged” newborn DBS that had been stored for up to 7 years. These DBS were from a different newborn screening program and included samples from 6 affected (full mutation) males.”

We added in Table2: “nl: normal, full: full mutation”

4. The fact that they include the Australian population, that is older than the 2000 samples from New York, implicates that they are working with two groups of samples with different technical characteristics. These samples should not be used as control group.
We agree with the reviewer. The fresh and aged DBS samples are different. As stated above (point 3), the retrospective study of aged DBS was not done as a control for the fresh 2,000 DBS but rather to evaluate the effect of long term storage of DBS on FMRP levels.

5. Finally, in the last paragraph of the discussion they state that is better for a newborn screening not to detect premutations. We agree with the authors although when a FXS is diagnosed, a cascade screening will be necessary for the rest of the family, which will implicate to detect premutations.

We agree with the reviewer. Even in absence of FX laboratory screening (either by DNA analysis or qFMRP) the clinical diagnosis of a full mutation FX individual would eventually result in a cascade screening for the rest of the family and detection of premutation carriers.

Level of interest: An article of limited interest
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests