Author's response to reviews

Title: Unique Immunologic Patterns in Fibromyalgia

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Christna Chap

Editor

BMC Clinical Pathology

Dear Editor:

Thank you for reviewing our manuscript MS: 2020487049761183 “The identification of patients with the clinical diagnosis of fibromyalgia using a multiplex cytokine assay”. We appreciate BMC Clinical Pathology’s interest in our study. We revised the manuscript and are resubmitting for publication. The revisions incorporate appropriate responses to the comments of the reviewers. The new title of the study has been changed to “Unique Immunologic Patterns in Fibromyalgia”.

In regard to the specific reviewer comments, we are providing the following:

Reviewer #1

1. All cytokines were tested in every subject. The N values in Tables 2-4 correspond to the number of subjects whose cytokine readings were within the detection range. The legend was added to each table explaining the meaning of N values.

2. High variability of cytokine values is typical for clinical studies. For example, see ref. Clin Exp Rheumatol. 2007;25:225-230. The important result of this study is that the cytokine values are statistically different in the group of patients.
compared to the group of controls. Patients with inflammation were excluded from the study.

3. We included in the paper the new Table 1 showing the clinical data on the patients. The patients who were studied all met specific criteria. Every Fibromyalgia (FM) patient had to have confirmation from two different physicians that they met the American College of Rheumatology criteria for the diagnosis of FM. And as we also stated, the FM patients could not have any other rheumatologic disorder, any auto-immune condition, any inflammatory disorder, any infectious disorder or any neoplastic disorder. They also were excluded if they were taking any anti-inflammatory medications or had taken any FM medications during two weeks before undergoing their blood draw. They also could not have been on any anti-neoplastic drugs or had used any illicit drug. Of the 110 FM patients, only 9 had been diagnosed by a licensed psychotherapist with depression. The statistical analysis was performed on the patients excluding those who had a confirmed diagnosis of depression and showed similar results, thereby proving that depression had a very limited impact on the cytokine profile of FM patients. The corresponding statements were added to the Results.

4. The characteristics of the healthy controls have been included in the Materials and Methods. They all lacked a history of any type of chronic or acute illnesses and none were using any medications, OTC or prescription drugs that had any know immunologic effects.

Reviewer #2

1. The blood was drawn from 10 am to 2 pm. It is known that the time of blood draw may affect concentrations of circulating cytokines in plasma. In our study the blood was taken during the same period of time from both patients and controls, so that the variations in cytokine values due to the time of withdrawal were averaged in both groups. Also, PBMC activity, which is the main focus of this paper, is unlikely to be affected by the time of the day.

2. Only one blood draw was performed for each patient. The blood was drawn in four tubes and was mixed together. PBMCs were isolated and cultured in triplicates for each challenge, including control cells. We added this information to the Materials and Methods.

3. The samples were shipped overnight at room temperature in insulated containers and PBMCs were isolated on the next day. Again, the same protocol was used for both patient and control samples. This statement was added to the text.

4. Fibromyalgia by definition lacks any consistent patterns regarding pain intensity, which we all know is a totally subjective process. And pain duration is a required criteria per the American College of Rheumatology definition for the diagnosis of FM, which we adhered to. Besides muscle tenderness, the vast majority of the FM patients suffered from chronic fatigue, sleep disorders as well as mental fogginess, thereby suggesting that these traits are part of the
fibromyalgia syndrome and therefore share the same cytokine pattern. Our findings proved that the immunologic basis of FM occurs independently of any subjective features. Hence, this illustrates the very strong clinical value of our test protocol. As to comorbidity, as was explained in response to the remarks relating to the reviewer #1, we carefully eliminated any possibility of confounding variables by excluding any type of similar or overlapping illness. We modified both the Results and the Discussion to address this issue.

Reviewer #3

1. To understand the intra-group relationships of cytokines in patients, we studied how expressions of individual cytokines correlated with each other by performing a pair-wise rank-based Spearman correlation test. The results were summarized in a new Table 5. All cytokines correlated well with each other with a coefficient of correlation $\# > 0.4$ (all were significant at 1% FDR), suggesting that cytokines dynamics was similar in individual patients. We modified the text in the Materials and Methods, Results and Discussion accordingly.

2. As was explained in the responses to the Reviewer 1 comments, we eliminated testing any FM patients who had any comorbid disorders. The only potential overlap was the nine patients with diagnosed depression but those individuals had no impact on the overall results, as was also explained in the responses to the Reviewer 1 comments. As explained in the responses to the Reviewer #2 comments, chronic fatigue as well as sleep disorders and mental fogginess are part of the fibromyalgia traits and a major population of the patients in our study suffers from these symptoms. Therefore, the samples from the patients having these traits share similar patterns of cytokine expression.

3. We used the t-test ($#=0.05$) to determine if the difference in means is statistically significant. This information was provided in Tables 2 and 3. The p-value represents the probability that the observed difference occurred by chance. We added a corresponding sentence to the Materials and Methods.

Reviewer #4

General comment:

The fact that the cellular responses to mitogenic activators were significantly lower in patients with FM vs. those in healthy controls suggests that cell-mediated immunity is impaired in FM patients. In the past, FM was claimed to be a rheumatologic, neurologic or psychiatric disease despite the fact that there were no objective links to any of those pathways. In this study we uncovered evidence that FM is instead an immunologic disorder. We modified the article to address this issue.

1. We amended the Abstract to show the decreases in cytokine concentrations in the patients.

2. We agree with the reviewer’s comment and modified the text accordingly.
3. We revised the title as suggested by the reviewer.

4. In response to the Reviewer’s questions we offer the following:
   a. As stated in the Materials and Methods, the American College of Rheumatology criteria were used for the diagnosis of FM.
   b. We included detailed clinical data on patients in Table 1.
   c. As was reiterated in our current responses to Reviewer #2, FM by definition does not have set patterns regarding pain locations and, in fact, the American College of Rheumatology has redefined FM as no longer possessing specific pain or tender regions.
   d. We added the information on the control subjects to the Materials and Methods.
   e. We addressed this comment in our responses to Reviewer #2. Because our results were not based on circulating cytokine levels, when the blood was drawn is not relevant.
   f. As we explained in our responses to Reviewer #1’s comments, we excluded any and all other potentially overlapping or confounding medical disorders, including infections.
   g. The inclusion and exclusion criteria were explained in the Materials and Methods section.
   h. We included the median detection ranges in Table 3.
   i. The cytokine measurements were performed according to the protocol provided by the manufacturer. Blank medium was used as a negative control. Cytokine standards were included in each test for the standard curve calculations. Pooled supernatants obtained from activated cell cultures served as a positive control. Cytokine concentrations were determined in both undiluted samples and in the samples diluted 1:20 in medium. We added this information in the Materials and Methods.
   j. The study was performed in a blinded manner. Blood samples were coded and shipped to the processing laboratory. We included this information in the Materials and Methods.

5. We conducted a preliminary study where we screened a larger panel of fifteen cytokines in a group of eighteen patients and compared their concentrations with those measured in eighteen matching control samples. Eight cytokines showed decreased values in patients. These cytokines were selected for this study of 110 patients and 91 controls.

6. In our opinion, the data representation in the form of a table is more informative. Due to the large number of samples, it is difficult to show the cytokine readings for each individual sample. We included the median cytokine
values as well as their ranges in each group to illustrate the variations of cytokine readings within the groups.

7. For statistical analysis, we used the Stats package from R software (ref. 1).

The risk of using the t-test on a non-normal population is the risk of losing some statistical power for smaller sample sizes (ref. 2). For sample sizes as large as in our case, the risk of not detecting a difference between the groups due to a non-normal distribution is minimal. The following are the two relevant conclusions from ref 2.

“The study on the whole shows that for practical purposes, the power of the t-test is not seriously invalidated even if the samples are from considerably non-normal populations.”

“Also, as is expected, our results show that with an increase in sample size, the effect of non-normality on the power of the t-test diminishes.”

In fact, we revalidated our statistical results with the Wilcoxon-Mann-Whitney test and obtained nearly identical results.

References:


8. Our Discussion summarizes what this clinical study revealed; that there are highly specific immunologic patterns which define FM. In addition, our findings uncovered evidence that cellular immunity is impaired in FM patients. We added this information in the Discussion section. We also modified the text related to the comparison to other studies as suggested by the reviewer.

9. As requested by the reviewer, abbreviations were corrected in the text

10. Table 3 has a different meaning than Table 4; it shows an increase in cytokine production upon stimulation with mitogens in control subjects, while Table 4 compares cytokine values in two study groups.

Thank you in advance for consideration for publication of this report.

Sincerely,

Frederick G. Behm, M.D.